Improving the diagnosis of tuberculosis – clinical evaluation of four new diagnostics

Inauguraldissertation

zur

Erlangung der Würde eines Doktors der Philosophie

vorgelegt der

Philosophisch-Naturwissenschaftlichen Fakultät

der Universität Basel

von

Klaus Reither

aus Rheinfelden-Eichsel

Deutschland

Basel, 2017

Originaldokument gespeichert auf dem Dokumentenserver der Universität Basel

edoc.unibas.ch

Genehmigt von der Philosophisch-Naturwissenschaftlichen Fakultät
auf Antrag von
Prof. Dr. Sebastien Gagneux (Fakultätsverantwortlicher und Dissertationsleiter)
und Dr. Jean-Pierre Zellweger (Korreferent).

Basel, den 21.03.2017

Dekan
Prof. Dr. Martin Spiess

To Eiman, Noah and Tamina

Table of Contents

Table of Contents iv
List of Figures vi
List of Tables viii
Acknowledgements ix
Summary 1
Abbreviations 4
1 Introduction 6
1.1 Background information 6
1.1.1 Short overview on tuberculosis biology and evolution 6
1.1.2 Global TB epidemiology 7
1.1.3 Poverty and TB 9
1.1.4 Challenges in TB control 9
1.1.5 Advancement and evaluation of novel diagnostic tools 11
1.2 The diagnosis of TB 12
1.2.1 Smear Microscopy 14
1.2.2 Culture 14
1.2.3 Chest radiography 15
1.2.4 Xpert MTB/RIF 16
1.2.5 Immune assays 18
1.2.6 Detection of volatile organic compounds 20
1.2.7 Symptom-based diagnosis and case definition in children 20
1.3 Study aims 22
2 Manuscripts 23
2.1 Evaluation of giant African pouched rats for detection of pulmonary tuberculosis in patients from a high-endemic setting 23
2.1.1 Abstract 24
2.1.2 Methods 26
2.1.3 Results 28
2.1.4 Discussion 30
2.1.5 Figures 34
2.1.6 Tables 35
2.2 Diagnostic accuracy of computer-aided detection of pulmonary tuberculosis in chest radiographs: a validation study from sub-Saharan Africa 39
2.2.1 Abstract 40
2.2.2 Introduction 42
2.2.3 Methods 43
2.2.4 Results 47
2.2.5 Discussion 49
2.2.6 Figures 54
2.2.7 Tables 58

2.3 Xpert MTB/RIF assay for diagnosis of pulmonary tuberculosis in children: a prospective, multi-centre evaluation 61
2.3.1 Abstract 62
2.3.2 Introduction 63
2.3.3 Methods 64
2.3.4 Results 66
2.3.5 Discussion 68
2.3.6 Figures: 71
2.3.7 Tables 74
2.4 Performance of the novel TAM-TB assay to diagnose active tuberculosis in children: a prospective, proof of concept, multi-centre study 77
2.4.1 Abstract 78
2.4.2 Introduction 79
2.4.3 Methods 80
2.4.4 Results 83
2.4.5 Discussion 85
2.4.6 Figures 89
2.4.7 Tables 90
2.4.8 Supplementary appendix 93
3 General discussion 100
3.1 Target product profiles 100
3.2 Detection rats – does this diagnostic approach meet TPP requirements? 102
3.3 CAD4TB - a potential triage test? 103
3.4 Xpert MTB/RIF – first step towards a smear microscopy replacement test for the diagnosis childhood TB? 105
3.5 TAM-TB assay – can an improved version become a biomarker test for paediatric TB? 106
3.6 TB diagnostic evaluation 108
3.6.1 Beyond accuracy studies 108
3.6.2 Framework for evaluation of new TB diagnostics 109
3.7 Concluding remarks 112
4 References 114
Curriculum Vitae 140

List of Figures

Introduction
Figure 1: *M. tuberculosis* infection: stages and outcomes.

Figure 2: Estimated TB incidence rate worldwide, 2015.

Figure 3: Desired decline in global TB incidence rates to reach the 2035 targets; WHO.

Figure 4: Current pipeline of TB diagnostics; FIND.

Manuscript 1
Figure 5: STARD flow chart for diagnostic threshold of ≥2 indications by rats.

Figure 6: ROC analysis for the detection of *M.tb* culture-positive individuals for different indication thresholds (per-patient analysis; all samples). (Standard error 0.03, 95 CI% 0.66-0.78).

Figure 7: ROC analysis for the detection of smear-positive (A) versus smear negative (B) *M.tb* culture-positive individuals for indication thresholds (per-patient analysis; all samples). (A: Standard error 0.03, 95 CI% 0.71-0.84; B: Standard error 0.06, 95 CI% 0.45-0.67).

Manuscript 2
Figure 8: Flow chart of individuals taking part in the study.

Figure 9: Distribution of CAD scores for patient groups A (s+/c+ *M.tb*), B (s-/c+ *M.tb*), C (s±/c+ NTM), D (s-/c- clin.TB), and F (Controls).

Figure 10: ROC analysis for the detection of *M.tb* culture-positive individuals.
——— A (s+/c+ *M.tb*), B (s-/c+ *M.tb*) vs. F (Controls): A_z=0.84 (0.80-0.88),
----- A (s+/c+ *M.tb*), B (s-/c+ *M.tb*) vs. C (s±/c+ NTM), E (EPTB), F (Controls): A_z=0.81 (0.77-0.85), p =0.28.

Figure 11: ROC analysis for the detection of *M.tb* culture-positive individuals by smear status.
——— A (s+/c+ *M.tb*) vs. F (Controls): A_z=0.90 (0.86-0.93),
----- B (s-/c+ *M.tb*) vs. F (Controls): A_z=0.67 (0.58-0.75), p <0.01.

Figure 12: ROC analysis for the detection of *M.tb* culture-positive individuals by HIV status.
——— HIV negative. A (s+/c+ *M.tb*), B (s-/c+ *M.tb*) vs. F (Controls): A_z=0.89 (0.85-0.94),
----- HIV positive. A (s+/c+ *M.tb*), B (s-/c+ *M.tb*) vs. F (Controls): A_z=0.79 (0.72-0.86), p <0.01.

Figure 13: Comparison of automated and human reading.
Sensitivity and specificity to distinguish groups A and B vs F. Line and shaded area: ROC curve and 95% CI for CAD4TB. The expert reader is represented by square symbols, the clinical officer by round symbols. The different fill of the symbols indicate different reading thresholds: empty symbols ='any abnormality', crossed symbols ='TB consistent abnormalities' and filled symbols ='abnormalities highly suggestive for TB'.

Manuscript 3
Figure 14: STARD flow diagram.

Figure 15: Venn diagram for positive culture, Xpert MTB/RIF and AFB smear microscopy results per patient.

Figure 16: Time to detection for participants in groups culture-confirmed and highly probable TB.

Manuscript 4
Figure 17: Study profile

Figure 18: Venn diagram of positive *Mycobacterium tuberculosis* culture, Xpert MTB/RIF, and TAM-TB-assay results, and TAM-TB-assay results

Supplementary appendix
Supplementary Figure 1: Flow diagram of enrolment and clinical classification in adult optimisation cohort.
Supplementary Figure 2: TAM-TB assay settings and performance in the adult optimisation cohort.

Discussion
Figure 19: Sensitivity and specificity of the four different diagnostic tests for the detection of TB (manuscript 1-4), shown in a ROC curve in relation to TPP 1-3 performance requirements.

Figure 20: Elements of the evaluation of new TB diagnostics.
(LOD=limit of detection; QOL= quality of life; RCT=randomised clinical trial)

List of Tables

Manuscript 1
Table 1: Patient classification.

Table 2: Patient characteristics and symptoms at recruitment.

Table 3: Diagnostic test performance of TB detection rats (per-patient analysis; all samples).

Table 4: Rat-positive samples (two or more indications) in group C (s ±/c+ NTM) with concurrent molecular test result (per-sample analysis).

Manuscript 2
Table 5: Classification of study population according to clinical and microbiological data.

Table 6: Summary statistics of study population.

Table 7: Median CAD scores and 90% central range.

Table 8: Performance of CAD4TB and human readers.

Manuscript 3
Table 9: Case definition categories.

Table 10: Baseline characteristics and demographic data of the enrolled children.

Table 11: Diagnostic Test Performance of Xpert MTB/RIF.

Manuscript 4
Table 12: Demographics and clinical characteristics of study participants by classification group

Table 13: T-cell activation marker–tuberculosis assay results by classification groups.

Table 14: Sensitivity, specificity, and predictive values of the T-cell activation marker–tuberculosis assay.
Data are n/N (%), unless otherwise indicated. Culture-confirmed tuberculosis and not tuberculosis were the reference standards for sensitivity and specificity. Calculation of predictive values was based on disease prevalence in the main paediatric cohort (n=290).

Table 15: Cases with discrepant culture and TAM-TB assay results: clinical characteristics at enrolment and follow-up visits by age
Positive tuberculin skin test suggested by a lesion of at least 10 mm in HIV-uninfected or at least 5 mm in HIV-infected children. TAM-TB=T-cell activation marker–tuberculosis.

Supplementary appendix
Supplementary Table 1: Clinical case categories of paediatric tuberculosis suspects (validation cohort).

Acknowledgements

First, I want to thank Marcel Tanner, Director emeritus of Swiss TPH, who gave me the opportunity to start clinical TB research in Bagamoyo, Tanzania, and who had great trust in the entire clinical TB research team and me. I would like to express my special appreciation and thanks to my supervisor Sebastien Gagneux, who is an outstanding and inspiring TB scientist. I would like to thank Jean-Pierre Zellweger for joining the PhD committee as a Co-referee.

Clinical research is teamwork. Therefore, special thanks go to the colleagues from Swiss TPH who were directly involved in the publications this thesis was based upon. Levan Jugheli, Swiss TPH's excellent TB laboratory specialist, whose contributions were essential for all studies. Important input in the field of TB immunology came from Damien Portevin and Claudia Daubenberger, who became the internal expert of the PhD committee. I would also like to thank Amanda Ross and Tracy Glass who gave crucial statistical support. Furthermore, I also want to name Marianne Breuninger, who impressed me by her hard work and professionalism in the early stage of her medical career. I am very thankful to the brilliant Andreas Steiner, who helped us in many problems regarding the study databases. I would particularly like to thank my dear fellow TB researchers at the Ifakara Health Institute (IHI). Francis Mhimbira, Frederick Haraka, Khadija Said, Elirehema Mfinanga and Jerry Hella are medical doctors and scientist who contributed significantly to the work of this thesis. They were and are together with the laboratory team led by Mohamed Sasamalo and Lujeko Kamwela (BSL 3 Lab), Sarah Mswata (Safety Lab) and Maximilian Mpina (Immunology Lab) the backbone of the clinical TB research at IHI. My special appreciations go to the several external research partners. I would like to mention some by way of example, without diminishing the contribution and merit of the others. Martin Nsubuga and Francesco Aloi, who led the team at the St. Francis Hospital Nsambya in Kampala, Uganda, were key partners in the TB CHILD consortium. Similarly, Nyanda Ntinginya, Andrea Rachow, Petra Clowes, and Christof Geldmacher and the teams from the Mbeya Medical Research Centre, Mbeya, Tanzania and the University of Munich, Germany, were extremely competent and experienced partners in our joint scientific work. Daniela Cirillo from the San Raffaele Scientific Institute, Milano, Italy, and Ben Marais from University of Sydney, Australia, - both renowned experts in the field- gave indispensible advice and guidance. I am grateful for the exciting collaboration with Bram van Ginneken and his team who are specialists in computer-assisted x-ray reading from Nijmegen, The Netherlands. Christophe Cox, Timothy Edwards, Christiaan Mulder and team were equally supportive when they introduced me into the world of TB sniffing rats in Morogoro, Tanzania.

I am grateful to the former CED of IHI, Salim Abdulla, the current CED of IHI, Honorati Masanja, and the Director of Swiss TPH, Jürg Utzinger, who constantly support clinical TB work in

Tanzania. Thanks to my colleagues Lukas Fenner and Thomas Zoller who discussed many scientific questions with me.

I am thankful for the funding from the following two sources. The European and Developing Countries Clinical Trials Partnership (EDCTP) funded the TB CHILD project [IP.2009.32040.007; "Evaluation of new and emerging diagnostics for childhood tuberculosis in high burden countries"]. The Rudolf Geigy Foundation, Switzerland, supported the TB Cohort project.

Finally, I would like to express my gratitude and my deepest respect to all study participants and their families and friends. Without their support, this research would not have been possible.

Summary

The United Nations Sustainable Development Goals target to end tuberculosis related deaths, transmission and catastrophic costs by 2030. In order to reach this very ambitious aim, a diverse set of various actions by country governments, civil society organisations and research communities is needed. Central components of the collective efforts are discovery, development and evaluation of new tools, interventions and strategies. Novel diagnostic tools are of particular interest, since an early and accurate diagnosis is considered key to improve tuberculosis control and to accelerate the fight against tuberculosis globally.

Therefore, the overall goal of this doctoral thesis was to evaluate four new diagnostic tests based on different approaches for tuberculosis detection. The clinical accuracy studies were conducted in sub-Saharan countries with high burden of tuberculosis disease.

This thesis is based on four manuscripts:

Manuscript 1: Evaluation of giant African pouched rats for detection of pulmonary tuberculosis in patients from a high-endemic setting

The first manuscript depicts the evaluation of trained giant African pouched rats for detecting *Mycobacterium tuberculosis* in sputum of 469 well-characterised patients with presumptive tuberculosis in a rural setting of Tanzania. The rats could detect tuberculosis with a sensitivity of 57% and a specificity of 81%. The diagnostic performance was negatively influenced by low burden of bacilli, but independent of the HIV status. Based on evidence collected in this and other similar studies, trained tuberculosis detection rats are not suitable as a standalone sputum-based diagnostic test. Research and development should focus on improving the diagnostic accuracy, and but also on issues such as scalability, deliverability and cost-effectiveness.

Manuscript 2: Diagnostic accuracy of computer-aided detection of pulmonary tuberculosis in chest radiographs: a validation study from sub-Saharan Africa

The CAD4TB software, a computer-aided system for detection of tuberculosis on chest radiographs, has been evaluated in 861 adults with suspected pulmonary tuberculosis in rural Tanzania. The area under the receiver operating characteristic curve for the detection of culture-positive pulmonary tuberculosis was 0.84, which indicates that CAD4TB can accurately differentiate between tuberculosis cases and controls. The performance of CAD4TB was better in HIV-negative compared to HIV-infected individuals. Overall CAD4TB outperformed a clinical officer reading x-ray images, but did not reach the accuracy of an expert. After adjusting, the

threshold for test positivity, higher sensitivity and acceptable specificity was achieved. This qualifies CAD4TB as a potential triage test for pulmonary tuberculosis in adults before further molecular confirmation test.

Manuscript 3: Xpert MTB/RIF assay for diagnosis of pulmonary tuberculosis in children: a prospective, multi-centre evaluation

The microbiological confirmation of tuberculosis in children is a challenge, because the collection of specimen is problematic. In particular, the paucibacillary nature of the disease rarely allows detection of microorganisms by smear microscopy, and culture -having also an imperfect sensitivity- is usually not available in high burden settings. Thus, diagnosis in children relies typically on clinical symptoms, tuberculin skin test, radiological findings, and history of tuberculosis contact.

The presented multi-centre evaluation of Xpert MTB/RIF in 451 children with presumptive pulmonary tuberculosis established an overall sensitivity of 68% and specificity of 100% compared to culture. Cross reactivity with non-tuberculous mycobacteria has not been observed. Xpert MTB/RIF detected ten children with smear negative but culture-positive for *Mycobacterium tuberculosis* and three additional cases in absence of mycobacterial growth on culture. Overall, the results indicate that Xpert MTB/RIF can diagnose pulmonary tuberculosis in children better than smear microscopy, but the sensitivity is suboptimal in comparison to culture. Therefore, Xpert MTB/RIF cannot be used as a standalone test to rule out paediatric tuberculosis because many children will still need empirical antituberculosis therapy in case of Xpert negative results. Yet, the technology is being further developed to a more sensitive format that will hopefully resolve the dilemma of diagnosing tuberculosis in children.

Manuscript 4: Performance of the novel TAM-TB assay to diagnose active tuberculosis in children: a prospective, proof of concept, multi-centre study

Non-sputum-based diagnostics are considered as promising solutions in the direction towards improved diagnosis of paediatric tuberculosis. The accuracy of a novel immunodiagnostic test - the TAM-TB assay- to identify children with active tuberculosis has been evaluated in a proof-of-concept study with 130 children in Tanzania. The assay uses the loss of CD27 expression on *Mycobacterium tuberculosis*-specific CD4 T cells as a marker of active tuberculosis. Standardised clinical case classifications based on microbiological and clinical findings served as a comparator. The TAM-TB assay detected culture-confirmed cases with a sensitivity of 83% and a specificity of 97%. Compared with *Mycobacterium tuberculosis* culture, the combination of Xpert MTB/RIF and TAM-TB, two assays with a turnaround time of less than 24 h, had a sensitivity of 94%, missing only one culture-confirmed case. Furthermore, five of eight children who were

culture-negative and TAM-TB assay positive had highly probable or probable tuberculosis. An analysis of the five clinical cases suggested that they probably did have active tuberculosis, suggesting that the TAM-TB assay is at least as sensitive as *Mycobacterium tuberculosis* culture. However, the flow cytometry based assay is currently too complex for routine use. Simplification, optimisation, miniaturisation, and broad clinical validation are necessary before TAM-TB can become part of diagnostic algorithms in paediatric tuberculosis.

This doctoral thesis also analyses the four clinically evaluated diagnostic approaches in the context of target product profiles for diagnostic needs of highest priority, as established by the World Health Organisation and key stakeholders. Currently, none of the diagnostics fully concurs with the requirements of the target product profiles. After further advancement, CAD4TB holds the potential for triage testing. A more sensitive version of the Xpert assay could become a smear-replacement test for the diagnosis of tuberculosis in children. A simpler and faster version of the TAM-TB assay would have prospects of becoming a non-sputum-based biomarker test.

In the last part of this doctoral thesis, accuracy studies are described in relation to current diagnostic evaluation pathways as an overarching concept. Special emphasis has been put on the important, but still underutilised studies on health impact and cost-effectiveness, which are needed beyond accuracy assessment. Further improvement of the development and evaluation of new diagnostic tests or strategies will bring the end of the global tuberculosis epidemic closer.

The results of this doctoral thesis improve our understanding of four diagnostic approaches and the key components for effective diagnostic evaluation. The lessons learnt will help to inform future clinical studies of new diagnostics for tuberculosis.

Abbreviations

AFB	Acid-fast bacilli
AIDS	Acquired Immune Deficiency Syndrome
Az	Area under ROC curve
CAD	Computer-aided detection
CD	Cluster of differentiation
CFP-10	Culture filtrate protein 10
CFU	Colony forming units
CI	Confidence interval
C_T	Cycle threshold
CXR	Chest X-ray
DEEP	TDR Diagnostics Evaluation Expert Panel
DNA	Deoxyribonucleic acid
DOTS	Direct Observation Treatment Short-Course
DST	Drug susceptibility testing
EDCTP	European and Developing Countries Clinical Trials Partnership
EPTB	Extrapulmonary TB
ESAT-6	Secreted antigenic target 6
FIND	Foundation for Innovative and New Diagnostics
GCP	Good Clinical Practice
GDP	Gross domestic product
GRADE	Grading of Recommendations Assessment, Development, and Evaluation
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
ICH	International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use
IFN-γ	Interferon gamma
IGRA	Interferon-gamma release assay
IHI	Ifakara Health Institute
IL	Interleukin
IP10	Interferon gamma-induced protein 10
IQR	Interquartile range
LJ	Löwenstein-Jensen
LTBI	Latent TB infection
M.	*Mycobacterium*
M.tb	*Mycobacterium tuberculosis*
MD	Medical doctor
MDGs	Millennium Development Goals
MDR-TB	Multidrug resistant TB
MFI	Median fluorescence intensity

MGIT	Mycobacterial Growth Indicator Tube
MTBC	*Mycobacterium tuberculosis* complex
NALC-NaOH	N-acetyl-L-cysteine-sodium hydroxide
NTM	Non-tuberculous mycobacteria
PACT	Polymyxin B, amphotericin, carbenicillin, trimethoprim
PANTA	Polymyxin B, amphotericin B, nalidixic acid, trimethoprim, azlocillin
PBMC	Peripheral blood mononuclear cell
PCR	Polymerase chain reaction
PEPFAR	President's Emergency Plan for AIDS Relief
PPD	Purified protein derivative
PTB	Pulmonary TB
QUADAS	Quality Assessment of Diagnostic Accuracy Studies
RIF	Rifampicin
ROC	Receiver operating characteristic
SD	Standard deviation
SEB	Staphylococcal Enterotoxin B
STAG-TB	Strategic and Technical Advisory Group for Tuberculosis
STARD	Standards for Reporting of Diagnostic Accuracy
TAM	T-cell activation marker
TB	Tuberculosis
Th	T-helper
TNF	Tumour necrosis factor
TPP	Target product profile
TST	Tuberculin skin test
UN	United Nations
USAID	US Agency for International Development
VOC	Volatile organic compounds
WHO	World Health Organization
XDR-TB	Extensively drug resistant TB
ZN	Ziehl-Neelsen

1 Introduction

1.1 Background information

1.1.1 Short overview on tuberculosis biology and evolution

Human tuberculosis (TB) is a communicable infectious disease caused by genetically related bacteria of the *Mycobacterium tuberculosis complex* (MTBC) [1]. The most frequent causative agent of TB in human beings is *Mycobacterium (M.) tuberculosis* [2]. Besides, *M. africanum* is causing almost half of the pulmonary TB cases in geographically restricted regions of Western Africa [3]. Other members of the MTBC, described as animal-adapted (*M. bovis, M. microti*) or of presumably environmental origin (*M. canettii*) [1,4] may cause sporadically TB in humans [5–9]. Among the latter three bacteria only *M. bovis* appears to be capable of spreading in a very small proportion of cases through human-to-human transmission [10].

TB has plagued humans for ages. Likewise, MTBC and modern humans have been coevolving for thousands of years [11]. The pathogen accompanied migrations of humans out of Africa and subsequently reached other continents before returning to Africa again [12]. During Neolithic Demographic Transition about 10,000 years ago, hunting was gradually replaced by farming and human demographics changed dramatically, most likely triggered by the controlled use of fire [13]. As a consequence, MTBC also expanded and evolved geographically together with its human hosts [12,14]. Today, the seven human-adapted lineages of MTBC are distributed worldwide. In accordance with the out-of-Africa hypothesis, only Africa harbours all of the seven lineages [15].

Characteristically, TB is an airborne disease transmitted from human to human by fine droplets produced during the pulmonary form of TB when coughing, sneezing or speaking [16]. These droplet nuclei are up to 5 μm in size and contain as few as 1-3 bacilli [17]. The largest respiratory droplets fall to the ground, but the smaller droplets can remain airborne for long periods of time and are eventually inhaled by another person [18]. The quantity of aerosolised *M. tuberculosis* is a reliable predictor of TB transmission, even better than established microbiological approaches as smear microscopy grade or culture [19]. TB can also be transmitted through other ways such as drinking of raw, unpasteurized milk (in the case of M. *bovis*) or congenital transmission [5,20].

In 90 to 95% of immunocompetent individuals, an infection with *M. tuberculosis* remains contained due to host defence mechanisms, resulting in latent TB infection (LTBI), in which the pathogen may stay in a quiescent state. For simplification purposes, LTBI is usually defined as an absence of clinical signs and symptoms of active TB disease and concurrent evidence of immunological response to mycobacterial proteins. However, LTBI does not refer to one defined

condition but rather to a spectrum of gradually varying host responses and differently replicating bacillary populations [21–24]. About 5% of immunocompetent individuals develop directly active TB ('primary progressive TB'; Figure 1). The lifetime risk of progression from LTBI to active TB is 5-10% with most individuals developing TB disease within the first five years after initial infection [25]. The risk of progression is higher when factors, e.g. co-infection with human immunodeficiency virus (HIV), diabetes mellitus, malnutrition, alcohol-related liver disease, silicosis, end-stage renal disease, indoor air pollution, smoking or intake of immunosuppressive medication (e.g. therapy with tumour necrosis factor (TNF)-α inhibitors) , perturb the immune system [23,24,26–28]. TB is most commonly characterised by necrotizing granulomatous inflammation of the lung. However, the disease can virtually involve any, so called extrapulmonary, site of the human body. Extrapulmonary TB constitutes between 15 and 50 per cent of all TB cases with a higher proportion in immunocompromised patients and in children [29].

In up to 50% of cases, early infection by *M. tuberculosis* may be cleared. Early clearance, presumably mediated by an effective innate immune response, leads to eradication of *M. tuberculosis* even before the adaptive immune response develops [30]. In another small proportion of cases, LTBI is probably cleared even several weeks after infection, described as 'acute resolving infection' [31].

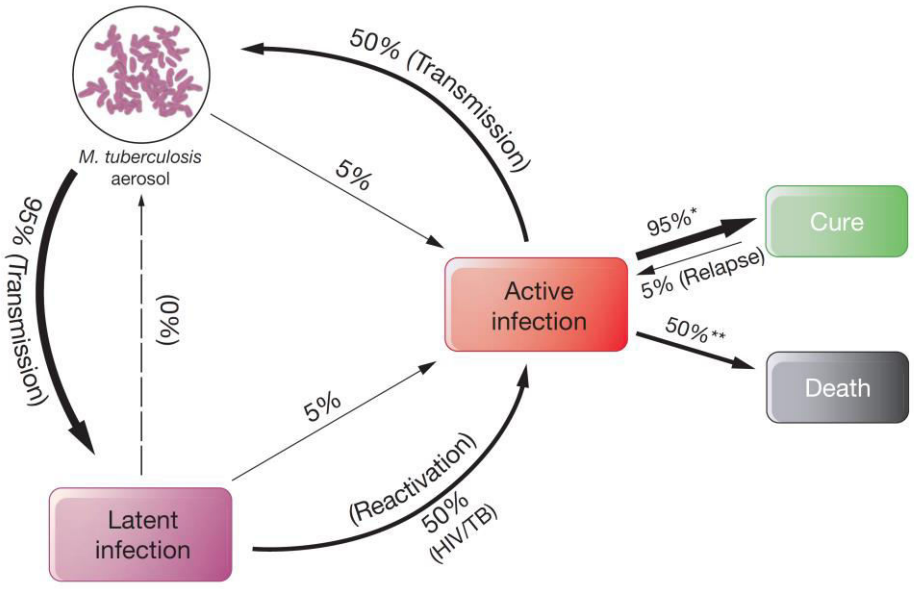


Figure 1: *M. tuberculosis* infection: stages and outcomes [26]**.**

1.1.2 Global TB epidemiology

TB has killed more people than any other infectious disease and is currently more prevalent in the world than at any other time in history [32]. One-third of the global population is potentially infected with *M. tuberculosis*. In 2015, the World Health Organization (WHO) estimated almost

10.4 million new active TB cases (Figure2) and 1.8 million deaths, primarily in low- and middle-income countries [33]. Underreporting, mainly in the private sector, and underdiagnosis, mainly in resource-poor settings, remain major problems leading to large detection and treatment gaps [33].

Today, TB kills someone approximately every 20 seconds. Thus, TB has surpassed HIV as a leading cause of death from an infectious disease worldwide. Both adults and children are seriously affected by the global TB epidemic [27,34,35]. The risk of developing active TB diseases after exposure differs by age; infants are at very high risk, the risk is less for children between 2 and 10 years of age and rises to a lifelong, high plateau beginning between 20 and 30 years of age [36]. The true global burden of childhood TB is difficult to assess, because an accurate reference standard for diagnosis is missing [37,38].

About 60% of all TB patients live in India, Indonesia, China, Nigeria, Pakistan or South Africa. HIV/AIDS and TB co-epidemics negatively affect each other, particularly in Sub-Saharan Africa, which is characterised by the most severe burden of active TB relative to population.

TB has become particularly dangerous to societies in Eastern Europe, Asia, and parts of Africa, due to the emergence of resistant, multidrug resistant (MDR) and extensively drug resistant (XDR) strains. MDR-TB is defined as resistance to at least rifampin and isoniazid and XDR-TB describes resistance to rifampin and isoniazid plus resistance to any fluoroquinolone and to at least one of three injectable second-line drugs (i.e., amikacin, kanamycin, or capreomycin) [39]. It is estimated that in 2015 approximately 480,000 MDR-TB and 100,000 rifampicin-resistant cases occurred, of which 45% were found in India, China and the Russian Federation. Only about 20% of all MDR-TB were correctly detected and only about 50% of the detected cases were successfully treated [33]. More than one out of every second country has reported at least one XDR-TB case.

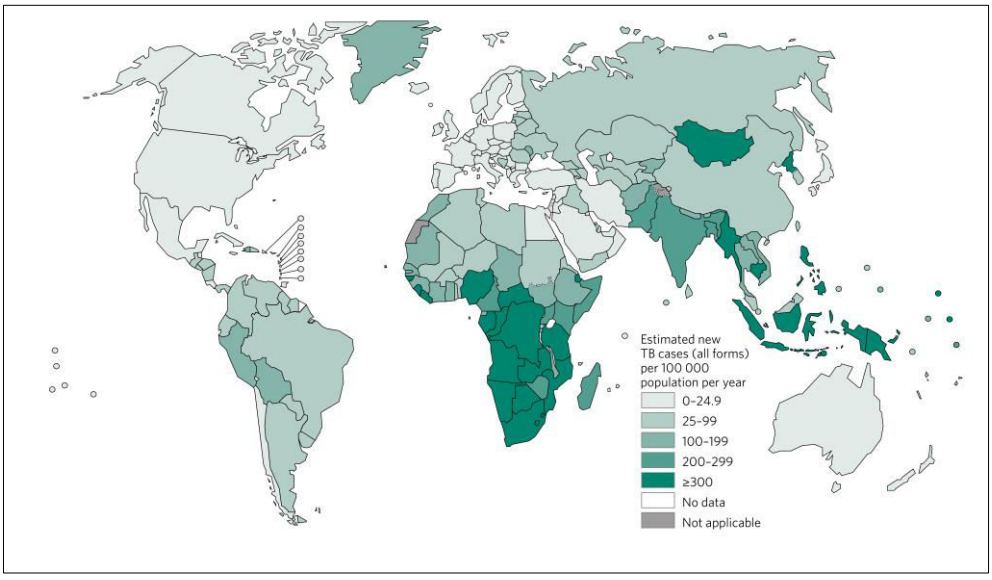


Figure 2: Estimated TB incidence rate worldwide, 2015 [33].

1.1.3 Poverty and TB

TB is a disease of poverty. People in resource-constrained settings have to live and work under difficult conditions. Overcrowding and poor ventilation, two catalysts of TB transmission, increase the likelihood of infection in those settings. Further risk factors such as malnutrition or inadequate diet, smoking, alcohol abuse prevail among the poorest. Limited health knowledge and a lack of empowerment to act on improvement of health conditions contribute further to the TB epidemic among the poor [40]. A global ecological analysis showed linear association between per capita gross domestic product (GDP) and incidence of TB, i.e. doubling of GDP is associated with almost 40% decrease in TB incidence [41]. Similarly, the country level human development index correlates with trends of TB incidence over time [42]. In the second half of the 19th and the first half of the 20th century, TB incidence decreased in high-income countries, e.g. in Europe, primarily due to improved socioeconomic conditions, better housing and more effective public health measures. Specific medical pharmacotherapies or the BCG vaccine became available late and did not play the most important role in the reduction of TB incidence [43–45].

TB also creates poverty. The economic impact of TB and treatment for patients and household members is often immense. In Africa, the pre-diagnostic costs - incurring between the onset of symptoms and the TB diagnosis- accounted in average for more than 10% of patients' annual income [46]. Different household coping strategies are necessary to deal successfully with the illness costs. However, particularly among the poor families these strategies are weak, e.g. because very poor households are often excluded from community support mechanisms such as friendship networks [47]. On country level, TB can have an enormous economic burden on the economy. In Africa, the estimated total expected GDP loss attributable to deaths by TB is 50.4 billion USD, approximately 1.37% of the combined GDP of the 47 WHO African Region countries [48].

1.1.4 Challenges in TB control

Since the WHO has declared TB a global emergency in 1993 [49] and the United Nations (UN) has launched the Millennium Development Goals (MDGs) in 2001 [50], some remarkable progress has been made in TB control. Globally, the TB mortality rate in 2015 was 47% lower than in 1990, with almost the entire improvement taking place between 2000 and 2015. Furthermore, diagnosis and treatment of TB saved 43 million lives, and the global cumulative TB incidence dropped by 18% between 2000 and 2014 [51]. Although overall the MDG target of halting and reversing the TB epidemic has been achieved in each of the six WHO regions and in 16 of the 22 high-burden countries, the global TB burden remains unacceptably high [51].

The new UN Sustainable Development Goals which were adopted in September 2015 specifically aim at 'ending the TB epidemic' by 2030. This means that TB incidence and death rates should be reduced by 80% and 90%, respectively [52]. Equally ambitious goals have been announced by the 'End TB Strategy' of the WHO: overall 90% reduction in incidence and 95% reduction in mortality by 2035 [53].

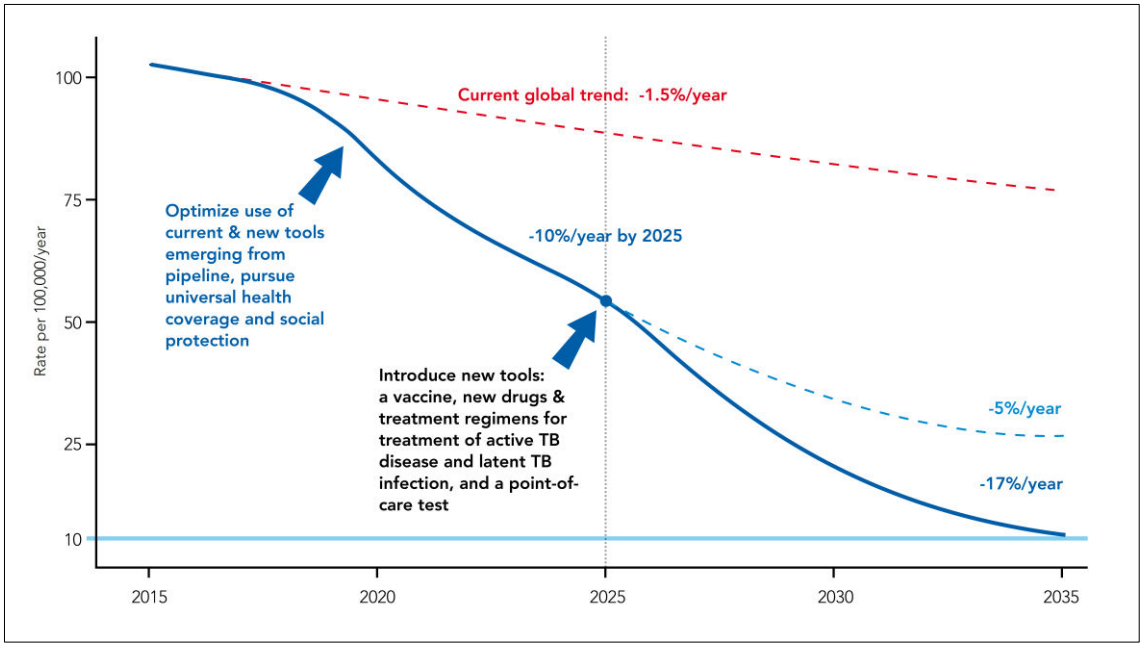


Figure 3: Desired decline in global TB incidence rates to reach the 2035 targets; WHO [53]**.**

Similarly, the Stop TB Partnership 'Global Plan to End TB 2016-2020 has the ambition to reach 90% of all people who need TB treatment, including 90% in key populations such as HIV-infected individuals or mining communities and achieving 90% treatment success [54].

TB eradication instead of TB control within the scheduled time-frame appears to be unattainable without major innovations in diagnostics, drugs, vaccines and prevention tools (Figure 3) [55–59]. Moreover, until this bold target of TB elimination becomes reality a spectrum of complementary measures are needed: health systems of the affected countries have to be strengthened; poverty and inequality, two major determinates of TB, need to be effectively reduced [60,61]; and all stakeholders in politics, health care, research and industry need to work in synergy towards a common goal of ending TB [62,63]. In addition, there is a need for better housing, transport and healthcare in fast growing urban conglomerations, where the majority of TB transmission takes place [64].

In absence of an effective vaccine, at present, the key to TB control relies in early diagnosis followed by effective therapy. A major challenge is the lack of cost-effective, widely available,

point-of-care diagnostic TB tests for adults and children. The development and the evaluation of such tests is on top of the research priority list [65]. Novel diagnostic TB tests should provide fast and reliable information on detection and drug resistance of *M. tuberculosis* with high sensitivity and specificity. This should lead to a reduction of patient and provider delays and eventually to a decline of transmission, morbidity, and mortality [66–68]. In this context, a mathematical decision-tree model predicted that 625'000 TB deaths can be annually prevented if a rapid, 100% sensitive and 100% specific test is accessible for everyone in need [69].

1.1.5 Advancement and evaluation of novel diagnostic tools

Innovation in TB diagnostics technology holds the potential to advance the diagnostic landscape and consequently, to improve detection and management of TB cases particularly in resource-poor countries. Efforts to develop better TB diagnostics and to improve laboratory services were triggered by the emergence of drug-resistant TB, particularly by the 2005 outbreak of XDR-TB in Kwazulu Natal in South Africa [70]. In the same time, additional resources became available, e.g. from US Agency for International Development (USAID), the President's Emergency Plan for AIDS Relief (PEPFAR), and UNITAID. Meanwhile, the Foundation for Innovative and New Diagnostics (FIND) has a central role in development and improvement of new TB diagnostics.

Studies on the accuracy of laboratory tests, medical imaging, or information from patient history or examinations have to be designed, conducted and analysed according to the following standardized guiding principles. The TDR Diagnostics Evaluation Expert Panel (DEEP) defines optimal design and conduct of diagnostic studies, with focus on operational aspects [71]. The Quality Assessment of Diagnostic Accuracy Studies (QUADAS) provides a quality tool for accuracy studies [72]. The Standards for Reporting of Diagnostic Accuracy (STARD) steering committee [73,74] mainly aims to improve reporting. Those guidelines ensure that the participants are correctly classified with regard to a certain target condition.

A target condition is usually an existing acute or chronic disease, the stage of the disease or the response to treatment. In a clinical accuracy study, the medical test, also called index test, is being compared to a reference standard to generate the main accuracy outcomes (sensitivity, specificity, positive and negative predictive values). If the reference standard does not accurately relate to the target disease (e.g. in the case of childhood TB or extrapulmonary TB), a composite reference standard, a latent class analysis or panel diagnosis is used to avoid bias [75,76]. The confidence intervals of these estimates are used to calculate the precision of the measurements. A receiver operating characteristic (ROC) curve can be created to illustrate the combination of sensitivity and specificity graphically for each possible test positivity cut-off. The area under the ROC curve gives a picture on the overall accuracy of the medical test.

The current theoretical concept for development and endorsement of TB diagnostics includes smaller clinical evaluation trials preceding larger so called demonstration studies, which are conducted at multiple sites to assess performance characteristics and early patient-important outcomes (e.g. turnaround times of test results) [77]. Evidence from these studies is being analysed and a group of experts gives recommendations to the WHO's Strategic and Technical Advisory Group for Tuberculosis (STAG-TB) after a new diagnostic tool has been thoroughly assessed through systematic reviews and meta-analyses based on GRADE (Grading of Recommendations Assessment, Development, and Evaluation) standards [78–81]. Recommendation based on evidence is eventually supposed to support scale-up at country.

In order to make endorsement and scale-up more efficient and open for revisions, a two-step process has been recently proposed starting with a technical recommendation, based on accuracy data and some cost and feasibility data. After 2 years, wider programmatic recommendations are supposed to follow with focus on patient-important outcomes, cost-effectiveness and operational issues in routine use. The evidence of both components should be critically appraised before complete scale-up [82]. Until now, WHO has not adopted the proposed framework, presumably because the GRADE process, which is used organization-wide within WHO as an evidence generating approach, is not suitable for the second step.

1.2 The diagnosis of TB

This chapter focuses on the diagnostic approaches, which were employed in the presented studies. It does not cover the whole landscape of diagnostic tests, which is much broader and displayed in Figure 4.

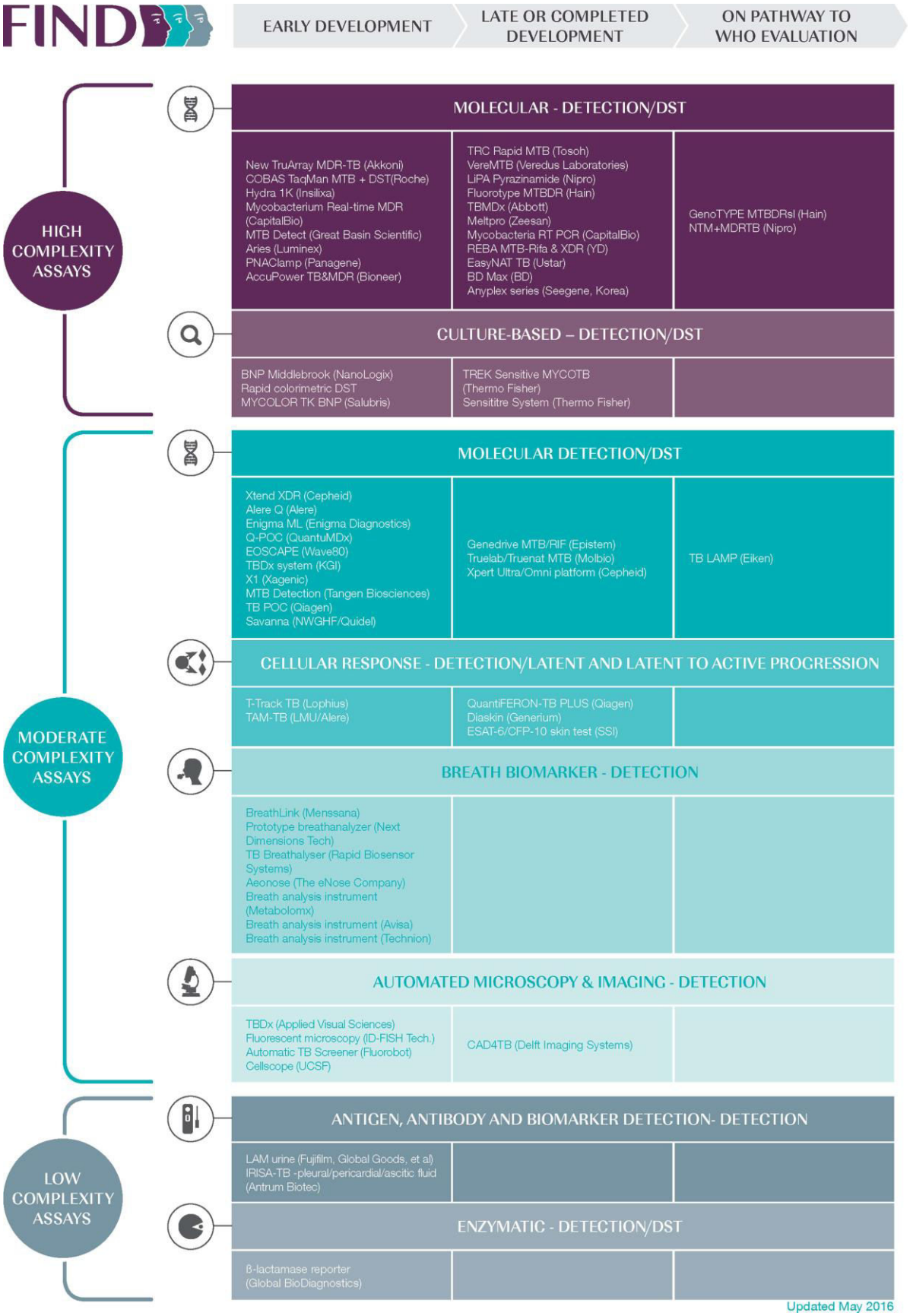


Figure 4: Current pipeline of TB diagnostics; FIND [67].

1.2.1 Smear Microscopy

In 1882, Robert Koch discovered that one specific micro-organism, which was named one year later *M. tuberculosis*, causes TB in its different manifestations, for instance miliary disease, caseous pneumonia, intestinal TB, or tuberculous lymphadenitis. On the evening of 24 March 1882, today's international "World TB Day", Koch made his landmark declaration at the monthly meeting of the Berlin Physiological Society. The lecture included more than 200 microscopic preparations showing the presence of the rod-shaped bacteria [83–85]. The staining methods were invented by Koch himself. At this occasion, microscopy was established as a key diagnostic modality for the visualisation of mycobacteria, which remains commonly used until now [86]. After Koch's discovery, various scientists have developed several improvements and modifications of the staining methods. Franz Ziehl and Friedrich Neelsen created a staining method with carbolfuchsin which evolved from Koch's original alkaline methylene blue-based method [87]. In a Ziehl-Neelsen stain, acid-fast bacilli appear as bright red rods. This method requires heat fixation (''hot staining'') to facilitate penetration of the cell wall. A modified method, published by Josef Kinyoun in 1915, is performed at room temperature after using higher concentration of the carbolfuchsin in the primary stain (''cold staining''). In the 1937, Hagemann described that acid-fast bacilli can also be identified through fluorescent dyes with an affinity for mycolic acids in the cell wall of the mycobacterium, as for instance auramine or a combination of auramine and rhodamine [56].

The inexpensive sputum smear microscopy is currently the most widely available method for the diagnosis of pulmonary TB [89]. A positive smear requires 5,000-10,000 acid-fast bacilli per µL sputum. Thus, microscopy after Ziehl-Neelsen staining detects pulmonary TB with a low sensitivity: <70 per cent in immunocompetent and usually < 50% in HIV infected individuals. Fluorescent microscopy, e.g. with auramine stains, is 10% more sensitive and allows due to the lower magnification, faster processing. The specificity, however, is relatively high (<94-100%)[90–94]. Apart from the bacterial load of the specimen, other factors such as proficiency and skills of laboratory staff, staining and sputum concentration methods as well as availability of reagents have an influence on the overall detection rate in a microscopy centre [95,96]. Self-evidently, smear microscopy cannot differentiate drug-susceptible from drug-resistant TB strains.

1.2.2 Culture

Robert Koch also contributed profoundly to the use of culture as a diagnostic tool for TB. In 1901, Koch presented the first mycobacterial culture colonies on solid growth media in glass tubes to the audience of the British Congress on Tuberculosis [97]. Since that time, culturing of

M. tuberculosis is widely considered the 'gold standard' for the diagnosis of TB disease. Additionally, culture is essential for phenotypic drug-resistance testing [98].

Solid, egg-based media (e.g. Löwenstein-Jensen (LJ), Ogawa, and Stonebrink) and liquid media (e.g. Middlebrook Bouillon, Kirchner) are used for culturing of mycobacteria. Liquid culture methods, e.g. the BACTEC Mycobacterial Growth Indicator Tube (MGIT) 960 system, are more sensitive than solid culture [99]. BACTEC MGIT 960 has a detection threshold of 1-10 colony forming units (CFU)/ml [100]. Though, in case of paucibacillary samples a definite diagnosis can often be made only after 6 or more weeks due to a long doubling time, which span between 16 and 69 hours [101]. The long incubation time pave the way for contamination with environmental bacilli, which is more pronounced in liquid than in solid culture, due to the higher nutrient concentration in liquid media [102]. Decontamination methods (e.g. NALC-NaOH or modified Petroff's method) and supplementation of antibiotics (e.g. PANTA or PACT) are applied to destroy as many of the contaminating bacteria as possible while harming as few mycobacteria as possible. Ideally, the methods are adjusted to laboratory and setting-specific requirements [102]. Compared to microscopy, culture is complex, costly, labour intense and requires special biosafety containment.

Despite those limitations, WHO recommended an endorsement and a phased implementation of automated liquid culture systems and drug susceptibility testing (DST) in low- and medium-income countries after reviewing performance data and results from demonstration studies on liquid culture systems [103]. A systematic assessment of a broader role-out of liquid culture system is still pending, but significant constraints and limited impact have been already reported from remote and resource-limited settings [104].

1.2.3 Chest radiography

Conventional and digital chest x-ray. Since the beginning of the twentieth century, conventional, film-based chest radiography has been an essential component of diagnostic algorithms for pulmonary TB in low and high burden settings [105,106]. The introduction of digital radiography has further extended the use of diagnostic imaging -at least in industrialized countries- because handling became easier, the exposure decreased, and high-quality digital images were now readily available and could be stored or transferred electronically. Furthermore, better hardware and advanced processing methods have improved the standards for image analysis [107]. Usually, digital x-ray units are relatively expensive and require specifically trained staff. Thus, innovative, less costly digital radiology solutions for low- and middle-income might make radiology globally available [108].

Chest x-ray is primarily used for the diagnosis of smear-negative pulmonary TB patients or for screening (triage test) before molecular testing (Xpert MTB/RIF), as a survey in the 22 countries

with highest burden of TB recently showed [109]. Chest radiography meets the requirement for a screening or triage due to its high sensitivity. TB related abnormalities can be identified in the radiograph in about 87-94% of pulmonary TB patients, any abnormality can be detected with a sensitivity of 94-98% [110,111]. However, chest x-ray should not be used as a final or stand-alone confirmatory approach, because of the modest specificity of around 73-75% for any abnormality and approximately 89% for abnormalities suggestive of TB [110–113]. Chest x-ray seems to be of little benefit for the detection smear-negative, culture-confirmed TB in HIV infected patients [114]. Moreover, the reproducibility of chest x-ray results is impeded by high levels of inter- and intra-reader variability [115–117]. In many high burden countries, the reading of x-rays is left to non-experts, because the numbers of radiologists in public services are very low [118].

Automated chest x-ray. Innovative software, such as CAD4TB from the Diagnostic Image Analysis Group at Radboud University Nijmegen, has been developed to overcome current operational obstacles by providing a reproducible and standardized way of analysing and reporting chest x-rays. The software determines whether a chest radiography shows evidence of pulmonary TB using a numerical probability score generated within one minute [119,120]. CAD4TB employs a detection system for the different textural, shape and focal abnormalities in pulmonary TB and transforms those into one score. The process is either based on a supervised learning approach that relies on large training databases, usually associated with time-consuming lesion annotation process, or with a multiple-instance learning process, which requires less supervision and has better adaptation capabilities for image processing by different devices [119]. CAD4TB has been developed according to industry standards and is able to process radiographs from different types of digital equipment by standardizing the images before processing [121].

1.2.4 Xpert MTB/RIF

In 2010, the WHO convened an Expert Group on the use of the within-cartridge PCR-based assay Xpert MTB/RIF using the GeneXpert Instrument System (Cepheid Inc., Sunnyvale, CA, USA). Policy recommendation were issued in early 2011 and the assay has been labelled as a ground-breaking diagnostic device [122,123]. After policy updates WHO currently recommends Xpert MTB/RIF as a first diagnostic test in adults and children suspected of having MDR-TB or HIV-associated TB (strong recommendation). The assay Xpert MTB/RIF may be used as the initial diagnostic test in all adults or children suspected of having TB (conditional recommendation acknowledging resource implications). Furthermore, the test should be used as the initial diagnostic test for cerebrospinal fluid specimens from patients suspected of having TB meningitis and may be used as a replacement test for usual practice for testing specific non-

respiratory specimens (e.g. lymph nodes) from patients suspected of having extrapulmonary TB [124].

Xpert MTB/RIF makes use of the molecular beacon technology [125] to identify DNA sequences amplified in a hemi-nested real time-PCR to detect the presence of MTBC and simultaneously of rifampin resistance in clinical specimens in less than two hours. Five overlapping nucleic acid hybridization probes, labelled with coloured fluorophores, are used for binding to an 81-bp core region, the so called rifampicin-resistance determining region, of the wild-type *rpoB* gene [126,127]. More than 95% of mutations associated with rifampin resistance are found in this region [128]. The molecular beacons hybridize only correctly with amplified wild-type *rpoB* gene sequences. *M. tuberculosis* is detected when fluorophores of two or more *rpoB*-specific molecular beacons give a signal with cycle threshold (C_T) values that are less than 39 and when the C_T values are in the same range (±2) [129]. As a central outcome of analytic performance studies the limit of detection (LOD) was determined at 131 CFU/ml (95% CI 106–176) in spiked sputum samples [126]. In comparison, culture has a LOD of 10-50 CFU/ml and smear microscopy only of ~ 10'000 CFU/ml [130]. Due to a tuberculocidal sample treatment reagent and the closed cartridge system, Xpert MTB/RIF decreases the risk of infectious-aerosol formation compared to sputum smear preparation, which is a prerequisite for a near patient use in absence of biocontainment facilities [131].

Numerous evaluation studies generated evidence on the diagnostic accuracy of Xpert MTB/RIF. In adults, Xpert MTB/RIF has a pooled sensitivity of 89% and a specificity of 99% for pulmonary TB diagnosis compared to culture as reference standard [132]. In children, the pooled sensitivities and specificities of Xpert MTB/RIF for tuberculosis detection were 62% and 98%, respectively, with use of expectorated or induced sputum samples and 66% and 98% , respectively, with use of samples from gastric lavage [133]. As an aside: Xpert MTB/RIF has shown in two studies sensitivities of 80% and 58%, and specificities of 93.8% and 93% for the diagnosis of lymph node TB in children against combined reference standards [134,135]. The value of Xpert MTB/RIF for diagnosing paediatric TB in blood is very limited [136].

The fact that Xpert MTB/RIF cannot differentiate between live and dead mycobacteria is disqualifying the assay for treatment monitoring [137]. The instrument has only limited point-of-care capability because it requires constant electricity supply, controlled temperatures and protection from dust, heat and humidity [138]. Moreover, results from accuracy studies do usually not include test failures, because the analysis relies on a valid index and reference tests. Studies from routine implementation, however, report an overall test failure of 7.2% and 10.6%, respectively and a need for frequent replacement of modules [139,140].

The GeneXpert technology has paved the way for next-generation test, which are more sensitive (Xpert MTB/RIF Ultra) and can be used as real point-of-care under challenging conditions (GeneXpert Omni).

1.2.5 Immune assays

Principles of TB immunology. The protective response against *M. tuberculosis* involves a multidimensional network of innate and adaptive immune responses. After entering the lung, *M. tuberculosis* bacilli reach the alveolar space and are ingested by alveolar macrophages. Subsequently, after one week, the bacilli are released and phagocytised by dendritic cells, neutrophils, interstitial and blood monocytes, as part of early innate immune response [141]. After phagocytosis the mycobacteria survive intracellularly by blockage of phagolysosomal fusion, spread to other cells, and inhibit apoptosis of the host cell [142]. It is assumed that those mechanisms lead to a delayed adaptive immune response, which is usually initiated several weeks after infection. After ingestion of the *M. tuberculosis*, the dendritic cells transport the pathogen to local lymph nodes to present the pathogen to naïve CD4+ and CD8+ T-cells in order to prime a T-helper-1 (Th1) type response [143,144]. The activated, naive CD4+ T-cells have a T-cell receptor that can recognize the antigens presented by class II molecules of the major histocompatibility complex at the surface of the dendritic cell [145]. The cellular interaction triggers the expansion of the antigen-experienced CD4+ T-cell to produce clones of effector cells capable to rapidly migrate via the blood stream to the site of infection and release cytokines [146]. The Th1 cell-mediated release of the cytokines interferon gamma (IFN-γ) and TNF-α is essential for activation of macrophages, formation of granuloma and the containment of *M. tuberculosis* [147,148]. Interleukin (IL)-12 serves as a key cytokine of the Th1 response by favouring the production of IFN-γ. However, a broad and diverse cytokine profile is necessary to sufficiently confer protection against TB [149]. Moreover, growing evidence suggests that a Th1/Th2 [150], as well as a Th1/TH 17 [151] balance is critical to protecting against *M. tuberculosis* by controlling the progression to active disease. In line with this notion, IL-17 and IFN-γ expression in Th1/Th17 lymphocytes correlates with the severity of the TB disease [152]. If antigens are cleared, the majority of T-cell effector cells will undergo apoptosis. Yet, subsets of memory CD4+ T-cells will remain for future response [153]. If the exposure to antigens persists or reappears, memory CD4+ T-cells will be quickly stimulated to proliferate and mediate effector cell functions. Consequently, only a small proportion of memory T-cells but a large proportion of effector T-cells circulates in the blood of patients with high antigen load during acute TB disease [146,154]. Effector and memory CD4+ T-cells can be differentiated by flow cytometry through the differential expression of activation as CD27, CD38, and HLA-DR or proliferation biomarkers as KI-67, which can be used for the distinction between LTBI and active TB diseases [146,155].

In the course of infection, a granuloma, referred to as tubercle, is usually formed with to contain the bacilli and may pass through different stages (solid, necrotic, caseous, fibrotic caseous) [156]. Typically, granuloma are composed of CD4+ and CD8+ T-cells, B-lymphocytes, macrophages, epitheloid cells, Langhans giant cells, which are mainly harbouring the mycobacteria. The necrotic centre of the granuloma has low oxygen concentrations and offers, therefore, a hostile environment for the bacteria [157]. A fibrotic wall with lymphoid follicular structures forms the external layer of the granuloma. The granuloma can persist for many years containing the mycobacteria in a dormant state. However, failure to contain the infection can result in discharge of pathogens with active clinical disease and transmission [158].

Interferon-gamma release assays. Conventional TB immunoassays, so called Interferon-gamma release assays (IGRAs), measure the IFN-γ release of T-cells following stimulation by antigens specific to the MTBC, i.e. secreted antigenic target 6 (ESAT-6), culture filtrate protein 10 (CFP-10) and TB7.7(P4). The specificity of this kind of immunodiagnostic tools, such as the commercialized IGRAs QuantiFERON®-TB Gold-Test and T-SPOT.TB assays, for active TB disease is greatly compromised by pre-existing immunity [159]. Many individuals may have been previously exposed to *M. tuberculosis* but did not progress towards active TB disease. Pre-exposed asymptomatic individuals may either have successfully cleared the infection or controlled the infection and remained latently infected with non-replicating mycobacteria. In both cases, pre-exposure lead to the formation of a memory immune response that will lead to positive IGRA results in the absence of disease. Consequently, IGRAs indicate a sensitization to *M. tuberculosis* and cannot differentiate between asymptomatic exposure or "latent" and active TB in children or adults [160]. As a consequence, WHO does not recommend the use of IGRAs for the diagnosis of TB or the diagnostic work-up of presumptive TB patients (including HIV-positive individuals) in low- and middle-income countries [161].

T-cell activation marker assays. Specific biomarker signatures are needed to discriminate immune memory due to asymptomatic infection, not requiring treatment, from responses elicited by an active disease. In the context of active TB disease the bacterial load is constantly stimulating the immune system. As a consequence the proportion of effector T-cells in comparison to memory T-cells is substantially increased during active TB disease. This phenomenon has been confirmed independently in clinical studies performed in adults looking at different phenotypic T-cell biomarkers such as CD38, HLA-DR, KI-67 or CD27 [162–165]. The diagnostic phenotypic biomarkers are, in contrast to IFN-γ per se, specifically up- or down-regulated during active TB disease but not during latent infection [165]. This results in a high discriminatory power in identifying latent and active TB and in an unprecedented diagnostic accuracy of the so called the T-cell activation marker (TAM-TB) assay. The TAM-TB assay

measures the CD27 phenotype -a cell surface protein- of CD4 T-cells producing IFN-γ in response to *M. tuberculosis* antigens by a standard intracellular cytokine staining procedure.

1.2.6 Detection of volatile organic compounds

VOC detection assays. The detection of unstable low molecular weight metabolites, so called volatile organic compounds (VOC), appears to be an attractive diagnostic strategy for the detection of many diseases including TB [166]. Pathological processes seem to influence the characteristics of VOC emitted from an organism or specimen, creating a specific signature odour [167]. The 'electronic nose' (E-nose) is capable of recognizing simple as well as complex odours and mimics the unique biological olfactory system and comprises an array of electronic chemical sensors with a pattern recognition system [168]. The system produces a distinctive classification based on the mixture of volatile organic compounds of fluids or organisms detected in the headspace gas. The current versions of the electronic nose, however, cannot yet adequately differentiate TB and non-TB sputum or breath samples at diagnostic levels, when applied in clinical cohorts [169,170]. The recent identification of individual volatile biomarkers specific for active pulmonary TB by gas chromatography/mass spectroscopy [171,172] hold promise for the development of a simple point-of-care breath test without the requirement for specialized laboratory resources. The first breath test for VOCs exhaled in picomolar concentrations provides results within six minute with a sensitivity of 71.2%, and a specificity of 72% [173].

VOC detection by animals. Not only diagnostic instruments but also animals can reliably identify the smell-print of different diseases [174]. For example, trained rats are at least as sensitive as the conventional Ziehl-Neelsen stain for detecting *M. tuberculosis* in sputum; moreover, they are able to process over 40 times more samples per day than a lab clinician [175]. TB detection rats are trained for so-called remote scent tracing after operant conditioning. Rats are placed in a chamber with 10 holes. Containers with sputum are put below the holes, which are open and closed by a sliding lid. In brief, trained rats smell the headspace vapour of sputum samples and give an indicator response in case the specimen contains *M. tuberculosis* [176].

1.2.7 Symptom-based diagnosis and case definition in children

TB can affect essentially all organs. Thus, the spectrum of symptoms is broad and often unspecific. Active pulmonary TB in adults, for example, is characterised by some or all of the following symptoms: cough, haematemesis, loss of appetite, weight loss, fever, and night sweats. In children cough, weight loss or failure to thrive, fever, and lethargy or reduced playfulness are leading symptoms for intrathoratic TB.

In absence of accurate and affordable diagnostics, physicians frequently use symptom-based approaches for TB diagnosis and case definition, particularly for children in absence of a practical reference standard [177–179]. However, TB among children cannot be accurately diagnosed because clinical features and microbiological tests -alone or combined- do not precisely predict true active TB disease [38]. A small proportion of children can even have a confirmed, transient *M. tuberculosis* bacteraemia following primary infection without developing an active TB disease [180].

Clinical case definitions for the classification of pulmonary TB in children have been developed based on consensus between experts in paediatric TB. Currently, this classification is the best option for TB diagnosis and reporting of TB diagnostic evaluations in children. It includes microbiological, radiological and immunological evidence but also criteria describing clinical signs or symptoms suggestive of tuberculosis, exposure to TB and treatment response [38]. However, those case definitions are potentially biased by subjective options and can only be an estimation of the disease status.

1.3 Study aims

This doctoral thesis investigates four different diagnostic modalities for the diagnosis of TB. Two clinical accuracy studies were conducted among adult presumptive pulmonary TB cases. The other two accuracy studies focused on children suspected of having pulmonary TB or pulmonary and extrapulmonary TB, respectively. Each accuracy study used microbiologically well-defined reference standards and additional patient classifications based on clinical and radiological findings.

Manuscript 1: The first study had the aim to assess the diagnostic performances of **giant African pouched rats** in detecting *M. tuberculosis* from sputum samples of adult presumptive TB patients recruited in rural Tanzania in a per patient and per sample analysis. One objective was to define the optimal threshold for the number of detection rats and analyse the diagnostic performance for different patient sub-groups. In addition, the interference with non-tuberculous mycobacteria has been investigated.
Manuscript 2: The second study analysed the diagnostic accuracy of the of the **chest x-ray reading software CAD4TB** for the diagnosis of pulmonary TB among symptomatic adults presenting at a rural health care and research facility in Tanzania. The performance analysis of the software included patient subgroups, e.g. with regard to sputum smear and HIV infection status, and compared to human readers with different proficiency in radiology.
Manuscript 3: The third study examined in an East-African multi-centre trial the diagnostic capability of **Xpert MTB/RIF** to diagnose pulmonary TB in children from Tanzania and Uganda. The performance characteristics of Xpert MTB/RIF were compared to smear microscopy and smear culture including an analysis of the time to detection and detection rate.
Manuscript 4: The fourth evaluation was a proof-of-concept study to assess the diagnostic performance of the **TAM-TB assay** to identify children with active TB in two research centres in Tanzania. The findings were compared to those of Xpert MTB/RIF and culture. The study also comprised a cases series detailing clinical characteristics of children with discrepant culture and TAM-TB assay results.

Apart from the evaluation of the diagnostic performance of the four diagnostic tests, the thesis will also provide an analysis of the findings in the context of newly developed target product profiles for TB diagnostic by the WHO. This is followed by a critical reflection on the significance and limitations of diagnostic accuracy studies and a general discussion on the need of a coherent concept for the evaluation of TB diagnostics, spanning the whole spectrum of analytical assessments, diagnostic accuracy studies, and evaluations of health impact and cost-effectiveness analyses.

2 Manuscripts

2.1 Evaluation of giant African pouched rats for detection of pulmonary tuberculosis in patients from a high-endemic setting

Klaus Reither[1,2,3], Levan Jugheli[1,2,3], Tracy R. Glass[1,2], Mohamed Sasamalo[3], Francis A. Mhimbira[3], Bart J. Weetjens[4], Christophe Cox[4], Timothy L. Edwards[4,5], Christiaan Mulder[4,6], Negussie W. Beyene[4], Amanda Mahoney[4,5]

[1] Swiss Tropical and Public Health Institute, Basel, Switzerland

[2] University of Basel, Basel, Switzerland

[3] Ifakara Health Institute, Bagamoyo, Tanzania

[4] Anti-Persoonsmijnen Ontmijnende Product Ontwikkeling (APOPO), Morogoro, Tanzania

[5] Department of Psychology, Western Michigan University, Kalamazoo, Michigan, USA

[6] Amsterdam Institute for Global Health and Development, Amsterdam, The Netherlands

Publication: PLoS One. 2015 Oct 7;10(10):e0135877.

2.1.1 Abstract

Background:

This study established evidence about the diagnostic performance of trained giant African pouched rats for detecting *Mycobacterium tuberculosis* in sputum of well-characterised patients with presumptive tuberculosis (TB) in a high-burden setting.

Methods:

The TB detection rats were evaluated using sputum samples of patients with presumptive TB enrolled in two prospective cohort studies in Bagamoyo, Tanzania. The patients were characterised by sputum smear microscopy and culture, including subsequent antigen or molecular confirmation of *Mycobacterium tuberculosis*, and by clinical data at enrolment and for at least 5-months of follow-up to determine the reference standard. Seven trained giant African pouched rats were used for the detection of TB in the sputum samples after shipment to the APOPO project in Morogoro, Tanzania.

Results:

Of 469 eligible patients, 109 (23.2%) were culture-positive for *Mycobacterium tuberculosis* and 128 (27.3%) were non-TB controls with sustained recovery after 5 months without anti-TB treatment. The HIV prevalence was 46%. The area under the receiver operating characteristic curve of the seven rats for the detection of culture-positive pulmonary tuberculosis was 0.72 (95% CI 0.66-0.78). An optimal threshold could be defined at ≥2 indications by rats in either sample with a corresponding sensitivity of 56.9% (95% CI 47.0-66.3), specificity of 80.5% (95% CI 72.5-86.9), positive and negative predictive value of 71.3% (95% CI 60.6-80.5) and 68.7% (95% CI 60.6-76.0), and an accuracy for TB diagnosis of 69.6%. The diagnostic performance was negatively influenced by low burden of bacilli, and independent of the HIV status.

Conclusion:

Giant African pouched rats have potential for detection of tuberculosis in sputum samples. However, the diagnostic performance characteristics of TB detection rats do not currently meet the requirements for high-priority, rapid sputum-based TB diagnostics as defined by the World Health Organization.

Introduction

Tuberculosis (TB) is the world's second deadliest infectious disease, which killed 1.5 million people in 2013 - approximately one person every 25 seconds [181]. A key priority for TB control is the accurate and early diagnosis in persons with active and potentially infectious TB to enable timely treatment that both cures patients and decreases transmission risk. The development of novel, accurate, robust, and rapid diagnostic capabilities will result in improved case detection, disease surveillance, healthcare delivery, and quality of future research.

The superior olfactory characteristics of animals have been formerly used for diagnosis of a variety of diseases. Trained dogs are capable of identifying pulmonary carcinoma in breath samples [182] or intestinal infections in stool samples [183]. Rats have a highly developed sense of smell. The number of functional olfactory receptor genes is about 3 times larger in rats than in humans [184]. Previous investigations suggest that trained giant African pouched rats (*Cricetomys gambianus*) are able to detect and indicate the presence of *Mycobacterium tuberculosis (M.tb)* in sputum samples giant African pouched [176,185–190]. The rats presumably detect a combination of volatile organic compounds specific to *M. tuberculosis*, rather than a single molecule [12]. Consequently, TB detection rats have the potential to become an alternative or a supplement to sputum smear microscopy which is characterised by low sensitivity although being the only widely used TB diagnostic in resource-limited settings [92].

Previous studies on TB detection rats provided proof of concept for the test [176] and showed its value as a tool for rescreening of samples from microscopy centres (second-line screening) by increasing TB case detection after microscopy by 31.4%, 44%, and 42.8% in 2008, 2009, and 2010, respectively [190]. In those studies the TB detection rats' performance was assessed against smear microscopy as the reference standard. A recent study has compared the accuracy of 10 rats in 910 sputum samples with the correspondent outcome from culture on solid media and subsequent multiplex polymerase chain reaction for species differentiation. The per-patient analysis showed that the mean sensitivity of the 10 rats used in this experiment was 70.5%, while the mean specificity was 80.5% [189].

However, the diagnostic potential of TB detection rats in respiratory specimen has been so far only evaluated in sputum previously evaluated in Direct Observation Treatment Short-Course (DOTS) centres accompanied by no or scarce clinical data, no follow up information, and without blinding of the involved investigators. For that reason, we have conducted the first prospective evaluation study on giant African pouched rats to detect TB in adult patients with symptoms of pulmonary tuberculosis which includes both comprehensive clinical and microbiological data. Rigorous evidence-based evaluation of diagnostic tests is essential prior to any clinical practice to avoid unwanted clinical

consequences due to misleading results of test accuracy and to limit healthcare costs by preventing unnecessary testing or avoidable follow-up investigations [191,192].

2.1.2 Methods

Study population. Individuals with signs and symptoms suggestive of pulmonary TB were prospectively recruited in two cohort studies (TB Cohort and TB CHILD), and followed up for at least 5 months. The recruitment took place at the Ifakara Health Institute, Bagamoyo, United Republic of Tanzania, between the 22nd of September 2010 and 8th of March 2012. Bagamoyo, a coastal town of 35,000 inhabitants, is located approximately 70 km north of Tanzania's largest city Dar es Salaam. Tanzania is one of the 22 high-burden countries with 295 prevalent bacteriological confirmed pulmonary TB cases per 100,000 [193] and 37% HIV infection in patients with TB [181].

Patients were eligible for the study if they presented with persistent cough of two weeks or more and at least one of the following TB associated findings: haemoptysis, chest pain, fever, night sweats, constant fatigue, recent unexplained weight loss, loss of appetite, malaise, or contact with a known TB case. Patients who received anti-TB treatment during the past year, were severely sick from TB or another disease, or did not reside within the study area were excluded from the study.

A minimum necessary sample size of 403 presumptive TB patients was calculated for the diagnostic study [194], assuming a prevalence of disease of 20%, an expected sensitivity and specificity of 70% and 80%, respectively, and a target accuracy of ±5% (i.e. confidence interval width of 10%) plus a type I error probability of less than 5%.

Classification of patients. The participants were categorised into six groups based on clinical and microbiological assessments, as shown in Table 1. The allocation to the groups was not mutually exclusive, e.g. due to mixed infection of *M.tb* and non-tuberculous mycobacteria (NTM). For the purpose of this analysis, the classification into group A, B, D, or G supersedes classification to group C.

Study procedures and laboratory methods. Clinical procedures at enrolment comprised medical history, physical examination, voluntary HIV counselling and testing, and chest radiography. Chest radiographs were interpreted by a trained radiographer for immediate patient management.

Two sputum samples, one spot and one early morning, were routinely collected and used for acid-fast bacilli smear, culture examination, and TB detection by giant African pouched rats. Following NALC-NaOH decontamination, each sputum sample pellet was subjected to microscopy after Ziehl-Neelsen staining. Sputum smear were graded according to the

concentration of the bacilli [195]. Each sample was inoculated on both liquid (BACTEC MGIT 960, Becton Dickinson, USA) and solid Löwenstein-Jensen (LJ) culture media. Positive cultures were confirmed by microscopy for acid-fast bacilli and for the presence of *M. tuberculosis* by MPT64 antigen and/or molecular tests (Genotype MTBC, CM, or MTBDRplus, Hain Lifescience, Germany). Genotype CM or AS (Hain Lifescience, Germany) were used for detection of NTM. GenoType MTBDRplus or phenotypic drug-susceptibility testing (BACTEC MGIT 960 SIRE kit, Becton Dickinson, USA) was employed for resistance testing. All tests were performed blinded to clinical or radiological information by qualified laboratory technical personnel according to Good Clinical Laboratory Practice. Results from established diagnostic procedures were made available to support clinical management according to national and international guidelines.

If the volume of the specimen was appropriate (≥ 2ml), an aliquot of 1ml was collected from each unprocessed sputum samples in sterile cryovials before decontamination. The cryovials were stored within 4 hours at minus 20°C. The specimens were transferred under controlled temperature in one shipment to the APOPO laboratory in Morogoro, United Republic of Tanzania. After thawing, a sterile phosphate buffered saline was added and subsequent heat inactivation (90°C water bath for 30 min) was performed prior to detection by the giant African pouched rats.

TB rats - training and detection sessions. Seven giant African pouched rats were used in succession for the detection of TB in the sputum samples. The animals had been trained prior to this study using operant conditioning to pause for at least 5 seconds over TB-positive samples (indicator response) and have passed an internal accreditation process under blind conditions. Training methods, standardisation of performance, quality control and experimental setup have been detailed before [176,186,196].
Prior to the detection sessions, training sessions with 196 sputum samples of known classification from the Ifakara Health Institute cohorts were carried out to allow adaptation to potential influence by factors specific to the site and the materials used (e.g. sputum collection container).

In the detection sessions, the personnel involved in the experiment at the APOPO laboratory were blind to the clinical, radiological, and mycobacteriological information related to the sputum samples. The samples (one or two per patient) were presented in detection sessions among samples that were being evaluated for routine second-line screening operations between 10th July and 28th August 2012.

Statistical analysis and reference standard. In the main per-patient analysis, a TB-positive test result was defined as a positive indication by at least one rat of at least one sample of the

patient. In this analysis, diagnostic test performance (sensitivity, specificity, predictive values and likelihood ratios) was calculated only in the groups with defined TB status (reference standard): group A (s+/c+ *M.tb*), group B (s-/c+ *M.tb*), and group F (controls). The performance has been analysed individually for each of the rats. Additionally, the performance of all 7 rats combined was analysed using the sum of the number of rats indicating a TB-positive test result (range 0 to 7) to determine the best threshold; e.g. a patient was considered test-positive if two or more rats indicated either of the samples of the patient as positive. Moreover, the diagnostic test performance was also assessed in a per-sample analysis. In this analysis the reference standard was defined as presence or absence of *M.tb* culture-positivity in the corresponding culture sample.
Receiver operating characteristic (ROC) curves and the areas under the curve (AUC) were calculated. Proportions were compared using logistic regression models and chi-square test. The statistical analysis was performed using Stata v13 (Stata Corp., College Station, TX, USA).

The presented diagnostic evaluation study followed guidelines of the TDR Diagnostics Evaluation Expert Panel (DEEP) and The Standards for Reporting of Diagnostic Accuracy (STARD) steering committee for assessing the test performance and operational features of diagnostics for infectious diseases in the respective target population [197,198].

Ethical considerations. The study protocol and the consent procedure were approved by the Institutional Review Board of the Ifakara Health Institute and the Medical Research Coordinating Committee of Tanzania. Written informed consent was obtained from a literate patient. In case of illiteracy, informed oral consent was attested by an independent witness in accordance with Good Clinical Practice (GCP) guidelines [199]. In both cases, the informed consent was documented on a paper-based, dated, signed and/or thumb-printed consent form. The study was conducted in accordance with the Helsinki Declaration [200] and GCP guidelines [199].

2.1.3 Results

A total of 480 individuals with symptoms suggestive of TB were enrolled; six children and adolescents of less than 15 years of age, one patient who was classified as having exclusively extrapulmonary TB and four patients without rat results were excluded from analysis. The 469 eligible study participants were assigned to the classification groups as displayed in Table 2.

About half of the participants were female (49%) and the overall HIV-prevalence was 46%. The proportion of HIV-infected participants was statistically significant higher in group B (s-/c+ *M.tb*) compared to all other groups (chi-square 22.21, $p<0.001$). Details on patient characteristics and symptoms at recruitment are shown in Table 2.

***M.tb*: per patient analysis.** The analysis of the combined diagnostic capability showed that sensitivity decreased with increasing threshold (indication by ≥1 to 7 rats) and, as an opposing trend, specificity increased. At the lowest and highest thresholds, sensitivities and specificities amounted to 71.6% (95% CI 62.1-79.8), 59.4 (95% CI 50.3-68.0) and 18.4% (95% CI 11.6-26.9), 97.7% (95% CI 93.3-99.5), respectively (Table 3).
In the ROC calculation, the optimal diagnostic threshold (minimal square of distance between the upper left hand corner of ROC and any point on the ROC curve) could be defined as ≥2 indications by rats. Fig 5 shows the STARD flow diagram for this optimal threshold. The area under the ROC curve, which determines the inherent validity of the diagnostic approach, was 0.72 (95 CI% 0.66-0.78) for the detection of *M.tb* culture-positive individuals (Fig 6). For the optimal threshold, this translates to a sensitivity of 56.9% (95% CI 47.0-66.3), specificity of 80.5% (95% CI 72.5-86.9), positive (PPV) and negative predictive value (NPV) of 71.3% (95% CI 60.6-80.5) and 68.7% (95% CI 60.6-76.0), and an accuracy for TB diagnosis of 69.6%.

The area under the ROC curve differed statistically significant for detection of smear-positive (p=0.016), *M.tb* culture-positive individuals and smear-negative, *M.tb* culture-positive individuals (0.78 vs. 0.56; Fig 7). For the optimal threshold, this translates to a sensitivities of 65.0% (95% CI 53.5-75.3) and 34.5% (95% CI 17.9-54.3) and specificities of 80.5% (95% CI 72.5-86.9) and 80.5% (95% CI 72.5-86.9) for the detection of smear-positive and smear-negative TB, respectively.

One sputum sample was collected from 122 (26%) patients and two samples were obtained from 347 (74%) patients. All available samples were tested by TB detection rats. The area under the ROC curve (graph not shown) did not differ if the rats tested only the first or both available samples (0.74 vs. 0.76).

There was no statistically significant difference in the performance of TB detection rats comparing the area under the ROC curves stratified by HIV status for group A (s+/c+ *M.tb*) and B (s-/c+ *M.tb*) and group F (control) as reference standards (chi-square 0.3466, p-value= 0.557).

Association analyses were performed under the assumption that ≥2 indications by rats represent the optimal threshold for TB detection. No statistically significant association was found between TB detection by rats and age, TB history, or having a cough, hemoptysis, sweating, or weight loss at recruitment. Having a fever at the time of recruitment was significantly associated with TB detection by rats (p=0.003).

The diagnostic performance differed considerably between the individual rats, e.g. a statistically significant higher sensitivity was achieved by rat no.6 (57.8%; 95% CI 48.0%-67.2%) compared

to rats no.2 and no.7 (31.2%; 95% CI 22.7%-40.8%). The specificity for six of the seven rats was above 80% (Table 3).

***M.tb*: per sample analysis.** In a per-sample analysis (n=819), using presence or absence of *M.tb* growth in the culture of the same sample as reference standard, the indicators of test performance, in particular the sensitivity, were overall poor. Depending on the threshold, the sensitivity ranged from 45.8% (95% CI 40.3-51.4) to 5.9% (95% CI 3.6-9.0) and specificity from 73.8 (95% CI 69.7-77.7) to 99.0 (95% CI 97.6-99.7). The area under the ROC curve for the per-sample analysis was 0.61.

A per-sample sub-analysis in group A (s+/c+ *M.tb*; n=134) showed a statistically significant association between grading of sputum smear and TB detection by rats with higher AFB results having more TB detection (chi-square 17.31, p=0.002).

NTM: sub-analysis

Group C (s ±/c+ NTM) encompassed patients with exclusively NTM in the sputum sample irrespective of a potential clinical relevance. In this group, 21 of 84 patients (25%) had a positive rat result (at least one sample with ≥2 indications). The per-sample analysis exhibited that 19.6% of the samples with concurrent identification of one or more NTM were rat-positive using the threshold of ≥2 indications. The proportion of rat-positive samples in the samples with the identified NTMs is displayed in Table 4.

2.1.4 Discussion

Giant African pouched rats can detect *M. tuberculosis* in clinical sputum samples with a fair sensitivity (56.9%) and moderate specificity (80.5%) compared to culture as reference standard if an indicator response from two or more out of seven rats is treated as a positive diagnostic indicator.

This is the first evaluation of the diagnostic performance of TB detection rats which includes microbiological investigations with liquid and solid culture, subsequent differentiation of species, combined with a comprehensive clinical data set from each patient. Solid culture (LJ) has been used as reference standard in previous studies on TB detection rats either without [176] or with identification by subsequent multiplex real-time PCR [175,189]. However, detailed clinical data were not available in any of those studies. Dissimilarities in study design impede the direct comparison between the presented and former studies. In one study with culture and multiplex PCR using 10 instead of 7 rats [189], the per-patient sensitivity was higher (81.9% vs. 56.9%) and the specificity was lower (64.5% vs. 80.5%) at a diagnostic threshold of ≥2 indications. The difference in diagnostic accuracy at the defined threshold is most probably influenced by the

different total number of rats used, resulting in a trade-off in sensitivity and a gain of specificity at the threshold of ≥2 in the presented compared to the previous study.

The presented data suggest that the performance of the TB detection rats depends on the bacterial load in sputum samples. TB was detected with a higher sensitivity in smear positive compared to smear negative, culture-positive TB patients. Moreover, the detection was significantly better in sputum samples with higher microscopy grade, a surrogate for high concentration of bacilli. In general, the diagnostic performance was equally good if only one or two samples were used for detection.

Interestingly, the sensitivity and specificity for detecting TB did not differ significantly between HIV-infected and HIV-uninfected patients, although in general sputa of HIV-infected patients have a lower burden of TB bacilli [201,202]. Further HIV-related differences in symbiotic or pathogenic respiratory and oral microorganisms [203–205] with different bouquets of volatiles, seem to have no impact on diagnostic performance of the giant African pouched rats.

The seven rats did not perform with the same diagnostic accuracy: specificity and particularly sensitivity varied considerably between the individual rats. The findings contrast those of preceding studies [176,185] which report consistent performance characteristics for each rat. Tentatively, different levels of operant conditioning training, but also other influences, e.g. related to the patient group, might be accountable for the observed variability.

In previous studies, the rats detected TB in the same sputum containers which were earlier also used for collection at the microscopy centres. Yet, in the presented study the specimens have been transferred to cryovials before frozen, thawed and subsequently evaluated by the rats. It remains speculative, if the background odour of the container used for sputum collection has any potential effect on the diagnostic performance of the rats. According to unpublished data, the intermediate freezing of specimens does not seem to have an influence on the diagnostic performance of the TB detection rats [189].

The odour of *M.tb* does not consist of a single compound but rather a combination of volatiles which is characteristic for *Mt.b* ('smellprint') and does, with regard to many volatiles, not overlap with compounds of non-tuberculous mycobacteria or other pathogenic and apathogenic microorganisms of the respiratory tract [190,206,207]. In approximately 80% of the samples with NTM only, the rats did not indicate for TB in the presented clinical evaluation. The findings suggest that trained giant African pouched rats can discriminate *M.tb*-specific volatile compounds from non-tuberculous odours to a certain degree. This has been also similarly

demonstrated in comprehensive experiments on cultured microbes and clinical sputa from microscopy centres [175,188].

Since one rat can screen 140 sputum samples in 40 minutes [176], large-volume second-line screening of sputum samples from microscopy centres in combination with a confirmatory test with high specificity could be a potential cost-effective application of detection rats technology. The high sample throughput and low cost make TB detection rats also a possible technology for active TB case finding in populations at high risk for TB, such as prison populations. However, the sensitivity would need to be further optimised, before TB detection rats can be used systematically for TB screening [208].

A limitation of the study is that the giant African pouched rats were primarily trained to perform an operant discrimination on high-intensity stimuli (high concentration of bacilli). This could explain the tendency towards superior detection in this kind of specimen. At the time of this publication, rats are now primarily trained sputum samples with low concentration of bacilli to improve performance with low concentration samples.

The objectives of this study were limited to the assessment of test accuracy in adult individuals with presumptive TB. Operational issues regarding a routine implementation of the technology on microscopy level and a potential scale up to different settings or countries with high burden of TB have not been addressed. In future, these research questions will become increasingly relevant, because until now breeding, training and diagnostic performance of TB detection rats has been only managed by APOPO, a non-governmental organisation using rats for TB detection in Tanzania and Mozambique.

In summary, giant African pouched rats have the potential for detection of tuberculosis in sputum samples. However, the diagnostic performance characteristics in this clinical evaluation were less favourable than those reported in previous diagnostic studies in less- or non-characterised patients [175,176,187,189].

Based on current evidence, rat technology as a standalone diagnostic test, does not fulfil the criteria for a rapid sputum-based test for detecting TB at the microscopy-centre level of the health-care system as it has been defined by the World Health Organization in 2014 at a stakeholder meeting on high-priority target product profiles [68,209].

Future efforts in research and development should be used to further improve the rat technology, to explore prospects for implementation and scale up and to determine its potential position in TB diagnostic or screening algorithms.

Acknowledgements

We thank all participants of the TB Cohort and TB CHILD projects. The TB Cohort project was supported by the Rudolf Geigy Foundation, Switzerland. The TB CHILD project was funded by the European and Developing Countries Clinical Trials Partnership (EDCTP) as part of project "Evaluation of new and emerging diagnostics for childhood tuberculosis in high burden countries" [IP.2009.32040.007]. The APOPO project was partially funded by UBS Optimus Foundation.

Competing interests

The authors have declared that no competing interests exist. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Funding

The TB Cohort project was supported by the Rudolf Geigy Foundation, Switzerland (http:// www.geigystiftung.ch/de/die-stiftung.html). The TB CHILD project was funded by the European and Developing Countries Clinical Trials Partnership (EDCTP) as part of project " Evaluation of new and emerging diagnostics for childhood tuberculosis in high burden countries " [IP.2009.32040.007] (http:// www.edctp.org/annualreport2010/grants_2010/lwilla.html). The APOPO project was partially funded by UBS Optimus Foundation (https://www.ubs.com/ global/en/wealth_management/optimusfoundation/ support/all-projects.html). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript

2.1.5 Figures

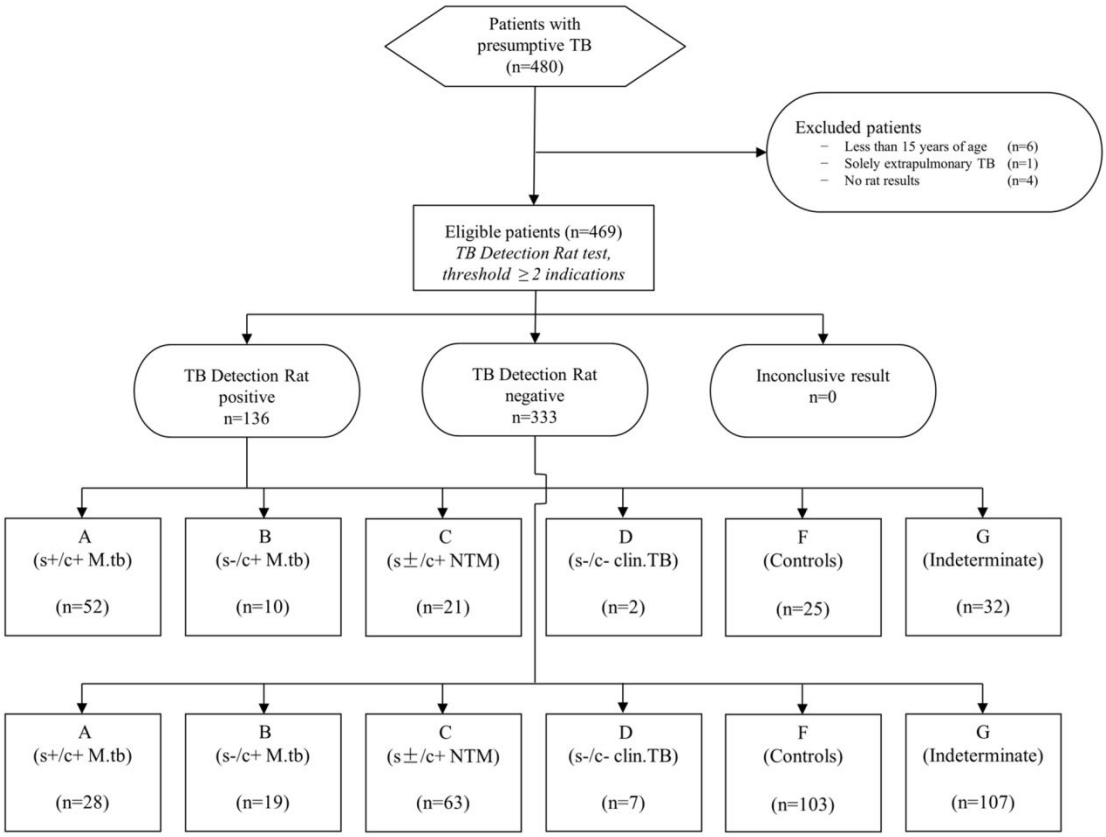


Figure 5. STARD flow chart for diagnostic threshold of ≥2 indications by rats.

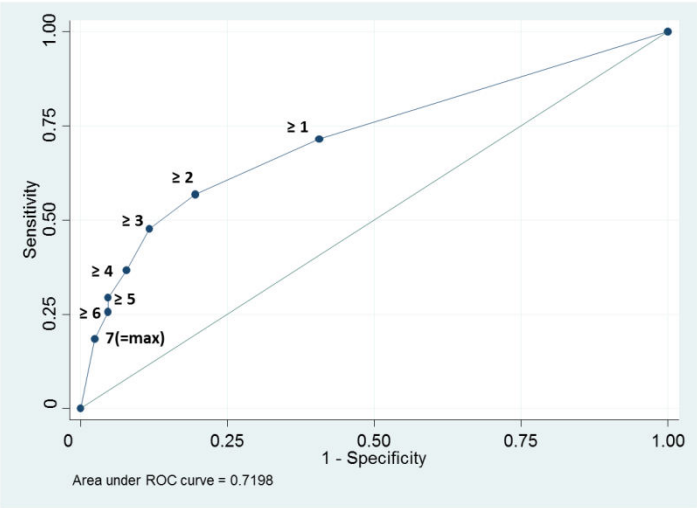


Figure 6. ROC analysis for the detection of *M.tb* culture-positive individuals for different indication thresholds (per-patient analysis; all samples). (Standard error 0.03, 95 CI% 0.66-0.78)

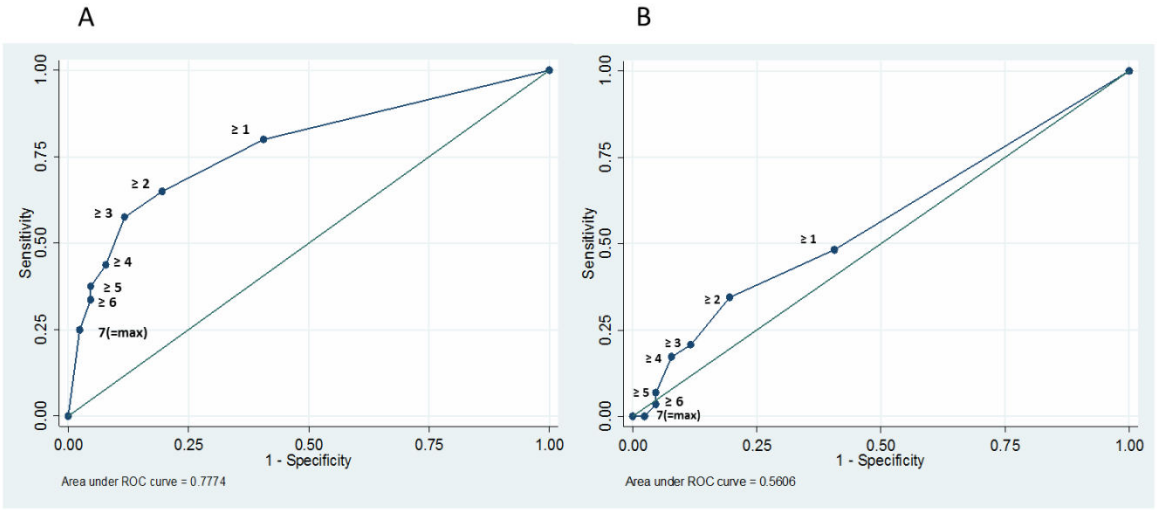


Figure 7. ROC analysis for the detection of smear-positive (A) versus smear negative (B) *M.tb* culture-positive individuals for indication thresholds (per-patient analysis; all samples). (A: Standard error 0.03, 95 CI% 0.71-0.84; B: Standard error 0.06, 95 CI% 0.45-0.67)

2.1.6 Tables

Table 1: Patient classification

Group	Description	Short name
A	Culture-positive, smear-positive, *M. tuberculosis;*	s+/c+ *M.tb*
B	Culture-positive, smear-negative, *M. tuberculosis*	s-/c+ *M.tb*
C	Culture-positive, non-tuberculous mycobacteria	s±/c+ NTM
D	Culture-negative pulmonary TB; strong clinical and radiological suspicion	s-/c- clin.TB
F	Smear-/culture-negative, sustained recovery up to 5th month	Controls
G	Loss to follow-up after recruitment or any other combination	Indeterminate

Table 2: Patient characteristics and symptoms at recruitment

	Missing data	All	Group A	Group B	Group C	Group D	Group F	Group G
			Culture-positive, smear-positive, *M. tuberculosis*	Smear-negative, culture-positive, *M. tuberculosis*	Culture-positive, non-tuberculous mycobacteria	Culture-negative pulmonary TB (strong clinical and radiological suspicion)	Controls, all smear- / culture-negative, sustained recovery up to 5th month	Loss to follow-up after recruitment or any other combination
Characteristics								
n (%)		469	80 (17.1)	29 (6.2)	84 (17.8)	9 (1.9)	128 (27.3)	139 (29.4)
Age Mean (SD)	0	41.6 (15.4)	36.8 (12.3)	42.3 (12.7)	43.2 (15.4)	44.9 (10.9)	42.3 (16.3)	42.3 (16.7)
Age Median (IQR)	0	38 (30-50)	35 (29-41)	39 (35-43)	42 (30-53.5)	45 (38-51)	39 (29.5-54.5)	39 (30-50)
Female sex - n (%)	1	231 (49.2)	25 (31.3)	16 (55.2)	51 (60.7)	5 (55.6)	64 (50.0)	70 (50.7)
Body mass index (kg/m^2) Median (IQR)	2	19.7 (17.7-22)	18.6 (16.9-20)	18.9 (17.4-21.8)	19.8 (17.7-22.4)	20.2 (16.1-22.2)	20.6 (18.2-23.3)	19.4 (17.6-21.7)
HIV positive - n (%)	31	203 (46.4)	26 (34.7)	24 (82.8)	40 (51.3)	3 (37.5)	48 (40.7)	62 (47.7)
History of TB - n (%)	1	74 (15.8)	12 (15.0)	2 (6.9)	16 (19.1)	4 (44.4)	13 (10.2)	27 (19.6)
Symptoms at recruitment								
Cough ≥ 2 weeks - n (%)	1	442 (94.4)	74 (92.5)	26 (89.7)	80 (95.2)	9 (100)	124 (96.9)	129 (93.5)
Night sweats - n (%)	1	217 (46.4)	46 (57.5)	12 (41.4)	37 (44.1)	7 (77.8)	48 (37.5)	67 (48.6)
Haemoptysis - n (%)	9	52 (11.3)	8 (10.0)	1 (3.5)	11 (13.6)	1 (11.1)	14 (11.3)	17 (12.4)
Fever - n (%)	1	241 (51.5)	49 (61.3)	18 (62.1)	44 (52.4)	5 (55.6)	60 (46.9)	65 (47.1)
Weight loss - n (%)	4	229 (49.3)	50 (62.5)	19 (65.5)	35 (41.7)	3 (33.3)	56 (43.8)	66 (48.9)

Table 3: Diagnostic test performance of TB detection rats (per-patient analysis; all samples)

Culture-positive TB cases (Group A&B) versus Controls (Group F)	Threshold (Number of rats indicating TB)	Sensitivity	Specificity	Positive Predictive Value	Negative Predictive Value	Positive Likelihood Ratio	Negative Likelihood Ratio
N=237		% (95% CI)	% (95% CI)	% (95% CI)	% (95% CI)	% (95% CI)	% (95% CI)
Rat 1		42.2 (32.8-52.0)	89.8 (83.3-94.5)	78.0 (65.3-87.7)	64.6 (57.1-71.6)	4.16 (2.37-7.28)	0.64 (0.54-0.76)
Rat 2		31.2 (22.7-40.8)	93.0 (87.1-96.7)	79.1 (64.0-90.0)	61.3 (54.1-68.2)	4.44 (2.23-8.83)	0.74 (0.65-0.85)
Rat 3		42.2 (32.8-52.0)	83.6 (76.0-89.5)	68.7 (56.2-79.4)	62.9 (55.2-70.2)	2.57 (1.64-4.03)	0.69 (0.58-0.83)
Rat 4		42.2 (32.8-52.0)	87.5 (80.5-92.7)	74.2 (61.5-84.5)	64.0 (56.4-71.1)	3.38 (2.03-5.61)	0.66 (0.56-0.79)
Rat 5		39.4 (30.2-49.3)	85.9 (78.7-91.4)	70.5 (57.4-81.5)	62.5 (54.9-69.7)	2.81 (1.72-4.57)	0.70 (0.60-0.83)
Rat 6		57.8 (48.0-67.2)	78.9 (70.8-85.6)	70.0 (59.4-79.2)	68.7 (60.5-76.1)	2.74 (1.89-3.97)	0.53 (0.42-0.68)
Rat 7		31.2 (22.7-40.8)	89.8 (83.3-94.5)	72.3 (57.4-84.4)	60.5 (53.2-67.5)	3.07 (1.71-5.52)	0.77 (0.67-0.88)
Mean		40.9 (37.5-44.3)	86.9 (85.2-88.7)	73.3 (71.7-74.8)	63.5 (62.0-64.5)	3.31 (3.03-3.59)	0.68 (0.65-0.71)
	≥1	71.6 (62.1-79.8)	59.4 (50.3-68.0)	60.0 (51.0-68.5)	71.0 (61.5-79.4)	1.76 (1.38-2.24)	0.48 (0.34-0.67)
	≥2	56.9 (47.0-66.3)	80.5 (72.5-86.9)	71.3 (60.6-80.5)	68.7 (60.6-76.0)	2.91 (1.98-4.29)	0.54 (0.42-0.68)
	≥3	47.7 (38.1-57.5)	88.3 (81.4-93.3)	77.6 (65.8-86.9)	66.5 (58.8-73.5)	4.07 (2.43-6.81)	0.59 (0.49-0.72)
All rats combined	**≥4**	36.7 (27.7-46.5)	92.2 (86.1-96.2)	80.0 (66.3-90.0)	63.1 (55.8-70.0)	4.70 (2.47-8.95)	0.69 (0.59-0.80)
	≥5	29.4 (21.0-38.8)	95.3 (90.1-98.3)	84.2 (68.7-94.0)	61.3 (54.2-68.1)	6.26 (2.72-14.42)	0.74 (0.65-0.84)
	≥6	25.7(17.8-34.9)	95.3 (90.1-98.3)	82.4 (65.5-93.2)	60.1 (53.0-66.9)	5.48 (2.36-12.74)	0.78 (0.69-0.88)
	7	18.4 (11.6-26.9)	97.7 (93.3-99.5)	87.0 (66.4-97.2)	58.4 (51.5-65.1)	7.83 (2.39-25.64)	0.84 (0.76-0.92)

CI= Confidence interval

Table 4: Rat-positive samples (two or more indications) in group C (s ±/c+ NTM) with concurrent molecular test result (per-sample analysis)

Molecular differentiation of non-tuberculous mycobacteria	≥2 rat indications
M. abscessus/M. immunogenum	0/1
M. asiaticum	3/10
M. avium	0/2
M. celatum	0/2
M. fortuitum 1	2/13
M. fortuitum 2/M. mageritense	1/12
M. godii	0/1
M. gordonae	1/2
M. intracellulare	4/15
M. malmoense	0/1
M. malmoense/M.haemophilum/M. patustre	0/2
M. scrofulaceum	1/7
M. scrofulaceum and M.celatum	0/1
M. simiae	2/5
M. smegmatis	0/2
M. szulgai/M.intermedium	1/1
High GC Gram positive bacterium	2/7
Mycobacterium species*	1/16

**Species could not be determined by Hain GenoType Mycobacterium CM/AS*

2.2 Diagnostic accuracy of computer-aided detection of pulmonary tuberculosis in chest radiographs: a validation study from sub-Saharan Africa

Marianne Breuninger[1,2,3], Bram van Ginneken[4], Rick HHM Philipsen[4], Francis Mhimbira[2], Jerry J Hella[2], Fred Lwilla[2], Jan van den Hombergh[5], Amanda Ross[1,6], Levan Jugheli[1,2,6], Dirk Wagner[3], **Klaus Reither**[1,2,6]

[1] Swiss Tropical and Public Health Institute, Basel, Switzerland

[2] Ifakara Health Institute, Bagamoyo, United Republic of Tanzania

[3] Center for Infectious Diseases and Travel Medicine, University Hospital Freiburg, Freiburg, Germany

[4] Diagnostic Image Analysis Group, Radboud University Medical Center, Nijmegen, The Netherlands

[5] PharmAccess International, Dar es Salaam, United Republic of Tanzania

[6] University of Basel, Basel, Switzerland

Publication: PLoS One. 2014 Sep 5;9(9):e106381.

2.2.1 Abstract

Background:

Chest radiography to diagnose and screen for pulmonary tuberculosis has limitations, especially due to inter-reader variability. Automating the interpretation has the potential to overcome this drawback and to deliver objective and reproducible results. The CAD4TB software is a computer-aided detection system that has shown promising preliminary findings. Evaluation studies in different settings are needed to assess diagnostic accuracy and practicability of use.

Methods:

CAD4TB was evaluated on chest radiographs of patients with symptoms suggestive of pulmonary tuberculosis enrolled in two cohort studies in Tanzania. All patients were characterized by sputum smear microscopy and culture including subsequent antigen or molecular confirmation of *Mycobacterium tuberculosis* (*M.tb*) to determine the reference standard. Chest radiographs were read by the software and two human readers, one expert reader and one clinical officer. The sensitivity and specificity of CAD4TB was depicted using receiver operating characteristic (ROC) curves, the area under the curve calculated and the performance of the software compared to the results of human readers.

Results:

Of 861 study participants, 194 (23%) were culture-positive for *M.tb*. The area under the ROC curve of CAD4TB for the detection of culture-positive pulmonary tuberculosis was 0.84 (95% CI 0.80-0.88). CAD4TB was significantly more accurate for the discrimination of smear-positive cases against non TB patients than for smear-negative cases (p-value<0.01). It differentiated better between TB cases and non TB patients among HIV-negative compared to HIV-positive individuals (p<0.01). CAD4TB significantly outperformed the clinical officer, but did not reach the accuracy of the expert reader (p=0.02), for a tuberculosis specific reading threshold.

Conclusion:

CAD4TB accurately distinguished between the chest radiographs of culture-positive TB cases and controls. Further studies on cost-effectiveness, operational and ethical aspects should determine its place in diagnostic and screening algorithms.

2.2.2 Introduction

The role and potential of chest radiography as a diagnostic and screening tool for pulmonary tuberculosis (PTB) has long been debated. As a rapid examination that can be interpreted on-site with a high sensitivity (between 74 and 90% for PTB related abnormalities, up to 97%, if any abnormality is considered [112,210–212]), it has a firm place in the diagnosis of pulmonary tuberculosis. However, the lower specificity, a lack of consistency in how results are reported, and high levels of inter- and intra-reader variability are matters of concern. Interpreting chest radiographs is complex and subjective: it is a two dimensional representation of a three-dimensional structure, and there are varied manifestations of PTB. The complexity of the interpretation code and the structure of the report form affect the result [213,214]. Different readers are also influenced by experience and professional training [116,215] and momentary factors like distraction, focus and tiredness.

In contrast, the automated reading of radiographs by computers is devoid of inter- and intra-observer variability. Research in this field started fifty years ago. Although early optimistic goals such as "fully automating the chest exam" [216] are still far from being achieved, at least one application, the automatic detection of masses and micro-calcifications in mammograms, has been successfully integrated in clinical routine to support radiologists in their decision [217].

Most of the research on computer-aided diagnosis (CAD) of chest radiographs focuses on the detection of nodules, but there are a number of research groups also working on promoting CAD in PTB. Among these, the Diagnostic Image Analysis Group at Radboud University Medical Center, Nijmegen, The Netherlands introduced CAD4TB, a software to determine whether a chest X-ray (CXR) shows evidence of PTB. CAD4TB underwent field tests in 2010 and has been developed since then. Previous software versions were comparable to clinical officers for detecting culture confirmed tuberculosis (TB) among 166 presumptive TB patients at a Zambian clinic (v1.08; area (Az) under the receiver operating characteristic (ROC) curve = 0.73) [218] and reached a sensitivity of 95% at a specificity of 57% in 95 CXRs of homeless people in London (texture abnormality detection system; Az= 0.86) [219].

A recently published review article on automatic screening for tuberculosis in chest radiographs by Jaeger and colleagues [220] concludes that even though proposed CAD algorithms seem to perform reasonably well when tested individually, no fair comparison can be made without testing the systems on the same, preferably large and publicly available dataset of well characterized patients. The authors further emphasise that there are hardly any validation studies from clinical or screening situations so far and therefore a lack of evidence on how the systems perform in the practical field.

We conducted the first validation study to assess the diagnostic accuracy of the most recent CAD4TB software (v3.07, updated release) on a large set of well characterized adult presumptive PTB patients from sub-Saharan Africa. We compared the performance of the automated reading with the results of human observers of different experience levels.

2.2.3 Methods

Study Population. This validation study was done on chest radiographs of participants from two cohort studies (TB Cohort and TB CHILD study) which have been conducted at the TB Clinic of the Ifakara Health Institute (IHI) in Bagamoyo, Tanzania. Tanzania has a high burden of active TB: according to the first national Tuberculosis Prevalence Survey in 2013 the prevalence is 295 cases per 100,000 population [193]. Bagamoyo, a town of 35,000 inhabitants, is located on the coast, approximately 70 km from the commercial capital Dar es Salaam.

Individuals presenting with clinical signs and symptoms suggestive of pulmonary TB to surrounding primary health care facilities were referred to the IHI TB Clinic. Patients who met the inclusion criteria and gave informed consent were consecutively enrolled into either the TB Cohort or TB CHILD study. In both studies the patients were followed up for 5-18 months. The main objective of the TB Cohort study was to generate a sound understanding of TB epidemiology in the Bagamoyo region while the TB CHILD study was conducted to assess performance characteristics of new TB diagnostics in adults and children. Written informed consent was obtained from all literate patients. In case of illiteracy, informed oral consent was attested by an impartial witness and documented with the patient's fingerprint according to ICH GCP guidelines, as approved by the IHI Institutional Review Board and the Medical Research Coordinating Committee of the National Institute for Medical Research, Tanzania. Patients who received anti TB treatment

during the last year, were severely sick or did not reside within the study area were not included. All adult patients from both studies were eligible for the CAD validation study if they initially presented with persistent cough of 2 weeks or more and at least one of the following TB associated findings: haemoptysis, chest pain, fever, night sweats, constant fatigue, recent unexplained weight loss, loss of appetite, malaise or contact with a known TB case.

Specimen collection & Laboratory methods. At enrolment, the participants answered a detailed questionnaire about their medical history and underwent a clinical examination, had a chest radiograph taken and sputum and blood samples were collected. All CXRs (resolution: 1760 x 2140 pixel) were taken with a Philips Cosmos BS radiography system, which operated combined with a Philips PCR System Eleva S processor. Two sputum specimens, one 'spot' and one early morning, were routinely obtained and used for acid-fast bacilli (AFB) smear and culture examination. All samples were decontaminated using the standard NALC-NaOH method, inoculated on both solid (Löwenstein-Jensen, LJ) and liquid (Mycobacterium Growth Identification Tube, MGIT) media and incubated at 37°C. Smears were performed from the decontaminated pellet, followed with Ziehl-Neelsen (ZN) staining. All positive cultures were tested by ZN microscopy for the presence of AFB, and *Mycobacterium tuberculosis* (*M.tb*) was confirmed by MPT64 antigen and/or molecular tests (Genotype MTBC, CM or AS; Hain Lifescience, Nehren). Interpretation of all microbiological tests was carried out blind to clinical information and radiological results. Voluntary HIV counselling and testing was offered to all participants. The laboratory work was carried out according to Good Clinical Laboratory Practice to guarantee objective standards, quality control and assurance.

Classification. All patients were classified by the study physicians (M.D., 1-3 years of clinical experience) in consultation with a senior physician (M.D., 20 years of clinical experience) into seven groups (table 5) according to all clinical and microbiological information available 5 months after enrolment. Allocation to the groups was not mutually exclusive. For the purpose of this analysis, it was agreed that classification to either group A (s+/c+ *M.tb*) or B (s-/c+ *M.tb*) supersedes classification to C (s ±/c+ NTM) or E (EPTB), and classification to either group G (Indeterminate) or D (s-/c- clin.TB) supersedes classification to group C (s ±/c+ NTM). Patients with resolved symptoms after 5 months, and who were

confirmed to be definitely free of TB (group F) will be referred to as 'Controls' in the following.

Reading of the chest radiographs. The computer-aided analysis of the CXRs was performed independently and blind to clinical information and radiological results, by the Diagnostic Image Analysis Group at Radboud University Medical Center, Nijmegen, The Netherlands. The images were processed with the latest CAD4TB software version (v3.07, updated release). CAD4TB is a software framework in which various subsystems for the detection of textural and shape abnormalities, for symmetry and correlation analyses operate at pixel and image level [221].

In CAD, the analysis is broken down to several computable steps [222]: First, radiographs are pre-processed to normalise image features like resolution and grey scale. During segmentation, the next step, the software seeks the anatomical orientation of the image by demarcating structures like the lungs, clavicles and ribs. The defined lung fields are then analysed for their shape, global symmetry and local texture. In addition, a global correlation with a typical normal CXR is determined. Scores generated by these subsystems are combined to an overall score for each image which summarises the result of the automated analysis as an abnormality score for the presence of active disease between 0–100.

In addition, the same set of images was read by two human observers: one experienced chest physician as expert reader and one clinical officer who had practical experience in reading chest X-ray exams in his role as District Tuberculosis and Leprosy Coordinator and had completed a one week course on "X-ray interpretation of tuberculosis and HIV-related opportunistic infections among people living with HIV" [223]. The two readers rated the images using the 'Tanzanian X-ray score', a template for a structured CXR report. At the end of their report, the readers were asked to choose between four possible conclusions:

1. normal.
2. abnormal, findings not suggestive for active TB (TB sequel possible).
3. abnormal, findings consistent with active TB, but TB sequel or other lung pathology possible.
4. abnormal, findings highly suggestive for active TB.

Three different reading thresholds were defined correspondingly, ranging from considering only 'abnormalities highly suggestive for TB' (conclusion 4) to 'TB consistent abnormalities' (conclusion 3+4) to 'any abnormality' (conclusion 2-4) for a positive test result.

The readings of chest radiographs were carried out retrospectively for both, the automated and the human interpretation, and had no influence on the diagnosis of the study participants. The human readers were only aware of the inclusion criteria of the study and the age of the patients, but were blinded to clinical information, bacteriological results as well as each other's results.

Data analysis. Culture-confirmed *M.tb* was used as a reference standard to assess the diagnostic accuracy of CAD4TB and the human readers for the diagnosis of PTB. Individuals whose state of disease could be definitely determined were included in the analyses: group A (s+/c+ *M.tb*) and B (s-/c+ *M.tb*) as true cases and group F (Controls) as definite non TB patients. Secondary performance analyses were carried out in which individuals of group C (s ±/c+ NTM) and E (EPTB) were considered additionally to group F (Controls) to be most likely free of pulmonary TB. Individuals of group D (s-/c- clin.TB) were classified partly due to an abnormal X-ray and were excluded from the analysis.

Receiver operating characteristic (ROC) curves and the areas under the curve (Az) were calculated based on the output of the software. Their 95% confidence intervals (CI) and p-values were computed using the De Long method [224]. The performance of the human readers was summarized by calculating sensitivities, specificities, positive and negative predictive values as well as diagnostic likelihood ratios and their 95% confidence intervals for reporting 'abnormalities highly suggestive for TB' (conclusion 4), 'TB consistent abnormalities' (conclusion 3+4) or 'any abnormality' (conclusion 2-4). The same performance measures were calculated for several exemplary cut-offs of the CAD4TB software. Proportions in different groups were compared using the chi-squared test. McNemar's test was applied to compare the specificity of CAD and humans at assumed levels of sensitivity. Mann-Whitney-Wilcoxon test was used to compare the CAD scores between different groups. All calculations were done using the statistical package 'R', version 3.0.0 [225] together with the extension packages 'pROC' [226], 'epiR' [227], 'ggplot2' [228], 'reshape2' [229] and 'plotrix' [230]. All data used for the analyses is

deposited in a public repository and can be accessed via http://dx.doi.org/10.6084/m9.figshare.936571.

Ethical considerations. The TB Cohort and TB CHILD study were approved by the IHI Institutional Review Board and the Medical Research Coordinating Committee of the National Institute for Medical Research, Tanzania.

2.2.4 Results

A total of 894 patients were enrolled in the CAD study. Thirty-three patients had to be excluded from analysis because of an incomplete enrolment visit, pregnancy or missing chest radiograph (figure 8). The final set of images for analysis consisted of 861 digital, posterior-anterior (PA) chest radiographs. Six of these radiographs were originally in a conventional film format and later digitized.

Group A (s+/c+ *M.tb*) and B (s-/c+ *M.tb*) included 194 (23%) of the study participants who were culture-positive for *Mycobacterium tuberculosis*. A further 233 patients (27%) presented with TB consistent symptoms, but proved to be culture-negative with a sustained recovery after 5 months and therefore were classified as group F (Controls) (figure 8).

Overall, the prevalence of HIV was 44%. There was a significant difference between groups ($p<0.01$) with the highest prevalence (73%, 95%CI 58-84%) in group B (s-/c+ *M.tb*) and the lowest (34%, 95%CI 27-43%) in group A (s+/c+ *M.tb*). The proportion of patients who reported a prior history of TB was 17% overall, but differed significantly ($p<0.01$) between classifications and was highest (48%, 95%CI 28-68%) among group D (s-/c- clin.TB) (table 6).

Culture-positive individuals (groups A (s+/c+ *M.tb*) + B (s-/c+ *M.tb*)) were significantly more likely to suffer from night sweats (60 vs. 38%), fever (63 vs. 49%) and weight loss (70 vs. 44%) than individuals classified as group F (Controls) (p-values <0.01). There was no evidence of a difference in the frequency of haemoptysis between these groups ($p=0.33$).

The distribution of CAD scores (figure 9) for groups A (s+/c+ *M.tb*) and D (s-/c- clin.TB) tends towards higher scores, this is less marked for group B (s-/c+ *M.tb*). The scores attained by individuals classified as group C (s±/c+ NTM) and F (Controls) are clustered around lower values, but can be found across the whole range. Around one third of the

individuals of group F (Controls) did attain a CAD score greater than 50. On the whole there is a considerable overlap in the distribution of CAD scores.On the whole there is considerable overlap in the distribution of CAD scores (table 7). The CAD scores in group B (s-/c+ *M.tb*) are significantly lower than those of group A (s+/c+ *M.tb*), and higher than those of group F (Controls), (p-values <0.01).

The automated reading software was able to distinguish between culture positive PTB cases (groups A (s+/c+ *M.tb*) + B (s-/c+ *M.tb*)) and non TB patients (group F (Controls)) with an area under the curve of 0.84 (95%CI 0.80-0.88). Including all *M.tb* culture-negative patients (group C (s±/c+ NTM), E (EPTB) and F (Controls)) as the negative reference standard, CAD4TB performed slightly, but not significantly, worse: Az of 0.81 (95%CI 0.77-0.85), p=0.28 (figure 10). CAD4TB displayed a greater ability to differentiate smear-positive (group A (s+/c+ *M.tb*)) than smear-negative (group B (s-/c+ *M.tb*)) diseased individuals against non TB patients (group F (Controls)): Az=0.90 (95%CI 0.86-0.93) against Az=0.67 (95%CI 0.58-0.75), p<0.01 (figure 11). Similarly, the software distinguished diseased individuals (groups A (s+/c+ *M.tb*) + B (s-/c+ *M.tb*)) from non TB patients (group F (Controls)) significantly more accurately among the HIV negative than among the HIV positive patient population: Az=0.89 (95%CI 0.85-0.94) against Az=0.79 (95%CI 0.72-0.86), p <0.01 (figure 12). Among group A (s+/c+ *M.tb*), B (s-/c+ *M.tb*) and F (Controls) there was no evidence of a difference in the performance of CAD4TB in between patients with and without history of TB: Az=0.84 (95%CI 0.80-0.89) against Az=0.79 (95%CI 0.65-0.92), p=0.42. The area under the curve of CAD4TB for the discrimination of group B (s-/c+ *M.tb*) against C (s±/c+ NTM) was 0.56 (95%CI 0.46-0.65).

We calculated a set of cut-offs of the CAD4TB score for our patient population (table 8). For example, a cut-off of ≥74 leads to a sensitivity and specificity of CAD of 77% (95%CI 71-83%) and 79% (95%CI 74-84%), respectively. Optimal values of sensitivity cannot be obtained without a considerable trade-off of the specificity, and vice versa.

Setting the CAD4TB cut-off to give sensitivity values achieved by human readers allowed us to compare the performance of automated and human readings (figure 13). There was no evidence of a difference between the specificities achieved by the software and both human readers reporting 'any abnormality' (p=0.49, 0.88). This was different for tuberculosis specific reporting thresholds: CAD4TB was significantly more specific (p=0.02) than the

clinical officer reporting 'TB consistent abnormalities', but did not reach the accuracy level of the expert reader (p=0.02).

A review carried out by a third reader (senior radiologist with extensive experience in TB) of the images (n=7) rated as false negative by CAD4TB at the exemplary cut-off (<74), but as true positive (conclusion 3 + 4) by both human readers, did not reveal any obvious pattern of abnormalities missed by CAD4TB.

2.2.5 Discussion

Automating the interpretation of a chest radiograph for the detection of active pulmonary tuberculosis leads to objective, reproducible results and a standardized way of reporting. The main finding of our study is that the automated reading software CAD4TB (v3.07, updated release) achieved a good diagnostic accuracy (Az=0.84 (95%CI 0.80-0.88)) on a large set of CXRs of presumptive TB patients from sub-Saharan Africa. The accuracy of CAD4TB was slightly, but not significantly worse in our secondary analysis using a binary classification of patients (*M.tb* culture-positive vs. negative) which we included for a better comparability with other diagnostic accuracy studies.

In our study, performance of automated and human reading was comparable when the observers considered 'any abnormality'. For a more TB specific reading threshold, however, the software outperformed the clinical officer significantly, but did not reach the accuracy of the expert reader. The software identified a significantly higher proportion of smear-positive compared to smear-negative, culture-positive individuals - most likely because smear-negative PTB patients tend to have more discrete or atypical radiographic features, especially in combination with HIV infection [231]. This assumption is substantiated by the fact that CAD4TB detected PTB cases significantly more accurately among HIV negative than HIV positive individuals.

Identifying cases of active PTB among symptomatic individuals with abnormal CXRs due to other pulmonary conditions (e.g. pneumonia) or sequelae of tuberculosis remains challenging for both human and automated readers. This fact manifests itself in low specificity values as a consequence of the considerable overlap in the distribution of CAD scores for the defined groups and the far higher proportion of patients who reported a history of TB among group D (s-/c- clin.TB).

One of the strengths of our study is the direct comparison of automated and human reading on the same set of images. Due to inter-reader variability in the interpretation of chest radiographs and the ability to include only one clinical officer and expert reader, the degree to which this comparison can be generalized is strongly limited. It is possible that other clinical officers or expert readers would have outperformed the software in our study. A second limitation of our study is the fact that it was conducted in only one high burden country and it would be preferable to repeat it in different settings to assess generalizability of the results.

HIV infection seems to influence the diagnostic accuracy of CAD4TB so our findings cannot be readily generalized to populations that differ significantly in their HIV prevalence. A further limitation constraint of the study is the high proportion (31%, group G) of patients who either could not be followed up sufficiently to comply with the precise classification criteria or that were still non-TB patients, but symptomatic after five months and therefore could not be classified as group F (Controls). However, since a heterogeneous patient group is concerned and the data can be most likely assumed to be missing at random, it can be postulated that study results were not substantially influenced.

The relatively high number of patients that were found to be culture-positive for NTMs (16%, group C) is not uncommon in the sub-Saharan African context [232–234]. This is probably largely due to contamination of culture samples either at patient level or from the environment as only few patients suffered from a pathogenetic relevant NTM infection that fulfilled the diagnostic criteria for a Nontuberculous Mycobacterial Lung Disease according to the American Thoracic Society [235]. The inability of CAD4TB to differentiate between patients of group B (s-/c+ *M.tb*) and C (s±/c+ NTM) might be due to the heterogeneity of group C (s±/c+ NTM).

Maduskar et al. evaluated the performance of a previous CAD4TB version and compared it to both, clinical officers rating the radiograph between 0-100 and the binary decision of an expert reader (as radiological reference) for the presence of TB consistent abnormalities [218]. We decided to use hierarchical reading thresholds as we believe that this reflects the common radiological practice in a setting like ours. The high accuracy achieved for the radiological reference (Az=0.91 (95%CI 0.86-0.95)) [218] is consistent with our finding that CAD4TB approaches values of sensitivity and specificity achieved by the expert reader. The

diagnostic accuracy of CAD4TB for the bacteriological reference was higher using the newer version in our study compared to previous CAD4TB versions used in the study of Maduskar and another small scale study [236]. This suggests advancement in the development of the software that is especially encouraging as we evaluated its performance on images obtained from a different X-ray machine than the one it was originally developed for.

Current national diagnostic algorithms for presumptive adult TB patients in many sub-Saharan African countries request between two to six negative sputum smear examinations and a failed treatment with a broad-spectrum antibiotic for 7 days before a chest X-ray is ordered [237–244]. According to recommendations of the World Health Organization (WHO), the CXR exam should even precede an administration of antibiotics in settings where HIV is highly prevalent and resources are constrained [245]. In both cases a thorough X-ray report and its integration with clinical information by a medically trained person is needed for the final diagnosis of smear-negative PTB. Our findings indicate that in this situation, the CAD4TB software could assist less experienced readers in their judgment. It could not entirely replace the human interpretation for radiographic questions beyond that of active tuberculosis, as the software was not designed to detect other pathologies. Its output, a single number, does not reflect the presence of abnormalities unrelated to TB, whose detection might be not less important or even prompt immediate action (such as pneumothorax). In addition, the high proportion of patients of group F (Controls) who did attain a 'false positive' high CAD score greater than 50 due to other pulmonary pathologies as pneumonia has to be taken into consideration.

By contrast, the very condensed output of the automated reading might be preferable for the binary decision in screening situations of either conveying a screened individual to confirmatory testing or to declare the absence of PTB. A strong feature of CAD4TB in its current stage of development is its continuous output which allows adjusting the reading threshold to the purpose of use, local epidemiology and availability of resources (such as the capacity to perform smear microscopy or the number of Xpert MTB/Rif cartridges).

It is not known whether active screening will have a positive effect on TB epidemiology [246], however the slow decline in incidence and case detection gap suggest that a more active approach could complement patient-initiated pathways [247]and enhance their efficacy. Among the broad spectrum of possible active case finding strategies, the new

comprehensive WHO guidelines for the systematic screening for active tuberculosis among certain risk groups, in which chest radiography found its firm place, if available, as a first or second screening step [208]. A robust CAD has the potential to enhance and facilitate the implementation of these recommendations by ensuring high test standards of objectivity, reproducibility and accuracy without straining personnel resources. A prerequisite for the CAD application is the availability of digital radiography which is not yet the case in most of resource-constrained high-burden settings. However, it has been identified as a key action point in a WHO Workshop to Scale Up the Implementation of Collaborative TB/HIV Activities in Africa earlier this year [248] as it has been shown to be feasible and to result in a significant better quality of chest radiography compared to conventional X-ray technology in countries with limited resources [249].

Prospective studies on cost-effectiveness, operational and ethical aspects of the use of CAD in different high burden countries are needed. Future research should also address the question whether the integration of a CAD output with clinical variables like symptoms and risk factors could result in a more accurate screening step.

In conclusion, the computer-aided diagnosis system CAD4TB is a reproducible and accurate test for the detection of pulmonary tuberculosis on radiographs in symptomatic patients. This prompts additional research on how its potential, both as assistance for clinical officers in the diagnostic interpretation of radiographs and as standalone triage test in systematic screening settings, can be exploited.

Acknowledgements

We would like to acknowledge the contribution of Mwinyikambi Salum to this study by reading the radiographs. We wish to thank all participants of the TB cohort and TB CHILD projects.

Competing Interests

Delft Imaging Systems (CAD4TBII, http://www.delftimagingsystems.com/tb-solutions/cad4tb) provided a research grant to Bram van Ginneken and Rick HHM Philipsen for the development of CAD4TB. There are no patents, further products in development or

marketed products to declare. This does not alter the authors' adherence to all the PLOS ONE policies on sharing data and materials.

Funding

This study was supported by the European and Developing Countries Clinical Trials Partnership (EDCTP) as part of the project ''Evaluation of new and emerging diagnostics for childhood tuberculosis in high burden countries'' (TB CHILD) [IP.2009.32040.007]. The development of the CAD4TB software was supported by the EDCTP as part of the project ''Evaluation of Multiple Novel and Emerging Technologies for TB Diagnosis in Smear negative and HIV infected Persons in High-burden Countries'' (TB NEAT study) [IP.09.32040.009]. Delft Imaging Systems (CAD4TBII, http://www.delftimagingsystems.com/tb-solutions/cad4tb) provided a research grant to Bram van Ginneken and Rick HHM Philipsen for the development of CAD4TB. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

2.2.6 Figures

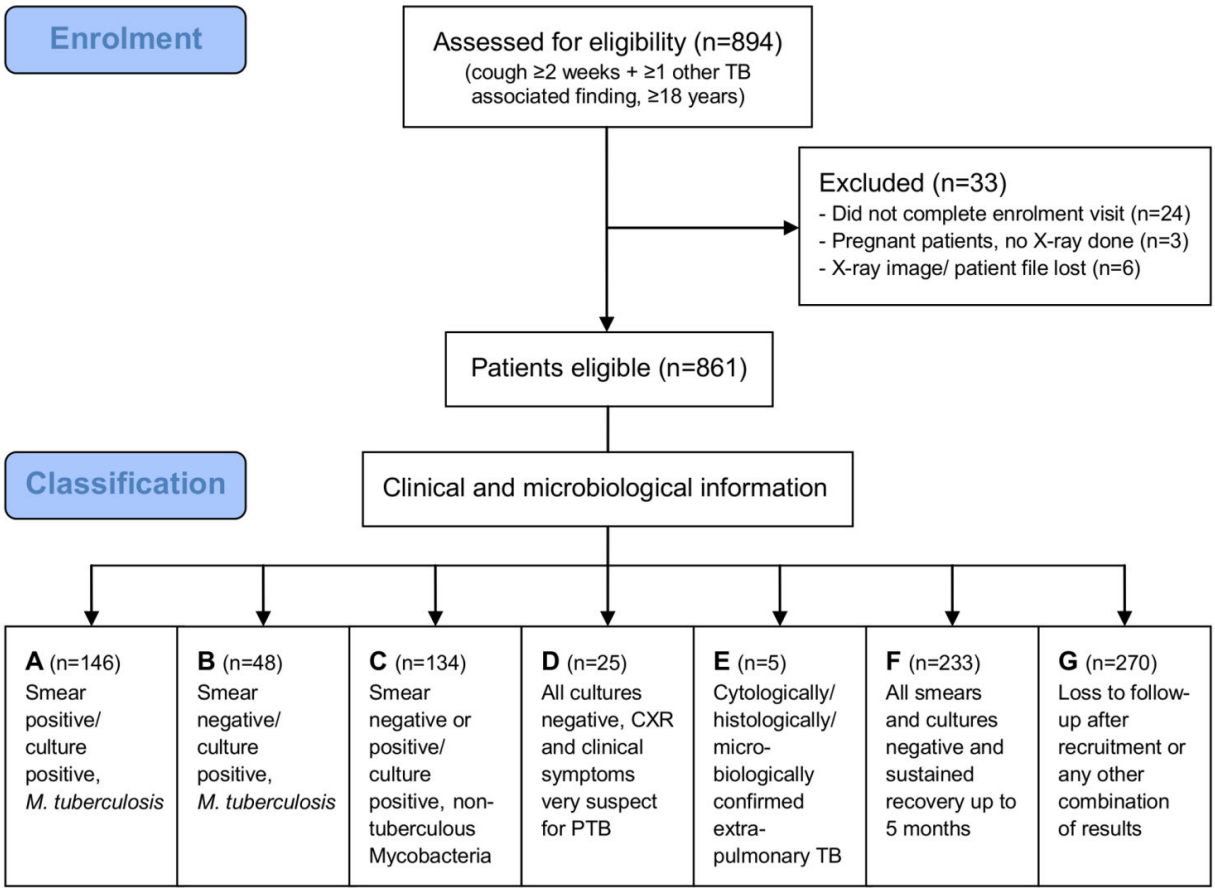


Figure 8: Flow chart of individuals taking part in the study.

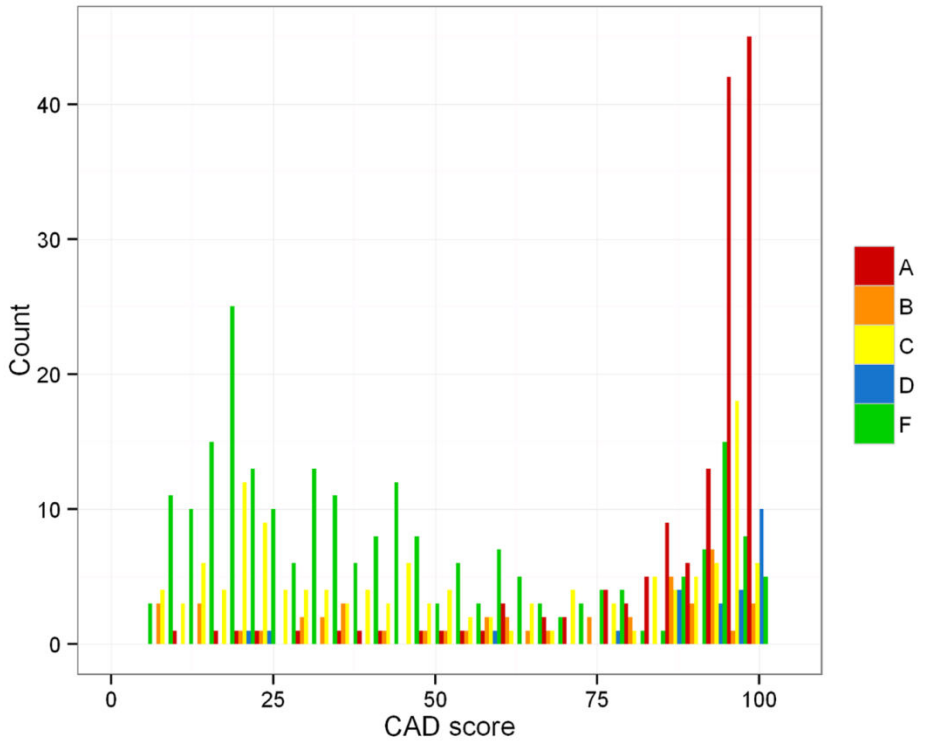


Figure 9: Distribution of CAD scores for patient groups A (s+/c+ *M.tb*), B (s-/c+ *M.tb*), C (s±/c+ NTM), D (s-/c- clin.TB), and F (Controls).

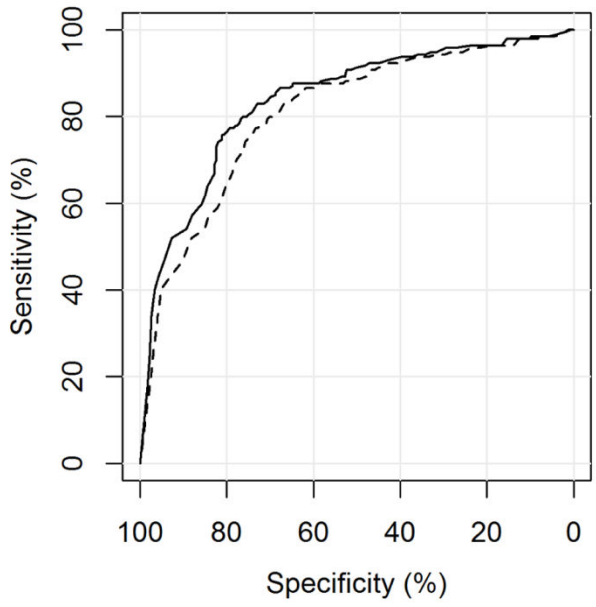


Figure 10: ROC analysis for the detection of *M.tb* culture-positive individuals.
—— A (s+/c+ *M.tb*), B (s-/c+ *M.tb*) vs. F (Controls): A_z=0.84 (0.80-0.88),
----- A (s+/c+ *M.tb*), B (s-/c+ *M.tb*) vs. C (s±/c+ NTM), E (EPTB), F (Controls): A_z=0.81 (0.77-0.85), p =0.28.

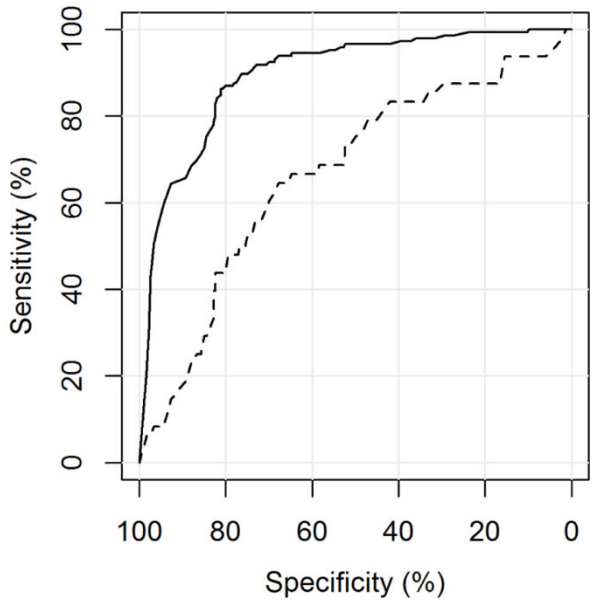


Figure 11: ROC analysis for the detection of *M.tb* culture-positive individuals by smear status.

——— A (s+/c+ *M.tb*) vs. F (Controls): A_z=0.90 (0.86-0.93),
------- B (s-/c+ *M.tb*) vs. F (Controls): A_z=0.67 (0.58-0.75), p <0.01.

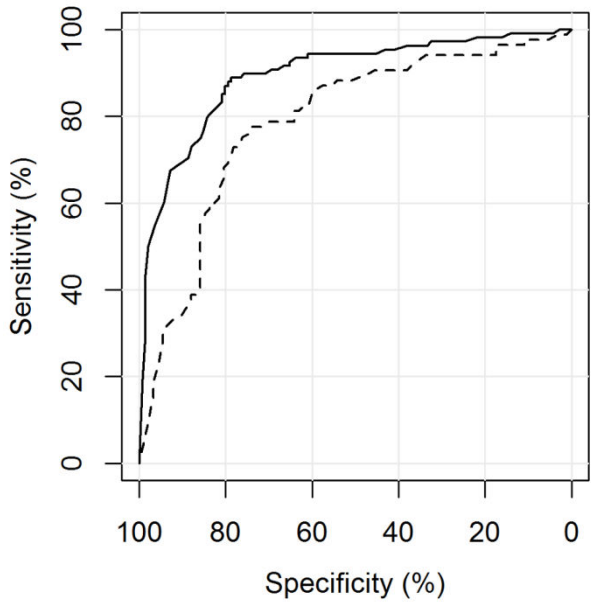


Figure 12: ROC analysis for the detection of *M.tb* culture-positive individuals by HIV status.

——— HIV negative. A (s+/c+ *M.tb*), B (s-/c+ *M.tb*) vs. F (Controls): A_z=0.89 (0.85-0.94),
------- HIV positive. A (s+/c+ *M.tb*), B (s-/c+ *M.tb*) vs. F (Controls): A_z=0.79 (0.72-0.86), p <0.01.

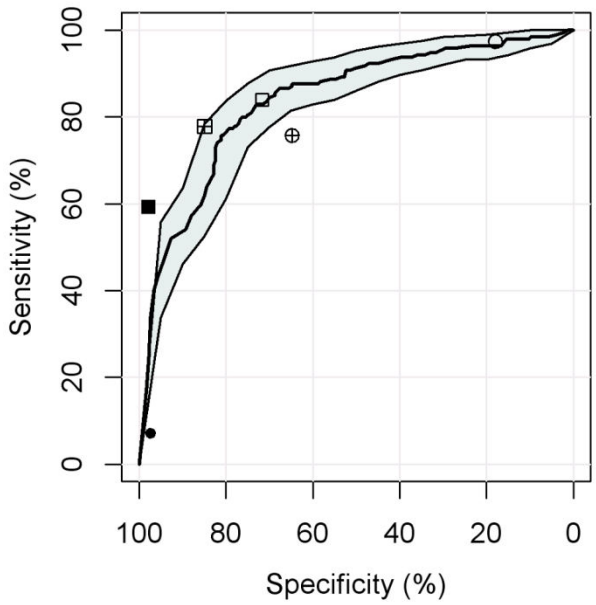


Figure 13: Comparison of automated and human reading.

Sensitivity and specificity to distinguish groups A and B vs F. Line and shaded area: ROC curve and 95% CI for CAD4TB. The expert reader is represented by square symbols, the clinical officer by round symbols. The different fill of the symbols indicate different reading thresholds: empty symbols ='any abnormality', crossed symbols ='TB consistent abnormalities' and filled symbols ='abnormalities highly suggestive for TB'.

2.2.7 Tables

Table 5: Classification of study population according to clinical and microbiological data.

Group	Description	Short form
A	Smear positive/ culture positive, *Mycobacterium tuberculosis*	s+/c+ *M.tb*
B	Smear negative/ culture positive, *Mycobacterium tuberculosis*	s-/c+ *M.tb*
C	Smear negative or positive/ culture positive, nontuberculous mycobacteria (NTM), irrespective of clinical relevance	s ±/c+ NTM
D	All cultures negative, CXR and clinical symptoms very suspect for PTB (clinically diagnosed TB)	s-/c- clin.TB
E	Cytologically/ histologically/ microbiologically confirmed extrapulmonary TB	EPTB
F	All smears and cultures negative and sustained recovery up to 5 months (e.g. resolved bronchitis or pneumonia)	Controls
G	Loss to follow-up after recruitment or any other combination of results (e.g. still symptomatic after 5 months)	Indeterminate

Table 6: Summary statistics of study population.

	No data	All	Group A	Group B	Group C	Group D	Group E	Group F	Group G
			s+/c+ *M.tb*	s-/c+ *M.tb*	s±/c+ NTM	s-/c- clin.TB	EPTB	Controls	Indeter-minate
Characteristic									
n (%)	0	861 (100)	146 (17)	48 (6)	134 (16)	25 (3)	5 (1)	233 (27)	270 (31)
Mean age (SD[1])	0	42 (15)	37 (13)	41 (13)	42 (15)	46 (16)	37 (10)	42 (16)	44 (16)
Female sex n (%)	0	433 (50)	48 (33)	25 (52)	76 (57)	12 (48)	4 (80)	122 (52)	146 (54)
HIV positive n (%)	4	379 (44)	50 (34)	35 (73)	66 (49)	10 (40)	2 (40)	92 (39)	124 (46)
History of TB n (%)	0	144 (17)	17 (12)	6 (13)	27 (20)	12 (48)	0 (0)	26 (11)	56 (21)
Symptoms at first visit									
Cough ≥ 2 weeks n (%)	0	820 (95)	139 (95)	45 (94)	129 (96)	23 (92)	4 (80)	227 (97)	253 (94)
Night sweats n (%)	1	426 (50)	92 (63)	24 (50)	64 (48)	19 (79)	3 (60)	89 (38)	135 (50)
Haemoptysis n (%)	10	91 (11)	11 (8)	4 (8)	16 (12)	5 (20)	0 (0)	24 (10)	31 (12)
Fever n (%)	0	470 (55)	91 (62)	31 (65)	78 (58)	15 (60)	3 (60)	114 (49)	138 (51)
Weight loss n (%)	6	447 (52)	101 (70)	34 (71)	60 (45)	10 (40)	4 (80)	101 (44)	137 (51)

[1]standard deviation

Table 7: Median CAD scores and 90% central range.

	All	Group A s+/c+ *M.tb*	Group B s-/c+ *M.tb*	Group C s±/c+ NTM	Group D s-/c- clin.TB	Group E EPTB	Group F Controls	Group G Indeter-minate
Median CAD score	61	97	67	48	97	83	34	57
90% central range	11-100	44-100	11-99	13-98	32-100	38-92	9-95	11-100

Table 8: Performance of CAD4TB and human readers.

	Threshold for test positivity	Sens.[1] [%] (95%CI)	Spec.[2] [%] (95%CI)	PPV[3] [%] (95%CI)	NPV[4] [%] (95%CI)	PLR[5] (95%CI)	NLR[6] (95%CI)
CAD4TB	≥23	95 (91-98)	33 (27-39)	54 (49-60)	89 (80-94)	1.42 (1.29-1.56)	0.16 (0.08-0.29)
	≥37	91 (86-94)	52 (46-59)	61 (55-67)	87 (80-92)	1.9 (1.65-2.19)	0.18 (0.11-0.28)
	≥56	85 (79-90)	69 (62-75)	69 (63-75)	85 (79-89)	2.71 (2.22-3.31)	0.22 (0.15-0.31)
	≥74	77 (71-83)	79 (74-84)	76 (69-82)	81 (75-86)	3.75 (2.88-4.88)	0.29 (0.22-0.37)
	≥89	62 (55-69)	85 (80-89)	77 (70-84)	73 (67-78)	4.12 (2.98-5.7)	0.45 (0.37-0.54)
	≥95	47 (40-54)	94 (91-97)	88 (80-93)	68 (63-73)	8.41 (4.86-14.56)	0.56 (0.49-0.64)
Expert reader	4	59 (52-66)	98 (95-99)	96 (91-99)	74 (69-79)	27.62 (11.52-66.26)	0.42 (0.35-0.49)
	3,4	78 (71-83)	85 (80-89)	81 (75-87)	82 (77-87)	5.18 (3.78-7.1)	0.26 (0.2-0.34)
	2,3,4	84 (78-89)	72 (65-77)	71 (65-77)	84 (79-89)	2.97 (2.4-3.67)	0.22 (0.16-0.31)
Clinical officer	4	7 (4-12)	97 (94-99)	70 (46-88)	56 (51-61)	2.8 (1.1-7.15)	0.95 (0.91-1)
	3,4	76 (69-82)	65 (58-71)	64 (58-70)	76 (70-82)	2.15 (1.78-2.61)	0.37 (0.29-0.49)
	2,3,4	97 (94-99)	18 (13-24)	50 (45-55)	89 (77-96)	1.19 (1.11-1.27)	0.14 (0.06-0.35)

[1]sensitivity [2]specificity [3]positive predictive value [4]negative predictive value [5]positive likelihood ratio [6]negative likelihood ratio

2.3 Xpert MTB/RIF assay for diagnosis of pulmonary tuberculosis in children: a prospective, multi-centre evaluation

Klaus Reither[1,2,3*], Christina Manyama[4], Petra Clowes[4,5], Andrea Rachow[5,6], Daniel Mapamba[4], Andreas Steiner[1,2], Amanda Ross[1,2], Elirehema Mfinanga[3], Mohamed Sasamalo[3], Martin Nsubuga[7], Francesco Aloi[7], Daniela Cirillo[8], Levan Jugheli[1,2,3], Fred Lwilla[3]

[1] Swiss Tropical and Public Health Institute, Basel, Switzerland

[2] University of Basel, Basel, Switzerland

[3] Ifakara Health Institute, Bagamoyo, Tanzania

[4] National Institute of Medical Research, Mbeya Medical Research Centre, Mbeya, Tanzania

[5] Division of Infectious Diseases and Tropical Medicine, Medical Center of the University of Munich (LMU), Germany

[6] German Centre for Infection Research (DZIF), Partner Site Munich, Germany

[7] St. Francis Hospital Nsambya / AISPO, Kampala, Uganda

[8] Emerging Bacterial Pathogens, San Raffaele Scientific Institute, Milano, Italy

Publication: J Infect. 2015 Apr;70(4):392-9.

2.3.1 Abstract

Background:

Following endorsement by the World Health Organisation, the Xpert MTB/RIF assay has been widely incorporated into algorithms for the diagnosis of adult tuberculosis (TB). However, data on its performance in children remain scarce. This prospective, multi-centre study evaluated the performance of Xpert MTB/RIF to diagnose pulmonary tuberculosis in children.

Methods:

Children older than eight weeks and younger than 16 years with suspected pulmonary tuberculosis were enrolled at three TB endemic settings in Tanzania and Uganda, and assigned to five well-defined case definition categories: culture-confirmed TB, highly probable TB, probable TB, not TB, or indeterminate. The diagnostic accuracy of Xpert MTB/RIF was assessed using culture-confirmed TB cases as reference standard.

Results:

In total, 451 children were enrolled. 37 (8%) had culture-confirmed TB, 48 (11%) highly probably TB and 62 probable TB (13%). The Xpert MTB/RIF assay had a sensitivity of 68% (95% CI, 50%-82%) and specificity of 100% (95% CI, 97%-100%); detecting 1.7 times more culture-confirmed cases than smear microscopy with a similar time to detection. Xpert MTB/RIF was positive in 2% (1/48) of highly probable and in 3% (2/62) of probable TB cases.

Conclusions:

Xpert MTB/RIF provided timely results with moderate sensitivity and excellent specificity compared to culture. Low yields in children with highly probable and probable TB remain problematic.

2.3.2 Introduction

Tuberculosis (TB) in children is increasingly recognized as a global public health concern. The difficulty of establishing an accurate TB diagnosis is the greatest challenge to patient management and impedes the assessment of the true disease burden and the development of treatment and vaccination strategies appropriate for effective disease control in children [250] [251].

The diagnosis of active tuberculosis in children is difficult because of non-specific clinical signs or symptoms, suboptimal recovery methods and low bacteriological yield, due to the paucibacillary nature of paediatric TB. Childhood TB can present in a similar way to conditions such as pneumonia, generalised infections, e.g. human immunodeficiency virus (HIV) infection, and malnutrition. Microbiological confirmation is rare with only 10-15% of sputum samples revealing acid-fast bacilli (AFB) upon smear microscopy and culture being positive in around 30-40% of paediatric cases with probable tuberculosis [252–254].

Mycobacterial culture is often not available in endemic regions due to limited laboratory capacities. Even when available, culture results are unable to guide timely treatment decisions due to slow turn-around times. This has particular relevance in young children at risk of rapid disease progression and dissemination [255]. In the absence of bacteriological confirmation the diagnosis of childhood TB is usually based on non-specific and subjective markers such as clinical symptoms, TB contact history, chest radiograph findings and tuberculin skin test (TST); often in combination with clinical scoring charts which have poor diagnostic accuracy [256]. Consequently, there is an urgent need to identify a practical, affordable, reliable and fast way to diagnose TB in children.

The Xpert MTB/RIF (Cepheid, Sunnyvale, USA) assay offers a substantial improvement in the diagnosis of adult TB compared to sputum smear microscopy. It was endorsed by the World Health Organisation (WHO) in 2010 and is strongly recommended as an initial diagnostic test in both adults and children suspected of multidrug-resistant tuberculosis (MDR-TB) or HIV/TB [257,258]. The assay uses real-time polymerase chain reaction to amplify a *Mycobacterium (M.) tuberculosis* complex-specific sequence of the *rpoB* gene and to detect the most prevalent *rpoB* mutations. Evaluation studies in adults demonstrated that Xpert MTB/RIF is fast, sensitive and specific for the simultaneous diagnosis of TB and identification of rifampicin (RIF) resistance [259,132].

A study of children suspected of having TB in Cape Town, South Africa, showed an overall Xpert MTB/RIF sensitivity of 74% compared to a positive culture from at least one induced sputum specimen [260]. Further studies showed similar test performance [261–264], but remain limited in size and geographic representation. The present study is, to our knowledge, the first prospective, multi-centre study on the test performance of Xpert MTB/RIF for the diagnosis of pulmonary TB in children in high-burden countries.

2.3.3 Methods

Study setting. This multi-centre study was conducted in one rural and two urban sites located in sub-Saharan African countries with high burden of tuberculosis and HIV: 1) the Ifakara Health Institute, Bagamoyo, Tanzania, 2) the NIMR-Mbeya Medical Research Centre, Mbeya, Tanzania, and 3) the St. Francis, Nsambya Hospital, Kampala, Uganda. The enrolment took place between 10 May 2011 and 13 September 2012.

Study participants and reference standard. At the three sites, children older than eight weeks and younger than 16 years of age with clinical signs of tuberculosis were consecutively enrolled and followed up after 2 and at least 5 months. One or more of the following inclusion criteria had to be met: a) persistent, non-remitting cough of more than 14 days not responding to a course of antibiotics, b) repeated episodes of fever within the last 14 days not responding to a course of antibiotics, after malaria has been excluded, c) weight loss or failure to thrive within the previous 3 months. Children who had received TB treatment in the past year were excluded from the study.

The patients were assigned to five case definition categories based on clinical, radiological, microbiological and molecular biological findings: culture-confirmed TB, highly probable TB, probable TB, not TB, or indeterminate (Table 9). The decision to start tuberculosis treatment was made in agreement with clinicians of the respective TB programme following national guidelines based on WHO recommendations.

Clinical procedures. At recruitment and follow up a detailed medical history and physical examination were carried out. Anthropometric data were collected at each visit and interpreted using WHO Child Growths Standards [265]. HIV testing (with pre- and post-test counselling), a tuberculin skin test (TST), and chest radiograph were performed at the recruitment visit. TST was considered positive if ≥5 mm in HIV-infected and ≥10mm in HIV-uninfected children. Chest radiographs were categorized as strongly indicative of TB,

uncertain TB or no TB by two independent experts, who were blinded to clinical information.

Specimen collection and laboratory procedures. Blood samples were collected at recruitment. Tests performed include the following: full blood count and differential, chemistry (total and direct bilirubin, creatinin, alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, albumin, gamma-glutamyltransferase, urea), HIV (two rapid tests or HIV-DNA PCR for ≤ 18 months of age; HIV ELISA as confirmatory test), and CD4 cell count, if HIV infected. The immunosuppression status was classified according to US Centers for Disease Control and Prevention classification [266]. We attempted to collect at least two induced or three uninduced sputum samples. However, in some cases the number and also the type of specimen collected varied according to the clinical requirements. Gastric lavage was only done in exceptional cases. Depending on the clinical picture, fine needle aspirates of enlarged lymph nodes or body fluids were collected additionally for further diagnosis. The specimen collection was done by trained study physicians or nurses according to standardized operating procedures.

Following standard NALC-NAOH decontamination, each induced and uninduced sputum sample pellet was subjected to microscopy using Ziehl-Neelsen staining. At least one sample was inoculated on liquid (BACTEC MGIT 960, Becton Dickinson, Sparks, USA) and on solid Loewenstein-Jensen culture (LJ) media. All positive cultures were tested by ZN microscopy for the presence of AFB. *M. tuberculosis* was finally confirmed by MPT64 antigen and/or molecular (Genotype MTBC or CM; Hain Lifescience, Nehren, Germany) tests. Drug susceptibility was tested from culture using either the BACTEC MGIT 960 SIRE kit (Becton Dickinson, Sparks, USA) or the GenoType MTBDRplus assay (Hain Lifescience, Nehren, Germany). Xpert MTB/RIF was performed in at least one sputum sample according to manufacturer's instructions. The reading of all microbiological and molecular tests was blind to clinical information and radiological results.

Statistical analysis. The analysis was carried out in R version 3.0.1 [267] and OpenBUGS version 3.2.1 [268]. To describe the diagnostic performance, we calculated the sensitivity, specificity, positive and negative predictive values. We also calculated positive and negative likelihood ratios. The confidence intervals for the diagnostic likelihood ratios were calculated using the method of Simel et al [269] via the R package epiR [227]. We

considered clustering of the data within the three sites by including a random effect in the models; this made no appreciable difference. The results presented are from analyses without the random effect, since small numbers led to some unstable results. Proportions were compared using logistic regression with a random effect for site. Continuous outcomes which were not normally distributed (such as age) were compared using the Kruskal-Wallis test. Accounting for clustering by site using a binary outcome and logistic model did not affect the conclusions. We calculated the z-scores for anthropometric variables using the WHO growth standards [265].

Ethics approval. The study protocol and related documents were approved by the local and national ethics committees relevant to each site (Institutional Review Board of the Ifakara Health Institute, Mbeya Medical Research and Ethics Committee, Medical Research Coordinating Committee of Tanzania, Uganda National Council for Science and Technology). Written, informed consent for enrolment in the study was obtained from a parent or legal guardian. In case of illiteracy, informed oral consent was attested by an independent witness. Children of more than seven years of age provided assent for participation. The study was conducted in accordance with the Helsinki Declaration [200] and Good Clinical Practice guidelines [199].

2.3.4 Results

A total of 493 children with symptoms suggestive of tuberculosis were enrolled at the three sites; 42 were excluded from data analysis. Figure 14 provides a STARD flowchart [73] of all study participants and their distribution within the five case definition categories in relation to Xpert MTB/RIF results. In all but one eligible patient valid Xpert MTB/RIF results were generated.

Table 10 reflects baseline characteristics and demographic data for each case definition category. There was a significant difference in the proportions of HIV across the five case definition categories (p=0.001); ranging from 27% in children with culture-confirmed TB to 71% in children with probable TB. The highest proportion of severely immunosuppressed HIV infected children was found in the probable TB (41%) and highly probable TB (39%) group. The nutritional status was impaired in all groups; 39% (133) of children below ten years of age had a weight for age z-score of less than -2.

Overall 100 /429 (23%) children were TST positive at enrolment; including 69% (18/26) of HIV-uninfected and 50% (5/10) of HIV-infected children with culture-confirmed TB.

Xpert MTB/RIF detected 25 of the 37 culture-confirmed TB cases and was negative in all children without TB. Thus, the sensitivity and specificity of Xpert MTB/RIF for culture-confirmed TB versus those without TB were 68% (95% CI, 50%-82%) and specificity of 100% (95% CI, 97%-100%), respectively (Table 11). Additionally, Xpert MTB/RIF was positive in 2% (1/48) of highly probable and in 3% (2/62) of probable TB cases. Xpert MTB/RIF detected 8 (80%) of the 10 HIV-infected children and 17 (63%) of the 27 HIV-uninfected children in the culture-confirmed TB group. The sensitivity dropped to 31% (95% CI, 21%-42%) when both culture-confirmed and highly probable TB cases were included in the reference standard.

Xpert MTB/RIF identified 10 additional culture-confirmed TB cases compared to smear microscopy (27% of all culture-confirmed TB cases) (Figure 15). In one culture-confirmed TB case smear microscopy was positive, but not the Xpert MTB/RIF assay. One child of the highly probable TB group with chronic cough not responding to antibiotic treatment, uncertain x-ray findings, intermittent fever and loss of appetite had one smear-positive sputum sample, neither confirmed by culture nor by Xpert MTB/RIF. The child recovered after anti-TB treatment.

Across all sites, 86% of children with culture-confirmed, highly probable or probable TB received anti-TB treatment. The low treatment coverage rate is largely caused by the fact that at the Ugandan site, 16 children classified as highly probable TB cases were not treated as TB patients after clinical judgement of the investigators. The children recovered from their symptoms without anti-TB treatment (one loss to follow-up). The study specific case classifications of these patients were based on reports of two radiographers who assessed the chest radiographs independently as 'strongly indicative of TB'.

The set of samples tested by Xpert MTB/RIF included 26 respiratory specimens with growth of non-tuberculous mycobacteria. None of those samples were Xpert MTB/RIF positive.

Of the 31 culture-confirmed or highly probable paediatric TB cases who had more than one sample with results by Xpert MTB/RIF, there was a high agreement between Xpert

MTB/RIF results for the first two induced or uninduced sputum samples (28/31 (90%), Kappa=0.77). The first sample identified 10 positive cases, the second sample one further child.

Time to detection, defined in this study as time from enrolment to the first positive test, was quicker by smear and Xpert MTB/RIF, whereas culture took longer but identified more of the patients (Figure 16). All positive test results from smear or Xpert MTB/RIF were received within 6 days, positive MGIT and LJ results were obtained between 6 and 135 days after enrolment.

There was no rifampicin resistance determined in the study population, neither by line probe assay or culture-based drug susceptibility testing (3 missing results) nor by Xpert MTB/RIF.

2.3.5 Discussion

The performance analysis of Xpert MTB/RIF for detection of pulmonary childhood tuberculosis established an overall sensitivity of 68% and specificity of 100% compared to culture. The results of this multi-centre study are almost identical to a WHO meta-analysis in children presumed of having TB with a pooled sensitivity and specificity of Xpert MTB/RIF against culture for samples of expectorated or induced sputum of 66% and ≥98%, respectively [258]. Consistent with results from adults and children, there was no evidence that HIV infection influenced sensitivity or specificity of Xpert MTB/RIF [259,261,262].

Xpert MTB/RIF detected 10 children with smear negative but culture-positive results, confirmed for *M. tuberculosis*. The enhanced yield compared to smear microscopy seems to be an important quality of Xpert MTB/RIF for the diagnosis of TB in children. Furthermore, no cross reactivity with non-tuberculous mycobacteria was observed in this study.

The Xpert MTB/RIF assay can be performed by a technician with minimal training at the same level of biosafety as microscopy and provides results from respiratory specimens after less than 2 hours. In a clinical context, as in the presented study, all Xpert MTB/RIF results were obtainable within the first six days after enrolment as were the results from the less sensitive AFB smear microscopy. Shortening time to detection is a substantial comparative advantage of Xpert MTB/RIF to culture and offers the opportunity for prompt clinical management of paediatric TB cases.

The dual ability of Xpert MTB/RIF to detect *M. tuberculosis* and to identify rifampicin resistance is a further asset of the device [132]. In this study, no conclusion could be drawn about the capacity of the assay to diagnose resistance, because rifampicin resistance was detected in none of the children by any of the applied methods.

The proportion of culture-positive children in this study (8%) was similar to the proportion reported from a recent South African study [264] where children were also recruited in outpatient facilities. The higher culture positivity rate in other paediatric studies might be attributed to a larger proportion of seriously ill inpatients [260–262].

Xpert MTB/RIF identified almost certainly three additional TB cases in highly probable and probable TB cases in absence of *M. tuberculosis* growth on culture. Conversely, the low detection rate of Xpert MTB/RIF in these two groups (2% and 3%, respectively) can be seen as a major limitation of the assay for the use as a single point-of-care diagnostic in children. The fact that Xpert MTB/RIF detected only a few children among clinically diagnosed paediatric TB cases is consistent with findings from previous studies [260–262,264] and a convincing argument for the development of even more sensitive molecular tests or novel immunodiagnostics. Both approaches might have a superior diagnostic value in children with low bacteriological load. This notion is supported by data showing that Xpert MTB/RIF does not detect TB in spiked sputum samples with less than 131 colony forming units per mL [126].

On the other hand, uncertainties regarding group specific clinical reference standards must be taken into consideration. In the present study it became obvious, that at one site two experienced radiographers most likely overestimated the radiological features in 16 chest radiographs which led almost certainly to a misclassification as highly probable TB cases. Consequently, the findings related to classification groups based on clinical and radiological definitions must be interpreted with some caution.

However, in day-to-day practice treatment decisions in children suspected of having TB will have to continue relying on clinical markers in the absence of more accurate, confirmatory diagnostic tools.

Although this study was not designed to assess the diagnostic advantage of an additional Xpert test in a second sputum sample, the results from culture-confirmed and highly

probable paediatric TB cases with more than one sample tested by Xpert MTB/RIF indicate that there might be an incremental yield with repeated testing. However, further systematic studies to assess the cost effectiveness of repeated Xpert MTB/RIF tests are needed.

One limitation of this study is the relatively small number of TB cases, which may lead to reduced power for subgroup analysis. Another limitation is that the clinical case classifications of the cited diagnostic evaluation studies are not entirely comparable to each other or to the group definitions in this study. In future, harmonised evaluation of new tuberculosis diagnostic technologies in paediatric populations should be facilitated by the use of unique clinical case definitions for classification of intrathoracic tuberculosis disease developed by an expert panel [178].

In conclusion, the findings of our study suggest that Xpert MTB/RIF can play an important role in diagnosis of pulmonary TB in children but should not be used as a stand-alone test to rule out childhood TB. The assay identified rapidly two-thirds of culture-confirmed paediatric TB cases with excellent specificity and provides, as shown in other studies, important information on rifampicin resistance. However, Xpert MTB/RIF was negative in the vast majority of children with highly probable and probable TB. In particular, but not exclusively, in those children, clinical and radiological assessment remains an essential part of the diagnostic pathway until better, more sensitive tools are available.

Acknowledgements

We wish to thank all children and their caregivers for participating in this study. This study was supported by the European and Developing Countries Clinical Trials Partnership (EDCTP) as part of the project "Evaluation of new and emerging diagnostics for childhood tuberculosis in high burden countries" (TB CHILD) [IP.2009.32040.007].

2.3.6 Figures:

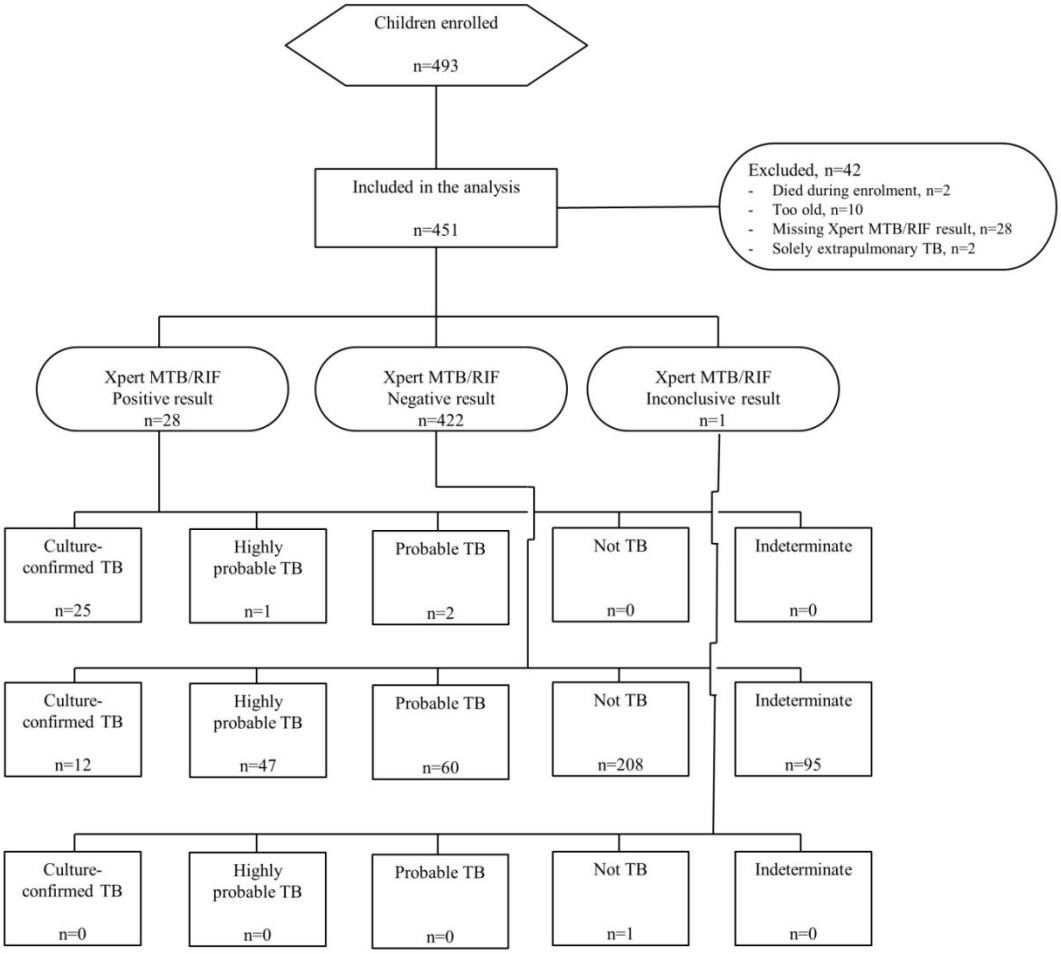


Figure 14: STARD flow diagram.

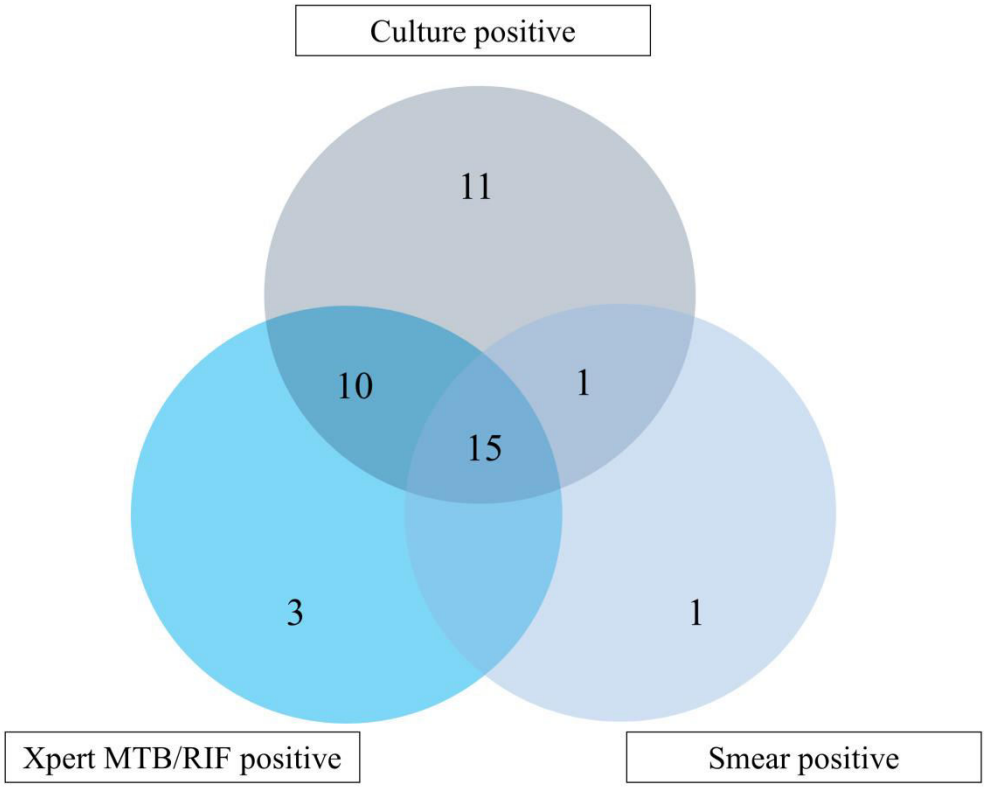
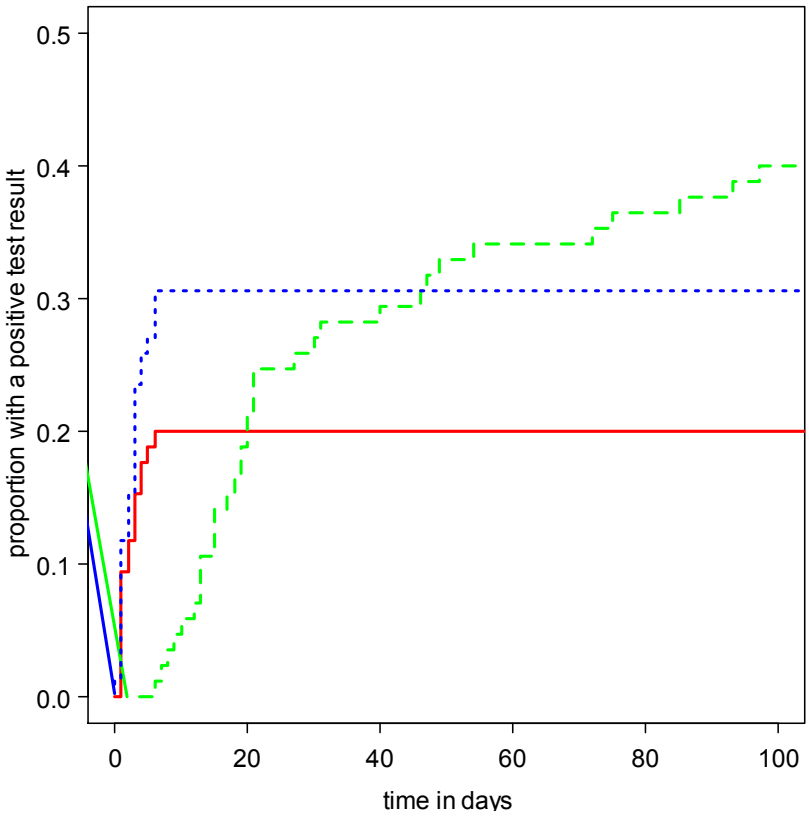


Figure 15: Venn diagram for positive culture, Xpert MTB/RIF and AFB smear microscopy results per patient.



—: AFB smear microscopy; ---: Solid and liquid culture combined; ----: Xpert MTB/RIF

Figure 16: Time to detection for participants in groups culture-confirmed and highly probable TB.

2.3.7 Tables

Culture-confirmed tuberculosis Symptoms suggestive of tuberculosis plus Liquid or solid culture positive for *M. tuberculosis*
Highly probable TB Symptoms suggestive of tuberculosis plus chest radiograph strongly indicative of TB (confirmed by two independent reviewers) *or* fluorescent or acid-fast bacilli on microscopy
Probable TB Symptoms suggestive of tuberculosis plus CXR uncertain for tuberculosis or discordance between independent reviewers *and* no alternative diagnosis established and complete symptomatic or radiographic resolution on TB treatment
Not TB Alternative diagnosis established *and* TB workup negative and well-being established after 3-6 months follow-up without TB treatment
Indeterminate Any other possible combination of results and/or loss to follow up after recruitment

Table 9: Case definition categories.

	All Children (n = 451)	**Culture-confirmed TB (n = 37)**	**Highly probable TB (n = 48)**	**Probable TB (n = 62)**	**Not TB (n = 209)**	**Indeterminate (n = 95)**
Age						
Median years (IQR)	5.6 (2.0, 9.8)	6.7 (2.5, 11.1)	4.1 (1.2, 9.0)	4.4 (1.5, 9.1)	5.7 (2.5, 9.8)	5.7 (1.7, 9.9)
Age groups						
0-5; 6-10; 11-15 years of age *n*	211; 133; 106	16; 10; 11	26; 13; 9	32; 16; 14	94; 64; 51	43; 30; 21
Unknown or missing data *n*	1	-	-	-	-	1
Gender						
Male *n* (%)	219/451 (49)	18/37 (49)	24/48 (50)	33/62 (53)	101/209 (48)	43/95 (45)
HIV status						
Positive HIV test *n* (%)	197/444 (44)	10/37 (27)	24/47 (51)	44/62 (71)	80/207 (39)	39/91 (43)
Unknown or missing data *n*	7	-	1	-	2	4
ART treatment						
Received ART at enrolment *n* (%)	98/197(50)	5/10 (50)	11/24 (46)	22/44 (50)	41/80 (51)	19/39 (49)
Unknown or missing data *n*	96	5	13	21	37	20
Immune suppression status of HIV infected children						
No suppression *n* (%)	78/188 (41)	5/10 (50)	6/22 (27)	17/44 (39)	37/75 (49)	13/37 (35)
Moderate suppression *n* (%)	59/188 (31)	2/10 (20)	7/22 (32)	9/44 (20)	25/75 (33)	16/37 (43)
Severe suppression *n* (%)	51/188 (27)	3/10 (30)	9/22 (41)	18/44 (41)	13/75 (17)	8/37 (22)
Unknown or missing data *n*	9	-	2	-	5	2
Median weight for age (< 10 years) Z score (IQR)	-1.6 (-2.9, -0.7)	-2 (-3.3, -1.6)	-1.7 (-2.6, -0.8)	-2.8 (-3.7, -1.8)	-1.2 (-2.4, -0.2)	-1.3 (-2.9, -0.7)
Weight for age < -2 (< 10 years) Z score *n* (%)	133/340 (39)	11/26 (42)	16/38 (42)	34/48 (71)	48/157 (31)	24/71 (34)
Median height for age (< 10 years) Z score (IQR)	-1.7 (-2.8, -0.6)	-2.2 (-3.4 -1.2)	-1.6 (-2.6, -0.6)	-3.1 (-3.7, -1.8)	-1.5 (-2.5, -0.3)	-1.3 (-2.5, -0.4)
TST result						
Positive in HIV uninfected *n* (%)	72/237 (30)	18/26 (69)	9/23 (39)	5/18 (28)	25/123 (20)	15/47 (32)
Positive in HIV infected *n* (%)	27/187 (14)	5/10 (50)	3/24 (13)	8/41 (20)	5/77 (7)	6/35 (17)
Positive with unknown HIV status *n*	0/7	-	0/1	-	0/1	0/1
TB contact in last 12 months						
Yes *n* (%)	130/413 (31)	14/33 (42)	9/41 (22)	20/54 (37)	58/196 (30)	29/89 (33)
Unknown or missing data *n* (%)	38	4	7	8	13	6

Table 10: Baseline characteristics and demographic data of the enrolled children.

Xpert MTB/RIF	**Sensitivity** **%** **(95 % CI)**	**Specificity** **%** **(95 % CI)**	**Positive Predictive Value** **%** **(95 % CI)**	**Negative Predictive Value** **%** **(95 % CI)**	**Negative Likelihood ratio*** **%** **(95 % CI)**
Culture-confirmed TB *versus* Not TB					
All children	68 (50, 82)	100 (97, 100)	100 (80, 100)	95 (91, 97)	32 (20, 52)
HIV infected	80 (44, 97)	100 (93, 100)	100 (52, 100)	98 (91, 100)	20 (6, 69)
HIV uninfected	63 (42, 81)	100 (96, 100)	100 (73, 100)	93 (87, 96)	37 (23, 61)

CI= Confidence interval.
*The positive likelihood ratio could not be computed since is given by sensitivity/(1-specificity). In all cases, the specificity was 1 (or 100%).

Table 11: Diagnostic Test Performance of Xpert MTB/RIF.

2.4 Performance of the novel TAM-TB assay to diagnose active tuberculosis in children: a prospective, proof of concept, multi-centre study

Damien Portevin[1,2], Felicien Moukambi[3,4], Petra Clowes[3,4], Asli Bauer[3,4], Mkunde Chachage[3], Nyanda E. Ntinginya[3], Elirehema Mfinanga[6], Khadija Said[6], Frederick Haraka[6], Andrea Rachow[4,5], Elmar Saathoff[4,5], Maximilian Mpina[6], Levan Jugheli[1,2,6], Fred Lwilla[6], Ben J. Marais[7], Michael Hoelscher[4,5], Claudia Daubenberger[1,2], **Klaus Reither**[1,2,6]† and Christof Geldmacher[4,5]†

1. Swiss Tropical and Public Health Institute, Basel, Switzerland

2. University of Basel, Basel, Switzerland

3. NIMR-Mbeya Medical Research Center, Mbeya, United Republic of Tanzania

4. Division of Infectious Diseases and Tropical Medicine, Medical Center of the University of Munich (LMU), Germany

5. German Centre for Infection Research (DZIF), Partner Site Munich, Germany

6. Ifakara Health Institute, Bagamoyo, United Republic of Tanzania

7. The Children's Hospital at Westmead, Sydney Medical School, University of Sydney, Sydney, Australia

† Corresponding and senior authors, contributed equally.

Publication: Lancet Infect Dis. 2014 Oct;14(10):931-8.

2.4.1 Abstract

Background:

The diagnosis of paediatric tuberculosis is complicated by non-specific symptoms, difficult specimen collection, and the paucibacillary nature of the disease. We assessed the accuracy of a novel immunodiagnostic T-cell activation marker–tuberculosis (TAM-TB) assay in a proof-of-concept study to identify children with active tuberculosis.

Methods:

Children with symptoms that suggested tuberculosis were prospectively recruited at the NIMR-Mbeya Medical Research Center in Mbeya, and the Ifakara Health Institute in Bagamoyo, Tanzania, between May 10, 2011, and Sept 4, 2012. Sputum and peripheral blood mononuclear cells were obtained for *Mycobacterium tuberculosis* culture and performance assessment of the TAM-TB assay. The children were assigned to standardised clinical case classifications based on microbiological and clinical findings.

Findings:

Among 290 children screened, we selected a subgroup of 130 to ensure testing of at least 20 with culture-confirmed tuberculosis. 17 of 130 children were excluded because of inconclusive TAM-TB assay results. The TAM-TB assay enabled detection of 15 of 18 culture-confirmed cases (sensitivity 83·3%, 95% CI 58·6–96·4). Specificity was 96·8% (95% CI 89·0–99·6) in the cases that were classified as not tuberculosis (n=63), with little effect from latent tuberculosis infection. The TAM-TB assay identified five additional patients with highly probable or probable tuberculosis, in whom M tuberculosis was not isolated. The median time to diagnosis was 19·5 days (IQR 14-45) for culture.

Interpretation:

The sputum-independent TAM-TB assay is a rapid and accurate blood test that has the potential to improve the diagnosis of active tuberculosis in children.

Funding

European and Developing Countries Clinical Trials Partnership, German Federal Ministry of Education and Research, and Swiss National Science Foundation.

2.4.2 Introduction

Tuberculosis in children is a serious public health problem. Recent estimates of the tuberculosis disease burden in children, based on the results of a systematic literature review and mathematical modelling, suggest that about 1 million children developed tuberculosis worldwide in 2010, including 280 000 incident cases in the African region.[270] Surveillance data for children remain imprecise because paediatric tuberculosis is often either underdiagnosed or overdiagnosed in high-burden countries.[271],[251],[272] The lack of accurate and rapid diagnostic methods contributes to tuberculosis morbidity and mortality in children and hampers the assessment of new drugs and vaccines in paediatric populations.[272]

Diagnosis of active tuberculosis in children poses a major challenge. Clinical symptoms of tuberculosis in children are often non-specific and resemble those of common paediatric illnesses, including pneumonia and malnutrition. Adequate respiratory specimens are difficult to obtain for bacterial confirmation, particularly in very young children who are unable to expectorate and in whom diagnostic yields are poor because of the paucibacillary nature of the disease[271],[273] Hence, the diagnosis is routinely made on the basis of a combination of clinical features, contact history, chest radiography, and tuberculin skin test, and often with scoring charts that have poor diagnostic accuracy.[274],[256]

The Xpert MTB/RIF assay enables timely, sensitive, and specific molecular detection of pulmonary tuberculosis and rifampicin resistance in adults,[275] but its value in young children is greatly reduced. In a recent meta-analysis, the calculated pooled sensitivity was 66% for Xpert MTB/RIF against culture in expectorated or induced sputa, or gastric lavage specimens from children with suspected tuberculosis.[258] WHO strongly recommends Xpert MTB/RIF as the initial diagnostic test in children suspected of having multidrug-resistant or HIV-associated tuberculosis, and only conditionally in all children suspected of having tuberculosis.[258]

Immunodiagnostic tests, such as the tuberculin skin test and interferon-γ release assays—ie, QuantiFERON-TB Gold and T-SPOT.TB—do not depend on the presence of *Mycobacterium tuberculosis* in collected samples. Although these tests have use in screening special risk populations for latent tuberculosis infection, they cannot enable the crucial distinction between active tuberculosis disease and latent tuberculosis infection.[276] Failure to accurately identify cases with active tuberculosis restricts the clinical application of these tests in endemic regions where latent tuberculosis infection is ubiquitous.

Results of previous studies in adults showed that flow-cytometric analysis of the CD27 on circulating *M tuberculosis*-specific T cells can discriminate active tuberculosis from latent

tuberculosis infection.[165],[164],[277],[278],[279] Loss of CD27 expression on M tuberculosis-specific CD4 T cells is a marker of active tuberculosis due to persistent antigenic stimulation [280] and probably relates to increased cellular homing to the site of disease. [281],[282] In a proof-of-concept study, we assessed the performance of the new T-cell activation marker–tuberculosis (TAM-TB) assay for the diagnosis of active tuberculosis in children with symptoms that suggest tuberculosis.

2.4.3 Methods

Study population. This prospective diagnostic assessment was done at two Tanzanian research sites—the NIMR-Mbeya Medical Research Center, Mbeya, and the Ifakara Health Institute, Bagamoyo. Children older than 6 months and younger than 16 years with signs or symptoms that suggested tuberculosis were enrolled from May 10, 2011, until Sept 4, 2012, and followed up for a minimum of 5 months. At least one of the following eligibility criteria had to be met: persistent, non-remitting cough for more than 14 days that did not respond to antibiotics; repeated episodes of fever within the past 14 days that did not respond to antibiotics, after malaria had been excluded; weight loss or failure to thrive during the previous 3 months; and signs and symptoms that suggested extrapulmonary tuberculosis. Children who received tuberculosis treatment in the past 12 months were excluded. The children were referred from peripheral health facilities and local hospitals. Because of cost restrictions, a subgroup of 130 children was selected from a larger cohort of 290 children to ensure testing of at least 20 children with culture-confirmed tuberculosis after prevalence estimation at the two sites. All other clinical information was masked from the investigators doing the selection with a list of patient identification numbers with corresponding disease classifications.

The Institutional Review Board of the Ifakara Health Institute, the Mbeya Medical Research and Ethics Committee, and the Medical Research Coordinating Committee of Tanzania approved the study protocol. We obtained written informed consent from a literate parent or legal guardian. In cases of illiteracy, informed oral consent was attested by an independent witness. Children older than 7 years provided assent for participation.

Classification and reference standard. Classification of children was based on the results of the clinical and microbiological assessment as culture-confirmed tuberculosis (culture-positive for M tuberculosis), highly probable tuberculosis (chest radiograph consistent with tuberculosis confirmed by two independent reviewers, histology or cytology typical for tuberculosis, or fluorescent or acid-fast bacilli on microscopy), probable tuberculosis (clinically suspected tuberculosis without objective findings as above), not tuberculosis (alternative diagnosis

established and clinical resolution without antituberculosis treatment), or indeterminate (any other combination; appendix). Culture-confirmed tuberculosis was used as the reference standard for sensitivity analysis and not tuberculosis as the reference standard for specificity assessment.

Clinical and laboratory procedures. Clinical procedures at enrolment comprised medical history, physical examination, HIV testing, CD4 T-cell count, interferon-γ release assay (QuantiFERON-TB Gold, Cellestis, Melbourne, VIC, Australia), and chest radiography. Chest radiographs were classified as strongly indicating, uncertain, or not tuberculosis by two independent experts from whom all clinical and diagnostic information was masked. Malnutrition was assessed on the basis of weight-for-age Z score in children aged up to 10 years (less than −2) and body-mass index in those older than 10 years (<2 SD below normal). If feasible, at least three induced or two expectorated respiratory specimens were obtained on consecutive days. Induced sputum was obtained in accordance with a standard protocol.[283] Fine-needle aspiration biopsies of enlarged lymph nodes were done when clinically indicated, as per standard protocol.[284]

After N-acetyl L-cysteine-sodium hydroxide decontamination, each sputum sample pellet was assessed with microscopy by use of Ziehl-Neelsen staining. At least one sample was inoculated on both liquid (BACTEC MGIT 960, Becton Dickinson, Franklin Lakes, NJ, USA) and solid media (Loewenstein-Jensen culture). Positive cultures were confirmed by use of microscopy, and subsequent MPT64 antigen or molecular tests (Genotype MTBC or CM, Hain Lifescience, Nehren, Germany) or both. GenoType MTBDRplus (Hain Lifescience) or phenotypic drug-susceptibility testing (BACTEC MGIT 960 SIRE kit, Becton Dickinson) was used for resistance testing. Xpert MTB/RIF (Cepheid, Sunnyvale, CA, USA) was done in at least one sputum sample according to the manufacturer's instructions. All tests, including TAM-TB assay, were done by trained laboratory technical staff masked to clinical information and radiological results.

Results from established diagnostic procedures were made available to support clinical management in accordance with national and international guidelines. Results of the experimental TAM-TB assay were not released.

TAM-TB assay. Details of the underlying biological principle of the TAM-TB assay, definition of cutoff values in an optimisation cohort of adults (n=87), and additional background information are provided in the appendix.

Briefly, the TAM-TB assay is used to measure the CD27 phenotype of CD4 T cells producing interferon γ in response to M tuberculosis antigens according to a standard intracellular

cytokine staining procedure.[278] *M tuberculosis-specific* CD4 T-cell responses were judged positive when at least five CD4 T-cell interferon-γ-producing events were detected, and the proportion of interferon-γ producing CD4 T cells after antigen stimulation was greater than 0·05% and was at least twice the frequency of that in the negative controls.

Well characterised samples from adults (appendix) were used to identify the CD27 median fluorescence intensity (MFI) ratio with optimum discriminatory power between latent tuberculosis infection and active tuberculosis. Cryopreserved peripheral blood mononuclear cells obtained at the baseline visit were stimulated for 12–16 h with a set of overlapping ESAT-6/CFP-10 peptides [21] (Elephants and Peptides, Potsdam, Germany) and purified protein derivative (Statens Serum Institut, Copenhagen, Denmark) before staining with fluorochrome-labelled antibodies. We compared the MFI of CD27 staining on interferon-γ-positive M tuberculosis-specific CD4 T cells to the CD27 MFI value for all CD4 T cells to define the CD27 MFI ratio for a sample (appendix). CD27 MFI ratio results were consistent in 17 independent quality control assessments of two batches of peripheral blood mononuclear cells (appendix).

Adult patients with tuberculosis had significantly higher CD27 MFI ratios than did control patients after ESAT-6/CFP-10 or purified protein derivative stimulation; area-under-operating-characteristic curves were 0·931% and 0·881%, respectively (appendix). We set CD27 MFI ratio thresholds at greater than 5 for ESAT-6/CFP-10 and greater than 13 for purified protein derivative responses to achieve a balanced TAM-TB assay sensitivity of 83·3% (95% CI 68·6–93·0) and specificity of 83·7% (69·3–93·2) in this optimisation cohort. Once these cutoff values were ascertained, samples from paediatric tuberculosis suspects were tested (validation cohort). TAM-TB assays were done at two independent laboratories according to identical standard operating procedures.

Statistical analysis. Calculation of medians, IQR, test accuracy measures (sensitivity and specificity), Kruskall-Wallis ANOVA with post-test correction (Dunn's), and Mann-Whitney and Fisher's exact tests were done with GraphPad Prism software (version 4.03).

Role of the funding source. The study was funded by the European and Developing Countries Clinical Trials Partnership, which had no role in study design, data gathering, analysis, and interpretation, or writing of the report. The corresponding author had full access to all the data and final responsibility for the decision to submit for publication.

2.4.4 Results

After assay optimisation in adults (optimisation cohort; appendix), the diagnostic performance of the TAM-TB assay was assessed in 130 children suspected to have tuberculosis (validation cohort); samples from 113 (87%) children were eligible for analysis. Figure 17 shows the study profile;[73] 17 (13%) children were excluded from the study because of inconclusive results. 18 (16%) of 113 eligible children had culture-confirmed tuberculosis, eight (7%) highly probable tuberculosis, 12 (11%) probable tuberculosis, 63 (56%) not tuberculosis, and 12 (11%) indeterminate cases (figure 17). A fine-needle aspiration biopsy of enlarged lymph nodes was done in five children; the final classifications were culture confirmed tuberculosis (n=3), probable tuberculosis (n=1), and not tuberculosis (n=1). All culture-confirmed tuberculosis cases were positive on assessment of a respiratory specimen, including one with additional cytomorphology that suggested tuberculous lymphadenitis. Nine (50%) of 18 children with culture-confirmed tuberculosis were sputum smear-positive. Six of eight patients with highly probable tuberculosis had chest radiographs that strongly suggested tuberculosis, one had chronic granulomatous lymphadenitis, and one was sputum smear-positive. Patients with probable tuberculosis had symptoms that suggested infection, which resolved completely on tuberculosis treatment, but radiographic signs and laboratory investigations were non-conclusive.

Table 12 shows the demographic and clinical characteristics of the children. The median age of the 113 children included in the analysis was 6·1 years (table 12). 33 of 113 children had HIV infection (table 12). According to the WHO immunological classification for established HIV infection, [286] 19 of the HIV-infected children had severe immunodeficiency (table 12). 37 of 38 children with culture-confirmed, highly probable, or probable tuberculosis received tuberculosis treatment, and one child died before the positive M tuberculosis culture result became available and treatment could be started. 33 of 37 children reported symptom resolution on treatment, one child with highly probable tuberculosis died, and three who were classified as culture confirmed, highly probable, or probable tuberculosis were lost to follow-up. No drug resistance was detected in the culture-confirmed cases.

Respiratory specimens could be collected from each child; 103 (91%) of 113 children provided at least three induced or two expectorated sputum samples. Figure 18 shows the overlap between children with a positive M tuberculosis culture, Xpert MTB/RIF, or TAM-TB assay, and table 13 shows the TAM-TB assay results. 15 of 18 children with culture-confirmed tuberculosis had a positive TAM-TB assay result (sensitivity 83·3%, 95% CI 58·6–96·4; table 14). Of the 63 cases classified as not tuberculosis, 61 had a negative TAM-TB assay (specificity 96·8%, 89·0–

99·6; table 14). Findings were similar at the two independent sites Bagamoyo and Mbeya (table 14). The sensitivity of the TAM-TB assay was 69·2% (48·2–85·6) when both culture-confirmed and highly probable tuberculosis cases were included in the reference standard.

Table 15 summarises the clinical characteristics, tuberculin skin test and QuantiFERON-TB Gold results, diagnosis at enrolment, response to antibiotic treatment, and clinical follow-up of all children with discrepant TAM-TB assay and M tuberculosis culture results. Of the eight children who were TAM-TB assay positive but culture-negative (figure 18), three cases were classified as highly probable tuberculosis, two as probable tuberculosis, two as not tuberculosis, and one as indeterminate; six were HIV infected (table 15). Of the three children with highly probable tuberculosis, one was sputum smear-positive at enrolment, one was culture-confirmed during follow up, and one had cytological signs (chronic granulomatous lymphadenitis) that suggested tuberculosis. In the two probable tuberculosis cases, broad-spectrum antibiotics had no effect, but symptoms resolved on tuberculosis treatment (table 15). The TAM-TB assay was positive in two HIV-infected patients who were classified as not tuberculosis (table 15). *Mycobacterium interjectum* was identified in one case with an induration of 10 mm on the tuberculin skin test and an indeterminate QuantiFERON-TB Gold result. The other patient was QuantiFERON-TB Gold-positive. One HIV-infected child who was classified as indeterminate, had a complex clinical picture with various disease episodes that never fully resolved. Of the three children who were M tuberculosis culture-positive, but TAM-TB assay negative, two were malnourished (table 15). The third child had a nephrotic syndrome and died before culture results were available (table 15).

Of the 63 children classified as not tuberculosis, 15 (24%) had a positive tuberculin skin test or QuantiFERON-TB Gold test at enrolment, suggesting probable latent tuberculosis infection. TAM-TB assay was positive in two (13%) of these 15 children. Xpert MTB/RIF enabled detection of 12 (67%) of 18 culture-confirmed tuberculosis cases, giving a sensitivity of 66·7% (95% CI 41·1–85·6) when two or more sputum samples obtained on consecutive days were analysed. The sensitivity was 55·6% (31·3–77·6) when only the first sputum sample was analysed. The specificity of Xpert MTB/RIF was 100% (94·2–100; data were missing for one person). Xpert MTB/RIF was positive in two of three children with culture-confirmed tuberculosis who were TAM-TB assay negative (figure 18). The sensitivity achieved with the combination of TAM-TB assay and Xpert MTB/RIF was 94·4% (72·6–99·1). The median time to detection by use of culture, defined as the period between enrolment and first M tuberculosis confirmation, was 19·5 days (IQR 14·0–45·0), whereas TAM-TB assay results were obtained within 24 h after recovery of peripheral blood mononuclear cells.

2.4.5 Discussion

The TAM-TB assay showed good sensitivity and excellent specificity with *M tuberculosis* culture as a reference standard. Specific detection of active tuberculosis in children was based on cutoff values set from the optimisation study in adults. Retrospectively lowering CD27 ratio thresholds to 2 for ESAT-6/CFP-10 and 7 for purified protein derivative stimulation, would further improve assay sensitivity from 83·3% to 88·9% (95% CI 88·0–99·4%) without affecting specificity with additional detection of highly probable (n=1), probable tuberculosis (n=1), and indeterminate (n=1) cases. Hence, cutoff values specifically optimised for children might further improve TAM-TB accuracy. Contrary to molecular-based assays like Xpert MTB/RIF and microbiological tests, the TAM-TB assay can be done on a readily available peripheral blood sample and is not limited by the paucibacillary nature of active tuberculosis in children. Consistent test performance at both study sites suggests the assay is robust and repeatable. Of greatest clinical relevance is that the TAM-TB assay provides an answer within a day of blood collection, which is important because early treatment initiation can be crucial in young children at high risk of disseminated tuberculosis disease.[271],[287]

Of the children with discrepant results, five of eight children who were M tuberculosis culture-negative and TAM-TB assay positive had highly probable or probable tuberculosis. The clinical classification used might have included some overdiagnosis. [272] International consensus definitions of intrathoracic tuberculosis were not available when the study was designed. [288] However, detailed case assessment suggested that these children probably did have active tuberculosis, suggesting that the TAM-TB assay is probably at least as sensitive as M tuberculosis culture.

Despite access to state-of-the-art tuberculosis diagnostics, most children enrolled in our study were treated for tuberculosis based on a combination of epidemiological and clinical findings. This shows the poor diagnostic usefulness of M tuberculosis culture in clinical practice, related to difficult specimen collection, suboptimum sensitivity in children with paucibacillary disease, and long turnaround times. Suboptimum sensitivity undermines the suitability of the test as a reference standard and complicates optimal assessment of diagnostic accuracy. Consistent with previous paediatric studies, Xpert MTB/RIF enabled the detection of culture-confirmed paediatric cases from expectorated or induced sputa with a sensitivity of 66·7% and a specificity of 100%.%.[289],[290],[291] Compared with M tuberculosis culture, the combination of Xpert MTB/RIF and TAM-TB, two assays with a turnaround time of less than 24 h, had a sensitivity of 94%, missing one case. This child had severe nephrotic syndrome and culture-confirmation was only achieved after the child had passed away. The combination of TAM-TB assay and Xpert

MTB/RIF enabled the detection of more tuberculosis cases than did culture when both culture-confirmed and highly probable tuberculosis cases were included in the reference standard.

The TAM-TB assay was highly accurate in identifying children without tuberculosis, including 13 children with a positive interferon-γ release assay or the result of the tuberculin skin test. Hence, the assay has a high specificity for tuberculosis disease in children despite immunological evidence of previous M tuberculosis exposure, consistent with data from the adult optimisation cohort and a previous study in adults.[279] Only two not tuberculosis cases were incorrectly assigned. Both children were infected with HIV, one had simultaneous infection with non-tuberculous mycobacteria and both had latent tuberculosis infection. These false-positive TAM-TB results were caused by a predominance of mycobacteria-specific effector memory T-cell responses in peripheral blood. In view of a positive CFP-10/ESAT-6 response in the child without non-tuberculous mycobacteria infection, the positive TAM-TB assay result might suggest recent M tuberculosis infection that did not progress to active disease during the period of observation. In the other case, the non-tuberculous mycobacteria (M interjectum) identified in the sputum might have elicited the positive TAM-TB assay result. Implementation of additional, highly immunogenic M tuberculosis-specific antigens in the next generation TAM-TB assay could further improve specificity and sensitivity.

The current version of the TAM-TB assay has several limitations, mainly related to cost and technical complexity. It needs advanced blood processing procedures, antigenic stimulations, flow cytometry equipment, and well trained staff. Refinement and simplification are in progress to optimise diagnostic performance and make it compatible with cytometers that are in widespread use for measurement of CD4 T-cell counts in HIV/AIDS-affected countries. The TAM-TB assay did not generate valid test results in 13% of the samples of peripheral blood mononuclear cells, but this might be attributed to reduced T-cell viability and decreased cytokine production after cryopreservation.[292] Ideally, the TAM-TB assay should be done on fresh whole blood samples—eg, in antigen-precoated tubes similar to the commercial QuantiFERON In-Tube system (Cellestis).[293] Low CD4 T-cell counts due to severe HIV infection might remain a problem even when fresh blood samples are used. The small sample size of this proof-of-concept study resulted in very wide confidence intervals and did not allow systematic assessment of test performance in very young, malnourished, or HIV-infected children who have recently been started on antiretroviral therapy. These factors might negatively affect the accuracy of the TAM-TB assay.[279],[294] Additional studies need to be done to specifically address test performance in these patient groups. The assessment of the TAM-TB assay in a study population enriched for culture-confirmed tuberculosis cases might

have been a source of bias, particularly for the analysis including the under-represented classification groups. Although the TAM-TB assay cannot provide information about drug susceptibility, use of concomitant Xpert MTB/RIF testing can address this limitation.

To our knowledge, this study is the first to assess the diagnostic performance of the novel TAM-TB assay in children (panel). Importantly, it was done in a region with a high tuberculosis incidence and recruited children with symptoms that suggested tuberculosis who represent the real-life diagnostic challenge in these settings. Despite a need for further refinement and testing in other regions with high burden of tuberculosis, our results suggest that the sputum-independent TAM-TB assay is a major advance for the rapid and accurate diagnosis of tuberculosis in children.

Panel: Research in context

Systematic review. As of June 10th 2014, searching PubMed using the terms "CD27", "diagnosis", "human" and "tuberculosis" returned 33 results. Seven clinical studies from five independent laboratories explicitly demonstrated the potential of monitoring CD27 expression on *M. tuberculosis*-specific CD4 T cells to diagnose active tuberculosis disease in adults. We did not find a report assessing the use of *M. tuberculosis*-specific T cell phenotype for the diagnosis of active childhood tuberculosis.

Interpretation. Our report is the first to assess the accuracy of using CD27 expression analysis for the diagnosis of paediatric tuberculosis. Immunological data were generated and analysed blinded to clinical parameters. The T cell activation marker (TAM)-TB assay showed excellent sensitivity (83·3%) and specificity (96·8%) when using *M. tuberculosis* culture positivity as a reference standard, and detected additional cases in culture negative children who were clinically suspected of having tuberculosis. Despite a relatively small sample size and potential bias originating from cohort enrichment in culture-confirmed tuberculosis cases, this proof of concept study demonstrates that immunodiagnostic tests that incorporate phenotypic characteristics of *M. tuberculosis*-specific T cells have the potential to improve rapid detection of active tuberculosis in children.

Conflicts of interest

We declare that we have no conflicts of interest.

Acknowledgments

We want to thank all children who participated in the study, and the children's carers. We thank Philipp Metzger for his support in the immunology laboratory. The authors gratefully

acknowledge funding from the European and Developing Countries Clinical Trials Partnership (EDCTP) for this study as part of the TB CHILD project (IP.2009.32040.007). Co-funding was provided by the German Federal Ministry of Education and Research (BMBF Grant 01KA1006), and the Swiss National Science Foundation (32EC30_131192/1). Christof Geldmacher`s salary is funded by the German Center for Infection Research (DZIF).

2.4.6 Figures

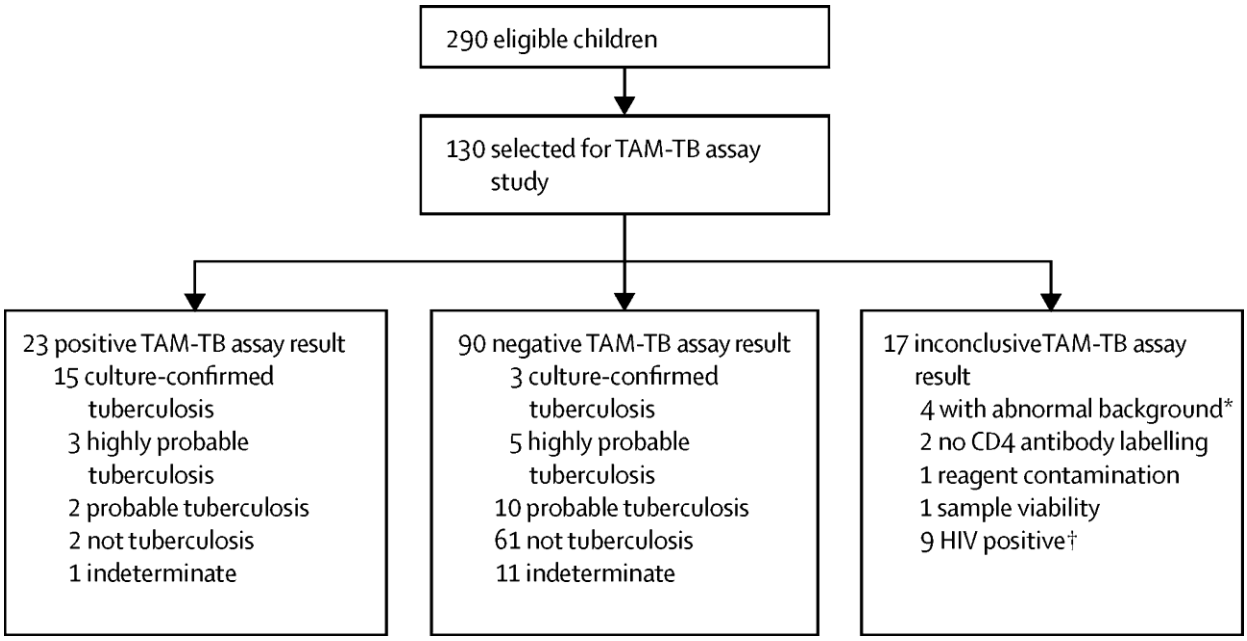


Figure 17: Study profile
TAM-TB=T-cell activation marker–tuberculosis. *Frequency of interferon-γ-positive CD4 T cells in the negative control greater than the median frequency plus 3 SD of the tested sample. †CD4 T cell count of less than 10 000 per μL and interferon-γ CD4 T cells less than 0·05%.

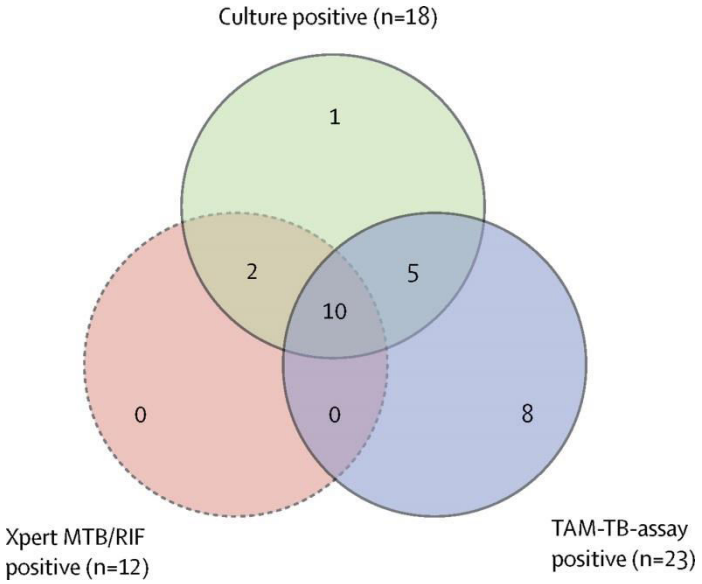


Figure 18: Venn diagram of positive *Mycobacterium tuberculosis* culture, Xpert MTB/RIF, and TAM-TB-assay results, and TAM-TB-assay results.

TAM-TB=T-cell activation marker–tuberculosis.

2.4.7 Tables

	All patients (n=113)	**Culture-confirmed tuberculosis (n=18)**	**Highly probable tuberculosis (n=8)**	**Probable tuberculosis (n=12)**	**Not tuberculosis (n=63)**	**Indeterminate (n=12)**
Age (years)	6·1 (2·1–10·3)	4·6 (1·5–12·7)	8·1 (5·2–11·9)	6 (1·8–10·5)	6·1 (2·0–9·8)	5·4 (2·2–10·5)
Female sex	52 (46%)	8 (44%)	5 (63%)	6 (50%)	27 (43%)	6 (50%)
Symptoms at enrolment						
Cough	108 (96%)	16 (89%)	6 (75%)	12 (100%)	62 (98%)	12 (100%)
Fatigue or lethargy	28 (25%)	8 (44%)	2 (25%)	2 (17%)	16 (25%)	0
Wheezing	16 (14%)	1 (6%)	1 (13%)	3 (25%)	9 (14%)	2 (17%)
Breathing difficulties	47 (42%)	10 (56%)	4 (50%)	8 (67%)	22 (35%)	3 (25%)
Fever	82 (73%)	16 (89%)	6 (75%)	9 (75%)	45 (71%)	6 (50%)
Chest pain	23 (20%)	1 (6%)	0	1 (8%)	18 (29%)	3 (25%)
Haemoptysis	4 (4%)	1 (6%)	0	1 (8%)	1 (2%)	1 (8%)
Enlarged lymph nodes	12 (11%)	5 (28%)	1 (13%)	1 (8%)	5 (8%)	0
Weight loss	55 (49%)	13 (72%)	3 (38%)	5 (42%)	32 (51%)	2 (17%)
Abdominal pains	20 (18%)	5 (28%)	2 (25%)	3 (25%)	9 (14%)	1 (8%)
Malnutrition	60 (53%)	12 (67%)	4 (50%)	5 (42%)	24 (38%)	5 (42%)
HIV infection	33 (29%)	4 (22%)	3 (38%)	7 (58%)*	15 (24%)	4 (33%)
WHO immunological staging						
Not clinically relevant	7/33 (21%)	0/4 (0%)	0/3 (0%)	2/7 (29%)	4/15 (27%)	1/4 (25%)
Mild	6/33 (18%)	1/4 (25%)	1/3 (33%)	2/7 (29%)	2/15 (13%)	0/4 (0%)
Advanced	1/33 (3%)	1/4 (25%)	0/3 (0%)	0/7 (0%)	0/15 (0%)	0/4 (0%)
Severe	19/33 (58%)	2/4 (50%)	2/3 (67%)	3/7 (43%)	9/15 (60%)	3/4 (75%)
On antiretroviral therapy	13/33 (39%)	2/4 (50%)	1/3 (33%)	2/7 (29%)	7/15 (47%)	1/4 (25%)
Positive tuberculin skin test	31/103 (30%)	13/17 (76%)	3/8 (38%)	2/11 (18%)	9/57 (16%)	4/10 (40%)
Positive interferon-γ release assay	27/110 (25%)	13/18 (72%)	1/7 (14%)	3/12 (25%)	8/61 (13%)	4/12 (33%)
Positive tuberculin skin test or interferon-γ release assay	47/112 (42%)	17/18 (94%)	3/8 (38%)	4/12 (33%)	15/63 (24%)	4/11 (36%)

Table 12: Demographics and clinical characteristics of study participants by classification group.

Data are median (IQR), number (%), or n/N (%).

*p=0·033 compared with not tuberculosis distribution (Fisher's exact test).

	Culture-confirmed tuberculosis (n=18)	Highly probable tuberculosis (n=8)	Probable tuberculosis (n=12)	Not tuberculosis (n=63)	Indeterminate (n=12)
Assay-positive cases	15 (83%)	3 (38%)	2 (17%)	2 (3%)	1 (8%)
Assay-negative cases	3 (17%)	5 (63%)	10 (83%)	61 (97%)	11 (92%)

Table 13: T-cell activation marker–tuberculosis assay results by classification groups.

	Bagamoyo (n=63)	Mbeya (n=50)	All patients (n=113)
Sensitivity (95% CI)	83·3% (35·9–99·6)	83·3% (51·6–97·9)	83·3% (58·6–96·4)
Positive/total	5/6	10/12	15/18
Specificity (95% CI)	95·6% (84·9–99·5)	100% (81·5–100)	96·8% (89·0–99·6)
Negative/total	43/45	18/18	61/63
Disease prevalence	7/115 (6%)	22/175 (13%)	29/290 (10%)
Positive predictive value	54·9%	100%	74·5%
Negative predictive value	98·9%	97·7%	98·1%

Table 14: Sensitivity, specificity, and predictive values of the T-cell activation marker–tuberculosis assay.

Data are n/N (%), unless otherwise indicated. Culture-confirmed tuberculosis and not tuberculosis were the reference standards for sensitivity and specificity. Calculation of predictive values was based on disease prevalence in the main paediatric cohort (n=290).

	Clinical case classification and reason for classification	**Tuberculin skin test or QuantiFERON**	**HIV status**	**First-visit clinical diagnosis, treatment, and response**	**Follow-up clinical diagnosis, treatment, and outcome**	**Clinical case classification and reason for classification**
Culture-negative and TAM-TB assay-positive cases						
15 years	Highly probable tuberculosis by radiograph	Positive	Negative	Pneumonia Amoxicillin No	Pulmonary tuberculosis later confirmed by culture and Xpert MTB/RIF 2 months of isoniazid, rifampicin, pyrazinamide, and ethambutol, and 4 months of isoniazid and rifampicin Resolved	15 years
7 years	Highly probable tuberculosis by sputum smear	Positive	Negative	Pneumonia Amoxicillin No	Pulmonary tuberculosis 2 months of isoniazid, rifampicin, pyrazinamide, and ethambutol, and 4 months of isoniazid and rifampicin Resolved	7 years
9 years	Highly probable tuberculosis by lymph node cytology	Positive	Positive	Lymphadenitis Ceftriaxone No	Tuberculosis lymphadenitis 2 months of isoniazid, rifampicin, pyrazinamide, and ethambutol, and 4 months of isoniazid and rifampicin Resolved	9 years
11 years	Probable tuberculosis by symptoms, resolution after treatment	Negative	Positive	Pneumonia Ceftriaxone No	Pulmonary tuberculosis 2 months of isoniazid, rifampicin, pyrazinamide, and ethambutol, and 4 months of isoniazid and rifampicin Resolved	11 years
1 year	Probable tuberculosis by symptoms, resolution after treatment	Positive	Positive	Chest infection Cefalexin No	Pulmonary tuberculosis 2 months of isoniazid, rifampicin, pyrazinamide, and ethambutol, and 4 months of isoniazid and rifampicin Resolved	1 year
5 years	Not tuberculosis, no treatment, and healthy after 5 months	Positive	Positive	Pneumocystis pneumonia plus *Mycobacterium interjectum* Co-trimoxazole Resolved	Recovered from initial diagnosis	5 years
7 years	Not tuberculosis, no treatment, and healthy after 5 months	Positive	Positive	Pneumonia, urinary tract infection Antibiotics Resolved	Intestinal helminths Albendazole Resolved, new dry cough 6–9 months after recruitment	7 years
10 years	Indeterminate	Positive	Positive	Bronchiectasis Metronidazole Moderate	Bronchiectasis, malaria Antimalarial Persisting cough, mediastinal lymphadenopathy	10 years
Culture-positive and TAM-TB assay-negative cases						
14 years	Culture-confirmed tuberculosis with *Mycobacterium tuberculosis* in sputum	Positive	Negative	Pulmonary tuberculosis, malnutrition 2 months of isoniazid, rifampicin, pyrazinamide, and ethambutol, and 4 months of isoniazid and rifampicin Unchanged	Pulmonary tuberculosis 2 months of isoniazid, rifampicin, pyrazinamide, and ethambutol, and 4 months of isoniazid and rifampicin Resolved	14 years
8 years	Culture confirmed tuberculosis	Positive	Negative	Nephrotic syndrome Furosemide,	Pulmonary tuberculosis (only retrospectively confirmed) No tuberculosis treatment	8 years

	with *Mycobacterium tuberculosis* in sputum			prednisolone Deterioration, hospital admission	Death	
2 years	Culture confirmed tuberculosis with *Mycobacterium tuberculosis* in sputum	Negative	Negative	Pulmonary tuberculosis, malnutrition 2 months of isoniazid, rifampicin, pyrazinamide, and ethambutol, and 4 months of isoniazid and rifampicin Unchanged	Pulmonary tuberculosis 2 months of isoniazid, rifampicin, pyrazinamide, and ethambutol, and 4 months of isoniazid and rifampicin Resolved	2 years

Table 15: Cases with discrepant culture and TAM-TB assay results: clinical characteristics at enrolment and follow-up visits by age

Positive tuberculin skin test suggested by a lesion of at least 10 mm in HIV-uninfected or at least 5 mm in HIV-infected children. TAM-TB=T-cell activation marker–tuberculosis.

2.4.8 Supplementary appendix

Defining optimal TAM-TB assay cut-off values for detection of active Tuberculosis disease in the adult optimisation cohort

Development of rapid, accurate and sputum-independent diagnostic tools for detection of pulmonary and extra-pulmonary tuberculosis and its differentiation from other conditions with similar clinical presentation is essential for improving the management of paediatric tuberculosis suspects. Current immunodiagnostic methods, such as the TST or IGRAs lack the ability to discriminate latent *M. tuberculosis* infection (LTBI) from active tuberculosis disease. While also based on the detection of *M. tuberculosis*-specific memory T cell responses, the TAM-TB assay incorporates phenotypic T cell characterization; downregulation of CD27 on Interferon gamma (IFN-γ)+ *M. tuberculosis*-specific CD4 T cells differentiates active tuberculosis disease from latent *M. tuberculosis* infection (LTBI).[165],[164],[277],[278],[279],[154] Loss of CD27 expression is a marker of late stage effector T cell differentiation and a consequence of persistent antigenic stimulation.[280],[295] The vast majority of peripheral blood CD4 T cells express CD27, whereas a CD27 negative phenotype is specific for the comparatively small fraction of mature effector memory CD4 T cells (Supplementary Figure 2B).[296]

In murine *M. tuberculosis* infection models, CD27-effector memory CD4 T cells preferentially home to non-lymphoid tissues, including the lung.[281] In humans, the CD27 negative phenotype is characteristic of CD4 T cells collected by bronchalveolar lavage.[297] Hence, with

active tuberculosis, CD27 down regulation on peripheral blood *M. tuberculosis* -specific CD4 T cells most probably reflects increased cellular homing to the site of disease.

Defining consistent cut-off values for CD27 expression is essential to assess the diagnostic value of this biomarker. CD4 T cells with intermediate CD27 receptor density are abundant and variable in peripheral blood, complicating the definition of a consistent cut-off value. In order to reduce test variability, we compared the median fluorescence intensity (MFI) of CD27-staining of IFN-γ^+ *M. tuberculosis*-specific CD4 T cells (after ESAT-6/CFP-10 and PPD stimulation) to the CD27 MFI value for all CD4 T cells to define the CD27 MFI ratio (Supplementary Figure 2B). We used well-characterized adult samples (the "optimisation cohort") to identify the CD27 MFI ratio with optimal discriminatory power between LTBI and active tuberculosis disease, before testing it in child tuberculosis suspects (the "validation cohort"). We tested samples from healthy HIV-uninfected IGRA- and IGRA+ adults and in patients with culture-confirmed pulmonary tuberculosis (HIV-infected and uninfected).

Study subjects

A case-control study was conducted in selected adults (n=87) from October 2010 to November 2012 in Bagamoyo and Mbeya, Tanzania (Supplementary Figure 1). Active pulmonary tuberculosis was defined by the presence of at least one positive *M. tuberculosis*-culture from sputum. Adult healthy controls had to be HIV-negative, free of any disease symptoms and without a history of previous tuberculosis. The healthy controls were further stratified by their interferon gamma release assay (IGRA) result (Bagamoyo: QuantiFERON; Mbeya: in-house ESAT-6/CFP-10 ELISPOT assay). In order to define the optimal CD27 MFI ratio cut-off values we included healthy controls who were either IGRA+ or responding to PPD stimulation. The study protocol was approved by the Institutional Review Board of the Ifakara Health Institute, the Mbeya Medical Research and Ethics Committee, and the Medical Research Coordinating Committee of Tanzania. Written informed consent was obtained from literate participants. In case of illiteracy, informed oral consent was attested by an impartial witness.

TAM-TB assay. Thawed PBMCs were rested for 2h and subsequently stimulated for 12-16h in the presence of anti-CD28/CD49d (BD Pharmingen, 2□g/ml final) and Brefeldin A (Sigma, 5mg/ml final) with two sets of antigens: (1) ESAT-6/CFP-10 peptide pools (2 µg/ml, Elephants & Peptides, Germany),[285] and (2) Purified Protein Derivative (PPD) for *in vitro* use (10 µg/ml, Statens Serum Institute, Denmark). Stimulation with Staphylococcal Enterotoxin B (SEB) served as a positive control (0·8 µg/ml final, Sigma-Aldrich, USA), and no antigen stimulation as a negative control. We used the following antibodies: anti-CD4-PerCP Cy5.5 (Oct4, ebioscience),

anti-CD8 Horizon V500 (RPA-T8, BD Pharmingen) and anti-CD27-APC H7 (M-T271, BD Pharmingen) and for intracellular staining in combination with BD Cytofix/Cytoperm™ Buffer and BD Perm/Wash™ Buffer (BD Pharmingen): anti-CD3-Pac Blue (UCHT-1, BD Pharmingen) and anti-IFNγ FITC (B27, BD Pharmingen).

Stained PBMCs were then acquired on calibrated BD FACSCanto II (Mbeya) or BD LSR II Fortessa (Bagamoyo) flow cytometers allowing the detection and CD27-based phenotypic characterization of *M. tuberculosis*-specific CD4 T cells. The data were analysed using FlowJo software version 9·0 (Tree Star Inc., USA).

Demographics

The median age of the culture-confirmed adult pulmonary tuberculosis cases (n=44) was 33 years (IQR 29-38·4), significantly higher than the median age of the IGRA-negative healthy controls (n=18; 21·3 years; IQR 19·7-29; p<0·01) but similar to the median age of IGRA-positive healthy controls (n=30; 30 years; IQR 18·1-38). The prevalence of HIV infection among adult tuberculosis cases was 61·9%.

Definition of the CD27 MFI ratio and Cut-offs values for the TAM-TB assay

As expected, neither detection of IFN-γ+ *M. tuberculosis*-specific CD4 T cells after ESAT-6/CFP-10 or PPD stimulation, nor their frequency, could discriminate healthy IGRA+ controls from confirmed tuberculosis cases (Supplementary Figure 2A). The TAM-TB assay included two criteria; a) the presence of *M. tuberculosis*-specific CD4 T cell responses and b) a predominant $CD27^{low}$ cell phenotype among *M. tuberculosis*-specific CD4 T cells. Positive *M. tuberculosis*-specific CD4 T cell responses were defined by: a) a frequency of CD4 T cells producing IFN-γ of ≥0·05%, b) a 2-fold increase compared to unstimulated controls and c) acquisition of at least 5 events identified as IFN-γ producing CD4 T cells on antigen stimulation (as above).

The TAM-TB test was considered negative ("no tuberculosis") if a positive *M. tuberculosis*-specific CD4 T cell response was not detected. When a positive *M. tuberculosis*-specific CD4 T cell response was detected, results were further stratified using the CD27 median fluorescence intensity (MFI) ratio illustrated in supplementary figure 2B. The CD27 MFI of *M. tuberculosis*-specific CD4 T cells was divided by the CD27 MFI of IFN-γ+ CD4 T cells (Supplementary Figure 2B). Hence, a lower CD27 MFI of the *M. tuberculosis*-specific CD4 T cell population in a given sample results in an increased CD27 MFI Ratio. Active tuberculosis was significantly correlated with an elevated CD27 MFI ratio (p<0·0001, Supplementary Figure 2D). Receiver operating characteristic (ROC) curves of responders confirmed a high probability that a randomly selected

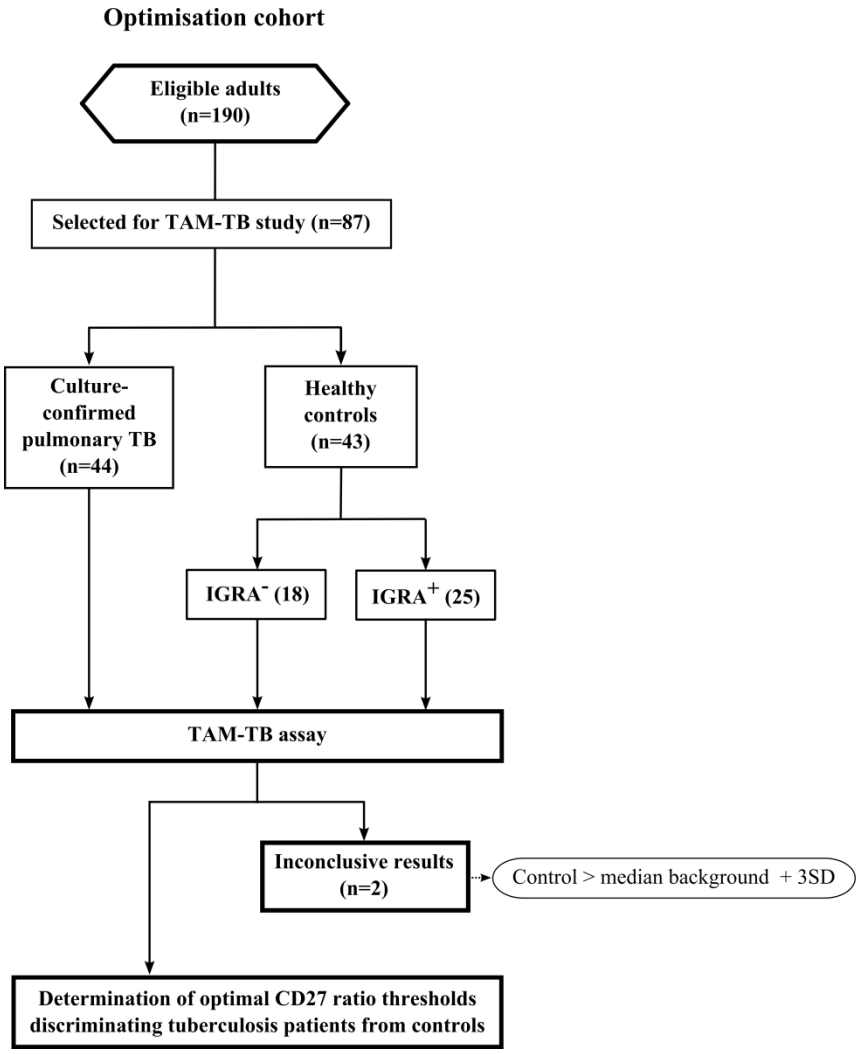
tuberculosis patient will harbour a significantly higher CD27 MFI ratio than a randomly selected control after ESAT-6/CFP-10 or PPD stimulation (area under the curve (AUC) = 0·931% and 0·881 respectively) compared to merely measuring the frequencies of antigen-specific CD4-T cells producing IFN-γ (AUC = 0·597 and 0·573 using ESAT-6/CFP-10 or PPD stimulation respectively) (Supplementary Figure 2C). Hence, further stratification of positive *M. tuberculosis*-specific CD4 T cell responses using the CD27 MFI ratio greatly enhances the discriminatory power by the TAM-TB assay and hence specificity for active tuberculosis disease. Importantly, CD27 MFI ratio results were consistent in 17 independent quality control experiments using two batches of Peripheral Blood Mononuclear Cells (Supplementary Figure 2E).

Optimal CD27 MFI ratio cut-off values for accurate detection of active tuberculosis were defined by comparing the CD27 MFI ratios from adults with culture-confirmed pulmonary tuberculosis and positive responses to ESAT-6/CFP-10 (n=33) or PPD (n=38) respectively, with those from adult healthy controls with detectable responses to ESAT-6/CFP-10 (n=17) or PPD (n=43). For ESAT-6/CFP-10-specific CD4 T cells, the optimal CD27 MFI ratio cut-off was defined as >5 and for PPD-specific CD4 T cells as >13. Using these threshold criteria, for both stimuli, the TAM-TB assay achieved a sensitivity of 83·3 % (95% CI, 68·6-93) and a specificity of 83·7% (95% CI, 69·3-93·2) in this adult "optimisation cohort".

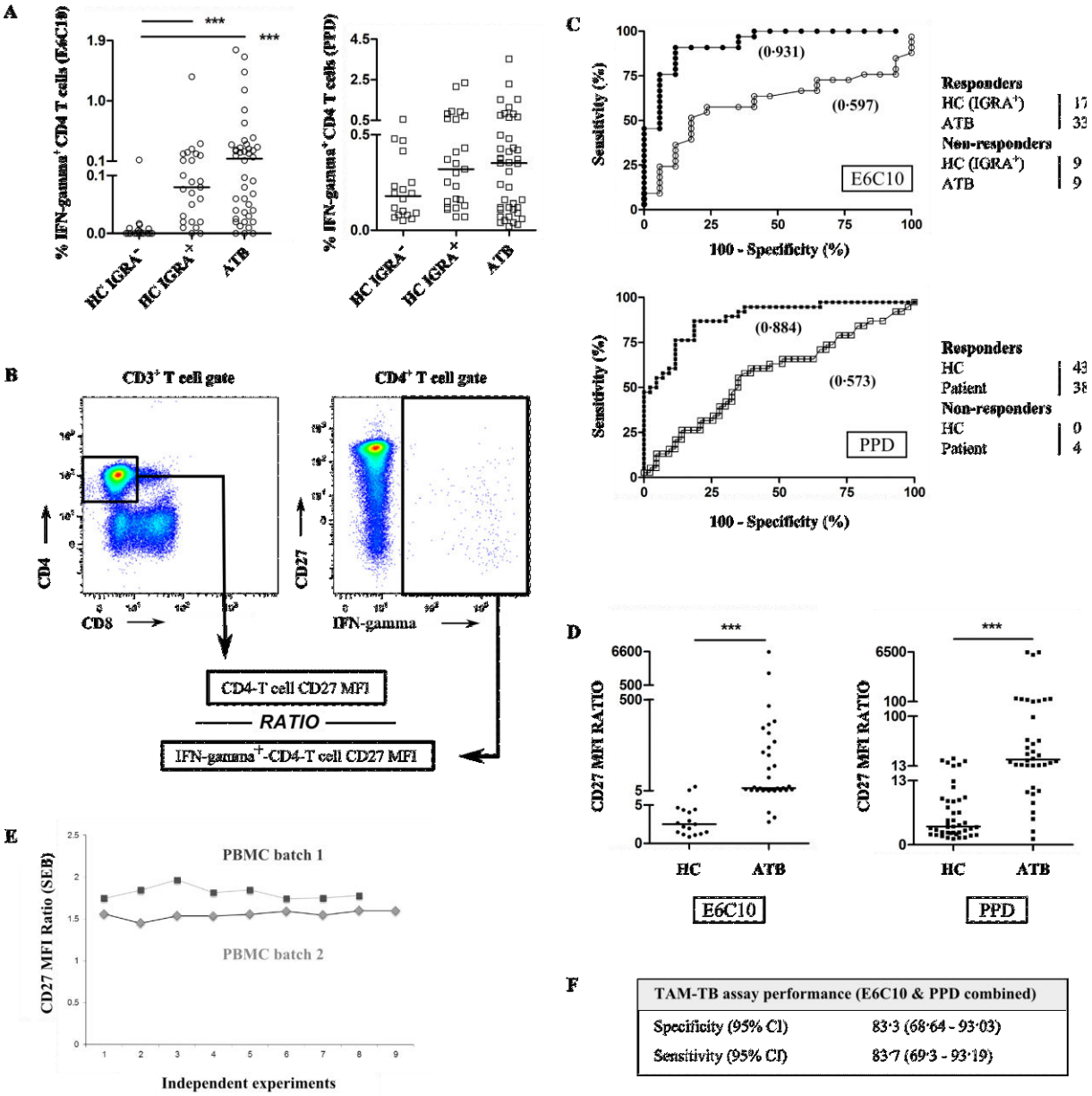
Conclusion

In summary, the novel TAM-TB assay is the first immune-based assay that has demonstrated the ability to differentiate LTBI from active tuberculosis disease. These results indicate that considering both ESAT-6/CFP-10-specific and PPD-specific CD4 T cell responses is the most sensitive approach. Further stratification using CD27 MFI ratios, with cut-off values of >5 for ESAT-6/CFP-10-specific and >13 for PPD-specific CD4 T cell responses optimized the ability to differentiate active tuberculosis disease from latent *M. tuberculosis* infection. These pre-determined cut-off values were then used to predict active tuberculosis in the prospective diagnostic evaluation study in Tanzanian children, reported in the main manuscript.

Supplementary appendix: Figures



Supplementary Figure 1: Flow diagram of enrolment and clinical classification in adult optimisation cohort.



Supplementary Figure 2: TAM-TB assay settings and performance in the adult optimisation cohort.

A) Scatter-plot of IFN-γ producing CD4 T cells frequencies upon ESAT-6/CFP-10 (left graph) or PPD (right graph) stimulation; one-way ANOVA with Dunn's multiple comparison testing correction, *** p<0·0001. B) Gating strategy for CD4 T cells and illustration of how the CD27 MFI ratio is calculated in the TAM-TB assay. C) Receiver Operating Characteristic (ROC) curves comparing the use of IFN-gamma producing CD4 T cells frequencies (open symbols) and the respective CD27 MFI ratio (closed symbols) using ESAT-6/CFP-10 (circles) and PPD (squares) stimulation respectively, to discriminate IGRA+ healthy controls from adult tuberculosis cases, (area under the curve). D) Scatter-plot of CD27 MFI ratio of IGRA+ healthy controls and active tuberculosis cases (ATB) responding to ESAT-6/CFP-10 (left panel) or PPD stimulation (right panel), two-tailed Mann-Whitney test, *** p<0·0001. E) CD27 MFI ratio results from 17 quality control experiments using two batches of Peripheral Blood Mononuclear Cells stimulated with

Staphylococcus enterotoxin B. F) TAM-TB assay specificity and sensitivity using >5 and >13 thresholds for ESAT-6/CFP-10 and PPD stimulation respectively.

Abbreviations used: PPD, purified protein derivative; EC6C10, ESAT-6/CFP-10; HC, healthy control; IGRA, Interferon Gamma Release Assay; ATB, active tuberculosis; MFI, median fluorescence intensity; QC, quality control.

Supplementary appendix: Tables

A. Culture-confirmed tuberculosis
Symptoms suggestive of tuberculosis plus Liquid or solid culture positive for M. tuberculosis.
B. Highly probable tuberculosis
Symptoms suggestive of tuberculosis plus or chest radiograph strongly indicating active tuberculosis and confirmed by 2 independent reviewers. or histology/Cytology showing typical morphology. or fluorescent or acid-fast bacilli on microscopy.
C. Probable tuberculosis
Cervical lymph node mass (greater than 2*2cm) with resolution on TB therapy. or abdominal mass or ascites with abdominal lymphadenopathy on ultrasound scan and resolution on TB therapy. or clinical picture of meningitis associated with CSF changes and/or CT scan findings consistent with TB (after other likely causes have been excluded). or suggestive symptoms, but CXR "uncertain" for active tuberculosis or discordance between independent reviewers; no alternative diagnosis established and complete symptomatic or radiographic resolution on TB treatment.
D. Not tuberculosis (controls)
Alternative diagnosis established, TB workup negative and clinically well after 3-6 months follow-up without TB treatment.
E. Indeterminate
Any other possible combination of results and/or lost to follow-up after recruitment.

Supplementary Table 1: Clinical case categories of paediatric tuberculosis suspects (validation cohort).

Abbreviations used: CSF, cerebrospinal fluid; CT, computed tomography; CXR, chest X-ray; TB, tuberculosis.

3 General discussion

3.1 Target product profiles

In recent years, key stakeholders attempted to identify and prioritise characteristics of TB diagnostics, which could close the existing global diagnostic gaps. It became apparent that a 'one-fits-all' approach is not realistic. Different novel TB technologies should meet different indications, namely diagnosis of active pulmonary TB, diagnosis of extrapulmonary TB and childhood TB, triage testing to spot individuals who need confirmation tests, diagnosis of latent TB, predictive biomarkers for progression to active TB, test for treatment monitoring, and drug susceptibility testing [67]. The development of accurate drug susceptibility tests should be in close alignment with emerging drug regimens to facilitate rational anti-microbial stewardship [298,299].

In 2013, participants of a TB Modelling and Analysis Consortium meeting gave important impetus to this development by identifying a list of unmet diagnostic needs which were ultimately converted into detailed target product profiles (TPPs) [300]. Experts in TB research, care, modelling, program work, patient advocacy and marketing graded different new diagnostic approaches using 10 criterions of the following thematic areas: prioritisation by key stakeholders, impact, market and implementation and scalability. Based on those results, interviews and literature search, four target product profiles for the high priority needs have been established [68,209], and a fifth is under development [301]:

(1) a point-of-care non-sputum-based test capable of detecting all forms of TB by identifying characteristic biomarkers or biosignatures (the biomarker test).
(2) a point-of-care triage test, which should be a simple, low-cost test that can be used by first-contact health-care providers to identify those who need further testing (the triage test),
(3) a point-of-care sputum-based test to replace smear microscopy for detecting pulmonary TB (the smear-replacement test),
(4) a rapid drug-susceptibility test that can be used at the microscopy-centre level of the health-care system to select first-line regimen-based therapy (the rapid DST test).

For each of the TPPs, optimal and minimal requirements for key characteristics have been defined, such as diagnostic sensitivity, specificity, possibility of treatment monitoring, type of power supply, maintenance and calibration, environmental conditions, and price per test. The general aim was to develop new low cost TB diagnostics with better performance features, which can be implemented at low level of the health care system.

The four diagnostics tests presented in this thesis, have the putative capability to serve different purposes for TB screening and detection in adults and children. Sensitivity and specificity data generated in these studies –especially from the TAM-TB proof-of-concept study– suggest that TPP performance requirements can almost be reached (Figure 19). However, the findings of the presented diagnostic accuracy studies (manuscript 1-4) need to be thoroughly analysed in the context of *all* TPP key requirements.

Therefore, each of the four diagnostic tests will hereinafter be critically scrutinized, and deficiencies in diagnostic or operational characteristics will be identified. Test accuracy studies do not happen in a vacuum, they are integral part of a diagnostic development strategy. Consequently, concepts for a framework of a harmonized, more effective diagnostic evaluation of TB diagnostics will be unrolled in the second part of the general discussion.

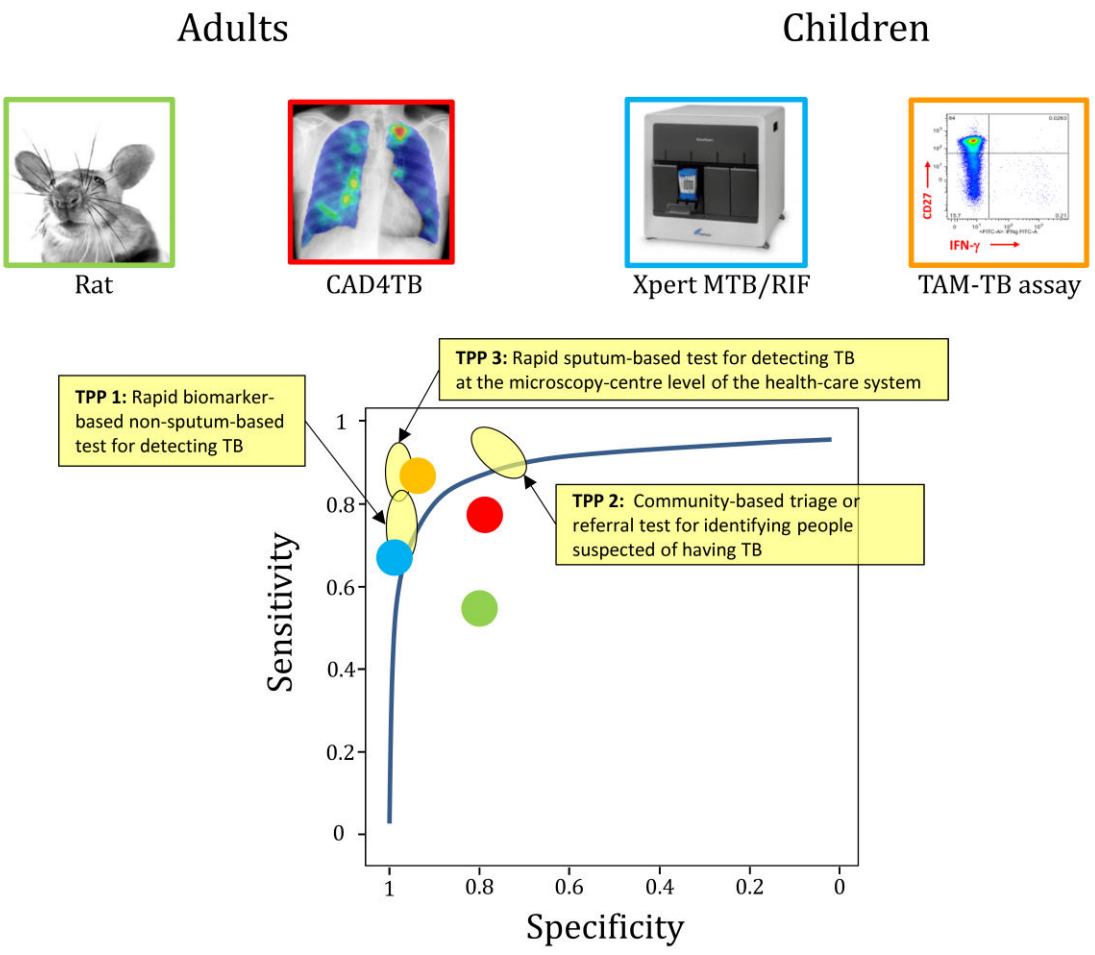


Figure 19: Sensitivity and specificity of the four different diagnostic tests for the detection of TB (manuscript 1-4), shown in a ROC curve in relation to TPPs 1-3 performance requirements.

3.2 Detection rats – does this diagnostic approach meet TPP requirements?

Owing to its uniqueness, the rat methodology and its public health usage attracts great international attention even beyond the scientific community [302,303]. However, a rigid and comprehensive clinical validation of the TB rat method is still pending. Only four studies, including the one presented in this thesis (manuscript 1) [304], have used microbiological reference standards to assess diagnostic accuracy [176,189,175]. The sensitivity and specificity of TB detection rats were overall below the values required for the smear-replacement test TPP and varied between the studies, presumably because of differences in index case definitions, reference methods and use of clinical data. More and standardized, prospective evaluations are mandatory to generate robust test accuracy data for TB detection rats.

TB diagnosis by rats has the advantage of high throughput, low costs per sample and no need for advanced laboratory-based technology. The rat technology is currently used for so-called 'second-line screening', which refers to screening of samples after they have been examined with smear microscopy. In this concept, rat-positive samples are concentrated and examined again by fluorescence smear microscopy for final confirmation. In Tanzania and Mozambique sputum samples from 21'600 and 9'048 presumptive TB patients, respectively, have been screened this way in 2014. The case detection rate has been increased in Tanzania and Mozambique by 39% and 53%, respectively. However, the rate of false-positive second-line indications, which can only be estimated in absence of a reference standard, seems to be high, as a low detection threshold has been chosen. Between 89%-91% of initially smear-negative, but rat-positive samples remain negative after fluorescence microscopy [305]. Since fluorescence smear microscopy alone increases the detection rate due to its higher sensitivity compared to conventional smear microscopy, the incremental yield of the TB detection rats might be rather small [93].

Although APOPO, the non-governmental organisation who has invented the rat technology, wants to extend the 'second-line' screening approach to other metropolitan areas, such as Addis Ababa [306], wide scalability might become a major operational barrier, because knowledge and expertise in animal behaviour training for TB detection is limited in Africa. Since the 'second-line' screening method relies on large numbers of patients, the rat chambers are meant to be placed in densely populated areas with thousands of samples expected per year. In Africa alone, there are more than 50 cities or conglomerates with one million or more inhabitants. It will be a mammoth task to provide training, maintenances, quality control and logistics in support of TB detection rat units in those areas. By comparison, operational challenges for the implementation of a newly developed lateral flow TB test are certainly much smaller. In addition, the perceptions of health care giver and patients need to be analysed in different cultural contexts to explore if

both groups have any reservation against accepting that an important test result has been generated by rats.

Overall, the TB rat technology does not meet the key criteria of any of the related TPPs, in particular because point-of-care usability at low health care level seems to be practically unachievable. Before this methods can find its niche for 'second-line screening' , questions on scalability and deliverability need to be addressed and answered by independent pragmatic studies, which should also include cost-effectiveness analyses.

3.3 CAD4TB - a potential triage test?

TB detection based on automated reading in chest radiographs through CAD4TB has reached an advanced stage of development. For example, technical solutions for pre-processing of imaging -e.g. bone suppression-, improved the ability to discriminate between normal and abnormal regions in an x-ray image. Similarly, new algorithms for the identification of certain TB manifestations, in particular methods for segmentation and detection of cavities and also pleural effusion, have been developed to advance the software for clinical use [121]. In 2015, the CAD4TB software (v 4.10) has been CE certified, and is now commercially available and operational in several countries [307].

However, a recent systemic review has reported that the current evidence from clinical validation studies on the use of CAD4TB is still insufficient, because only a small amount of studies with microbiological reference standard, including the presented study (manuscript 2) [308], have generated important data on their own. However, those data have limited generalizability, mainly because of a broad methodological variability (e.g. definition of thresholds) [309]. Thus, so far WHO has not provided any recommendation on using computer assisted x-ray systems [310].

Consequently, it remains an important task for research to determine if CAD4TB can be implemented in health systems with different financial resources and TB epidemiology. Next to candidate biomarkers, such as C-reactive protein [311], procalcitonin [312], neopterin [313] or IP10 [314], automated reading of digital x-rays using CAD4TB also seems to qualify as a triage test because of good test sensitivity, capability for high throughput and relatively low costs per test.

Costs and cost-effectiveness are key factors for the use of new TB diagnostics as triage tests, particularly in resource-constrained countries. Even when subsidized for poor countries, cost per e.g. Xpert MTB/RIF examination is 15-25 USD (10 USD for the test cartridge) [315]. Examining all patients presenting with cough with such tests is therefore not affordable. However, this kind of enhanced case finding is expected to substantially increase the TB detection rate [316]. A triage strategy, in which an inexpensive triage test is performed first, and

if positive, followed by a more expensive confirmatory assay, could substantially reduce diagnostic costs for the health system or the patient [315] and improve early access to diagnosis. Triage tests need to have high sensitivity while specificity results from a trade-off between cost and operational efficiency.

In most settings, chest x-ray remains an essential component of diagnostic algorithms for pulmonary TB. Screening algorithms starting with chest x-ray screening provide greater case detection compared to symptom screening [111,317]. The introduction of digital imagining has made chest x-ray more reliable and simplified diagnostic test, with images available after a few seconds. This allows high throughput at low cost of 1 USD per examination including investments. With its broad range of diagnostic application and presently declining equipment cost, digital x-ray is ready to be rolled out in health facilities of many high burden countries [310], where currently mainly conventional systems are used [109].

Cost-effectiveness and affordability of a potential triage algorithm e.g. with CAD4TB needs to be analysed in the setting and in the population it is intended to be applied [318]. In a South African urban setting, a simulation study could demonstrate that diagnostic algorithms using automated digital chest radiography as triage for the use of Xpert MTB/RIF substantially reduce costs, increase daily throughput and misses on a few TB cases [319].

However, a cost-effectiveness model on triage testing in Uganda using chest x-ray (without CAD4TB) and C-reactive protein concluded that both test are only cost-effective in populations with high TB prevalence (5%) and a high risk (1% per month) of death from untreated TB. For lower-risk populations a triage test needs to be cheaper or more specific [320].

According to the WHO expert panel on new TPP, a community-based triage test should have an overall sensitivity between >95% (optimal requirement) and >90% (minimal requirement), and a specificity of >80% and >70%, accordingly. In our clinical evaluation study, CAD4TB (v3.07, updated release) showed a good diagnostic accuracy (Az=0.84). However, the required target sensitivity and specificity levels were not attained. By lowering or by increasing the cut-off value, either sensitivity or specificity was in the TPP range. The combination of 77% sensitivity and 79% specificity was considered as a 'well-balanced' result, but still below the TPP requirements (Figure 19). Other evaluation studies comparing previous version of CAD4TB microbiological reference standards, showed even slightly lower diagnostic accuracy [218,321].

Furthermore, chest x-ray is also important for the detection of paediatric TB. In young children, the diagnosis of TB can usually not be confirmed microbiologically, as in most adults, because sputum is difficult to obtain and disease is often paucibacillary. Consequently, the development of CAD4TB for children is a next logical step. The development process has to observe peculiarities of paediatric radiology and should be guided by the NIH consensus on clinical case definitions for classification of intrathoracic TB disease in children [288]. Radiographic signs of

TB in children are in many aspects different to those in adults and depend largely on age and immune status [36,271]. In contrast to single x-ray pictures in adults, CXRs of children are typically assessed by using both frontal and lateral views [322]. New CAD4TB software for children might have potential to become an important supportive diagnostic tool for the diagnosis of childhood TB.

In conclusion, further and better-harmonised validation studies on CAD4TB with standardized protocols, pre-specified threshold scores and microbiologically well-defined reference standards are needed. Before any recommendation to policy is possible, pragmatic, prospective trials on cost-effectiveness and impact on patient outcomes are required to assess if CAD4TB is a suitable triage test for Xpert MTB/RIF or other molecular tools.

3.4 Xpert MTB/RIF – first step towards a smear microscopy replacement test for the diagnosis childhood TB?

Especially in resource-constrained settings, the detection of childhood tuberculosis relies mainly on clinical symptoms, history of exposure, TST and chest-x-ray, if available. Due to its poor sensitivity in detecting TB in children, smear microscopy plays only a minor role in children and needs to be replaced by a more precise diagnostic tool. The hopes that Xpert MTB/RIF would become the easy-to-use and accurate diagnostic for pulmonary TB in children have not been fully realised. Our study (manuscript 3) [323], which also informed a systematic review and meta-analysis published in *The Lancet Respiratory Medicine* [133], showed consistent with the other paediatric evaluation studies that Xpert MTB/RIF can diagnose pulmonary TB better than smear microscopy in children. However, Xpert MTB/RIF detected only two thirds of children with sputum culture-positive for *M. tuberculosis*. On the other hand, the specificity was excellent.

While in adults, Xpert MTB/RIF is detecting pulmonary TB with very good or excellent performance characteristics, i.e. in HIV uninfected individuals with 89% sensitivity and 99% specificity, the performance in children remains suboptimal. This is because children often have a paucibacillary disease with bacterial load most likely below detection limit. The respiratory samples have often reduced quality and quantity, and the recovery of respiratory and non-respiratory samples are specially problematic in young children [133,324,325]. Nonetheless, Xpert MTB/RIF provides results on the same day, faster than culture and even faster than smear microscopy. Currently, children with culture-positive TB are often not started on treatment because the confirmation is not available in time [326]. Thus, the time advantage of Xpert MTB/RIF has the potential to prevent delayed TB treatment.

The suboptimum diagnostic sensitivity of Xpert MTB/RIF for pulmonary TB in children disqualifies the instrument of becoming a microscopy replacement test, which should -according to the TPPs- have at least a sensitivity of 80%. Furthermore, the challenging environmental

conditions prevailing at microscopy centre in countries with the highest TB burden, do not allow a smooth operation of the current version of Xpert MTB/RIF, as experienced by many national TB programmes during roll-out [138,139].

However, the next-generation molecular test platform, GeneXpert Omni, combined with the Xpert MTB/RIF Ultra cartridge system (Cepheid, Sunnyvale, CA, USA) has the potential to become the desired smear replacement test. GeneXpert Omni is a robust instrument, which offers a cloud-based connectivity for TB surveillance. The small and portable tool has a rechargeable battery to provide power for 4 hours. These operational characteristics allow detection of TB in children as well as in adults in remote areas in resource limited settings with relatively low patient throughput.

The new Ultra cartridge can detect TB with sensitivities similar to standard liquid culture making it suitable for adults infected with HIV and most likely for children. Ultra has a limit of detection of 5 CFU/ml compared to 50 CFU/ml of Xpert MTB/RIF. The Ultra has also enhanced ability for detection of rifampicin resistance [327].

In conclusion, even if the current version of Xpert MTB/RIF does not meet the TPP criteria for smear microscopy replacement at peripheral facilities, it has been the central starting point for the development of next-generation molecular tests. These innovative tools will reach most likely the primary health care level and might be able to diagnose paediatric TB with an acceptable accuracy.

3.5 TAM-TB assay – can an improved version become a biomarker test for paediatric TB?

The novel TAM-TB assay measures the activation status in *M. tuberculosis*-specific T-cells. Effector T-lymphocytes pass through different stages of differentiation during and after exposure to *M. tuberculosis*-specific antigens and each of the stages has a characteristic pattern of surface makers, such as CD27 [146]. Late effector T-cells have experienced substantial antigenic stimulation and, therefore, they show minimal or no CD27 expression. Accordingly, down-regulation of CD27 on *M. tuberculosis* CD4 T-class is a biomarker for active TB [279]. In our proof-of-concept study in children (manuscript 4) [328], TAM-TB assay had an excellent specificity (96.8%) and an unprecedented high sensitivity (83.3%), which exceeded those of all known molecular TB diagnostic test, including Xpert MTB/RIF. The assay seems to have diagnostic performance characteristics at least similar to those of culture. However, in contrast to culture, results of the TAM-TB assay are already available after 24 hours.

Due to paucibacillary nature of paediatric TB, non-sputum based tests capable of detecting pulmonary and extrapulmonary manifestations of TB are essential for a better diagnosis in children, who carry approximately 11% of the global TB burden [271]. In 2014, experts defined

research priorities to improve TB case detection, surveillance, healthcare delivery, and effective advocacy for children at a meeting at the National Institutes of Health (NIH), US [329]. The experts agreed that the following characteristics for new diagnostic biomarker of all types of TB in children should have the following characteristics: i) the biomarker should be detectable in readily obtainable matrix, e.g. blood; ii) the identification of TB should be possible with high sensitivity and specificity, iii) the test should distinguish between latent and active TB, and iv) the assay should be deployable close to point-of-care. Our research team could demonstrate that the TAM-TB assay complies with the first three requirements defined in the NIH blueprint paper.

Unfortunately, the current TAM-TB assay does not meet the fourth criterion. Complexity and sophistication of the current flow cytometer version do not allow any point-of-care usability. In contrary, this version involves time-consuming preparation and incubation of peripheral blood mononuclear cells (PBMC) and adherence to biosafety regulations because of a bioaerosol infection risk. Moreover, highly trained staff and an expensive high-end polychromatic flow cytometer are required.

Consequently, there is a need to miniaturise and optimise methodologies of T-cell phenotypic biomarker measurements for paediatric TB. The improvement process should comprise testing of additional antigenic reagents (derived from epitope-mapping studies) and combined cytokine profiles (i.e. TNF-α [330] in combination with IFN-γ) to optimize test sensitivity and specificity in particular for the diagnosis TB in children with advanced HIV infection. Furthermore, reduction of the incubation time by innovative fluorescent in situ hybridization of cytokine mRNA would be an important optimisation step [331,332]. Another advancement could be the development and standardisation of a whole blood format [155], which will render advanced flow cytometer equipment and biosafety facilities for the preparation of the PBMC unnecessary. Finally, phenotypic T-cell biomarkers other than CD27 should be considered for identification of active TB infection in children; in particular CD38, HLA-DR, and KI-67 are promising candidates as previously shown in adults [162–165]. As a final product, a phenotypic T-cell biomarker assay could make use of the existing flow cytometry platforms for CD4 T-cell count in resource-poor settings to avoid the implementation of an additional, costly piece of equipment to the health systems. Alternatively, a new and robust instrument, as Alere Inc. apparently intends to develop [333], could advance the use of phenotypic T-cell markers for the diagnosis of TB.

Only after simplification, optimisation, miniaturisation, and successful clinical validation in large paediatric cohorts of the assay, TAM-TB assay or better a 'phenotypic T-cell marker assay' can become a promising candidate for a non-sputum-based test capable of detecting all forms of TB by identifying characteristic biomarkers or biosignatures, as delineated in the first TPP.

3.6 TB diagnostic evaluation

The four studies presented in this thesis belong to the category of test accuracy studies, one of the components in the diagnostic evaluation pathway. In the following, the status quo of the TB diagnostic evaluation process will be discussed, including a critical view on the role of diagnostic accuracy studies.

3.6.1 Beyond accuracy studies

Novel tests are helpful only if they lead to better patient outcomes [334]. Test accuracy is considered to be a surrogate for patient important outcomes [335]. The underlying theory is that better information on presence or absence of a disease or another target condition will result in better outcome. However, this assumption is not always valid, taking the range of other factors as operational context or financial implications into consideration. Consequently, cross-sectional or cohort studies on test accuracy are important in the evaluation pathway, but they cannot replace individual- or cluster-randomised controlled pragmatic trials, which are needed to assess the impact of a new diagnostic tool or strategy on patient-important outcomes [336]. Although randomised diagnostic trials require often a tailored design, they provide highest quality evidence base to support recommendations to policy with the least risk of any kind of bias [337].

In TB research, studies beyond test accuracy are relatively rare, even though expert guidance, as the multi-layered approach 'Impact Assessment Framework', have been developed to help generating the urgently needed policy-relevant data for new tests or test strategies [338,339].

The case of Xpert MTB/RIF, a technology considered a major breakthrough in TB control, illustrates remarkably that excellent test accuracy data do not necessarily result in improved patient-relevant outcomes. Mathematic models demonstrated that Xpert MTB/RIF will substantially change TB morbidity and mortality through improved case-finding and treatment [340]. Modelling also predicted that the use of Xpert MTB/RIF is a cost-effective diagnostic strategy for high burden countries with the potential to considerably decrease the TB burden [341].

However, data from nine published intervention trials on patient-relevant outcomes in comparison to smear microscopy showed that Xpert MTB/RIF does not have a significant impact on patient morbidity or mortality [342–350]. In almost all studies, this finding does not change when stratified by HIV status. A beneficial impact has only been seen in the early phases of the TB diagnosis and treatment cascade, i.e. yield of bacteriologically confirmed cases and patient volume [351]. Effects on the later stages of patient care were not detected. One explanation is that clinicians in these pragmatic trials were aware of the diagnostic used in each arm. Consequently, the low predictive value of smear microscopy motivated the caregiver to more

empiric treatment in the microscopy arm, which ultimately undermined the potential effect of Xpert MTB/RIF [352]. Trial design issues, in particular under-powering for mortality and morbidity outcomes in some trials, might have further distorted the picture of patient-important outcomes. Still, no publication could so far prove that the use of Xpert MTB/RIF for the diagnosis of pulmonary TB has a beneficial effect on morbidity or mortality. In addition or in contrast to several models, the roll-out of Xpert MTB/RIF is associated in 'real life' with very high costs in many settings, including those with placement of Xpert MTB/RIF at point-of treatment or with low test volumes [353–355].

It remains uncertain, if today's knowledge regarding patient-important outcomes would have changed the global and expensive scale-up of Xpert MTB/RIF in currently 122 countries. Yet, it seems undisputed that from now on diagnostic research on Xpert MTB/RIF and upcoming diagnostic technologies should focus more on health impact, operational aspects related to infrastructure as well as biosafety, and cost-effectiveness. The process must embrace the entire cascade of care to improve also accessibility, referral, linkage to care, patient follow-up and infection control [356–358]. Results from accuracy studies and short-term outcome assessments alone cannot be the basis for complete scale-up at country level. Additionally, there should be an accepted procedure within WHO to allow modification or withdrawal of existing recommendations based on post-endorsement evidence regarding patient-important impact [359].

3.6.2 Framework for evaluation of new TB diagnostics

Development and testing of new TB drugs and vaccines follows a clear hierarchical order of different stages from discovery, preclinical development to clinical development divided in phase I, II, II and IV [65,360]. Comparable models have been proposed for the clinical evaluation of diagnostics [361–363], but they are still not within reach. Principles for a phased and standardized approach for the evaluation of diagnostics can be described using the following criteria, either hierarchically or stepwise [361,364–366,356,82,367]:

(1) technology capability – analytical validity assessed under laboratory condition
(2) diagnostic accuracy – ability to diagnose a disease or predict future disease
(3) operational aspects – infrastructure and biosafety requirements
(4) place in clinical pathway –target population, context of use (replacement, add-on, or triage)
(5) accessibility – access especially for poor and vulnerable individuals
(6) diagnostic impact – influence of the test on diagnosis and diagnostic processes
(7) therapeutic impact – influence of the test on treatment decision
(8) patient outcome – influence of the test on health or quality of life
(9) cost-effectiveness – costs in relation to health effects.

This scheme can be further simplified to a three-step framework as displayed in figure 20.

New diagnostics

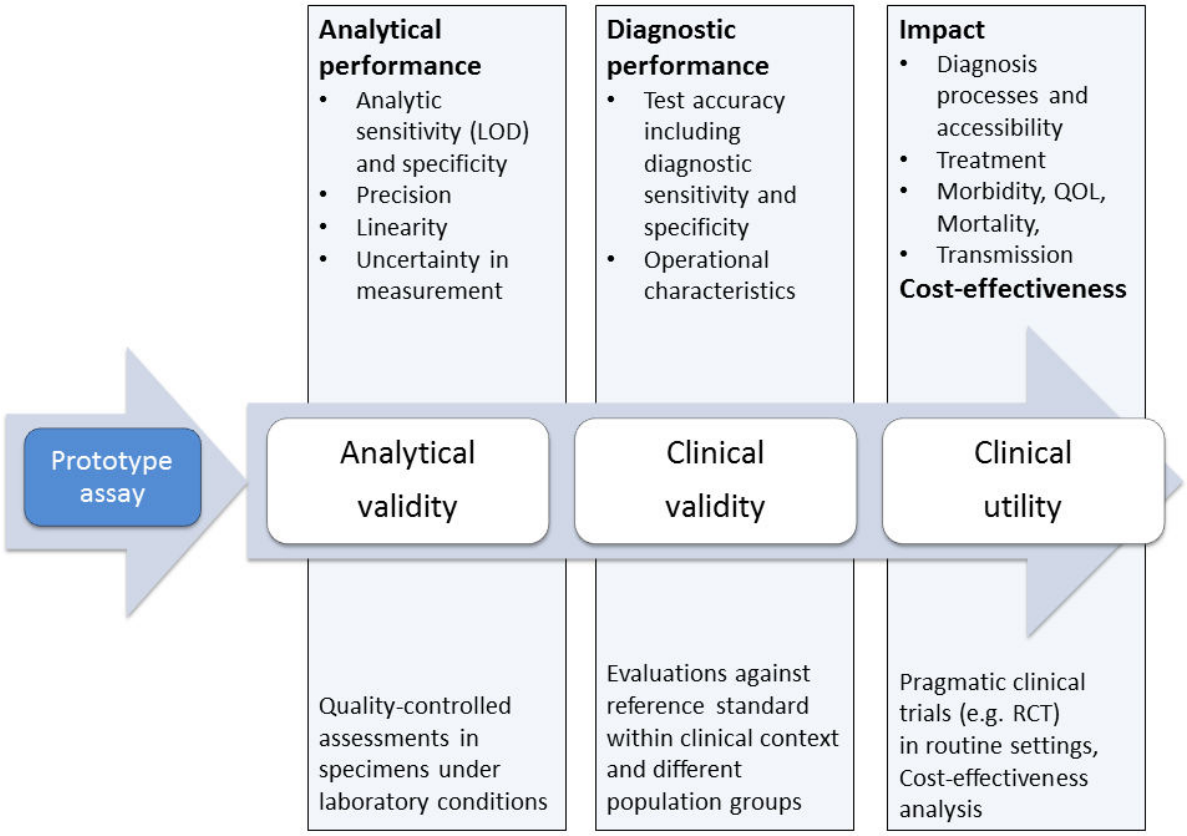


Figure 20: Elements of the evaluation of new TB diagnostics.
(LOD=limit of detection; QOL= quality of life; RCT=randomised clinical trial)

The current practice of evaluating new TB diagnostics, however, is rarely guided by an overarching concept. Diagnostic studies frequently lack methodological rigor [191]. Indeed, diagnostic research consists often of a patchwork of heterogeneous studies of low quality with regard to methodology and reporting. Regularly, TB diagnostic evaluation studies do not observe essential elements such as selection of a representative population, blinding or adequate reference standards [339]. They are frequently executed by scientific or industry groups, which rarely are interlinked or follow mutual goals.

On the other hand, even TB diagnostics studies complying with quality standards are often of limited value because results between the various uncoordinatedly conducted small-scale trials are not comparable due to diverse methodologies. The evaluation study on CAD4TB presented in this thesis serves here as an example. As mentioned, the current evidence from validation studies on CAD4TB is still not sufficiently generalizable, because only a small amount of studies - including the presented evaluation study- used a microbiological reference standard, but also very different study methods (e.g. definition of thresholds) [309]. Retrospectively, it seems to be

a rather poor use of resources to conduct those CAD4TB studies independently from each other. Synergy and harmonised study protocols would have delivered evidence in a more efficient manner.

To improve the quality of TB diagnostic evaluations, the existing tools for quality control and assurance should be used rigorously and in a binding way. To begin with, clinical trials and diagnostic studies follow very similar underlying principles for ethics and scientific quality standards, ICH Good Clinical Practice (GCP) and ISO 1415:2011 GCP, respectively [199,368]. The implementation of ICH GCP in clinical trials conducted in resource-limited setting is challenging and needs to be to some extent adapted to local requirements [369]. Nevertheless, investigators seem to perceive ICH GCP as helpful tools ensuring international standards [370]. Little is known, however, about the use of ISO GCP guidelines in diagnostic studies, most likely because regulation for this kind of studies are weak or entirely neglected in low-income countries [371].

Furthermore, research on TB diagnostics is supposed to follow strictly the existing guidelines and recommendations on reporting, quality assessment and design, namely STARD, QUADAS, and DEEP. As shown in a study on quality and reporting of diagnostic accuracy studies, however, only about 20% of scientific journals required the authors to use STARD when submitting diagnostic accuracy study manuscripts [372]. In future, scientific journals and peer reviewers should consequently encourage adherence or reject manuscripts of studies, which do not comply with those essential guidelines. Only strict adherence to guidelines will eventually improve conduct and reporting, as exemplified to some extent with the CONSORT guidelines for clinical trials [373].

Finally, there is a need for synergized TB research efforts with consensus on adequate trial designs and endpoints for each category of diagnostic [374,375,209]. Together with the research and end-users community, an internationally recognised institution or authority closely connected to the related funding agencies should ideally form a global agenda on new TB diagnostics and should oversee the conduct of the most relevant trials on new TB tests. Research priorities based on global consensus would also better find its way into calls of the major research funders. Due to its normative mandate to assess and endorse new technologies in a GRADE process, WHO seems to be an appropriate authority to develop this kind of 'WHO-approved' research agenda to avoid the flood of diagnostic studies which are currently a burden to the scarce resources for research, but do not necessarily answer questions of public health relevance. In contrary, the patchwork of clinical TB diagnostic trials delays testing and implementation of TB diagnostics to the detriment of the million patients in need of better tools. Given the fact that the TB pandemic is creating a public health crisis in many parts of the world, it seems unjustifiable that competing academic groups or commercial test developer continue

with uncoordinated test development. Only synergistic research efforts on novel TB diagnostics will help to reach the ambitious TB-related UN Sustainable Development Goals.

3.7 Concluding remarks

TB is a major public health threat. Despite certain achievements in disease control in the past, elimination of TB on a global scale is still out of reach. Intensified research on innovative tools, specifically new drugs, vaccines and diagnostics, is an important pillar of the worldwide strategy to fight TB. Since BCG, the only available TB vaccine, has only modest efficacy in children and none in adults, the main strategy for controlling the TB epidemic remains early diagnosis and treatment. Consequently, the development, evaluation, adoption and roll-out of better and affordable diagnostic tests should remain a high priority

The presented diagnostic accuracy studies of very different diagnostic tests make an important contribution to search for better TB diagnostics.

Animals can be used for detection of VOCs originating from specific pathogens. Trained giant African pouched rats can identify and indicate the presence of *M. tuberculosis* in sputum samples. However, currently assessed test accuracy does not allow an exclusive use for TB screening or detection.

CAD4 TB, a computer-aided system for detection of pulmonary TB on chest radiographs, has the potential to be utilized as a triage test for an accurate but more costly molecular test. Future research with CAD4TB should focus on this aspect.

Xpert MTB/RIF detects pulmonary TB in children better than smear, but not as well as *M. tuberculosis* culture. Due to its suboptimal sensitivity, the assay is not suitable as a standalone test for the diagnosis of pulmonary TB in children. Next-generation molecular tests, particularly Xpert MTB/RIF *Ultra*, will most likely show a higher sensitivity for the detection of *M. tuberculosis* in sputum samples of children.

The novel TAM-TB assay showed an unprecedented high sensitivity and an excellent specificity for the detection of TB in children. The flow cytometry-based immunoassay, which measures the loss of CD27 expression on *M. tuberculosis-specific* CD4 T cells, as the potential to become an non-sputum biomarker test, given that further technical development reduces its complexity and enhances its point-of-care applicability.

In order to improve the currently unstructured and uncoordinated evaluation process of TB diagnostics, a coherent concept reaching from analytical assessments, diagnostic accuracy

studies, to evaluations of health impact and cost-effectiveness analyses is urgently needed. Decisive key qualities should be strict adherence to international quality standards as well synergic efforts of all groups involved preferably overseen by an international guiding authority.

4 References

1. Gagneux S. Genetic diversity in Mycobacterium tuberculosis. Curr Top Microbiol Immunol. 2013;374:1–25.

2. Cole ST, Brosch R, Parkhill J, Garnier T, Churcher C, Harris D, Gordon SV, Eiglmeier K, Gas S, Barry CE, Tekaia F, Badcock K, Basham D, Brown D, Chillingworth T, Connor R, Davies R, Devlin K, Feltwell T, Gentles S, Hamlin N, Holroyd S, Hornsby T, Jagels K, Krogh A, McLean J, Moule S, Murphy L, Oliver K, Osborne J, Quail MA, Rajandream MA, Rogers J, Rutter S, Seeger K, Skelton J, Squares R, Squares S, Sulston JE, Taylor K, Whitehead S, Barrell BG. Deciphering the biology of Mycobacterium tuberculosis from the complete genome sequence. Nature. 1998 Jun 11;393(6685):537–44.

3. de Jong BC, Antonio M, Gagneux S. Mycobacterium africanum--review of an important cause of human tuberculosis in West Africa. PLoS Negl Trop Dis. 2010;4(9):e744.

4. Koeck J-L, Fabre M, Simon F, Daffé M, Garnotel E, Matan AB, Gérôme P, Bernatas J-J, Buisson Y, Pourcel C. Clinical characteristics of the smooth tubercle bacilli "Mycobacterium canettii" infection suggest the existence of an environmental reservoir. Clin Microbiol Infect Off Publ Eur Soc Clin Microbiol Infect Dis. 2011 Jul;17(7):1013–9.

5. Grange JM. Mycobacterium bovis infection in human beings. Tuberc Edinb Scotl. 2001;81(1–2):71–7.

6. van Soolingen D, Hoogenboezem T, de Haas PE, Hermans PW, Koedam MA, Teppema KS, Brennan PJ, Besra GS, Portaels F, Top J, Schouls LM, van Embden JD. A novel pathogenic taxon of the Mycobacterium tuberculosis complex, Canetti: characterization of an exceptional isolate from Africa. Int J Syst Bacteriol. 1997 Oct;47(4):1236–45.

7. van Soolingen D, van der Zanden AG, de Haas PE, Noordhoek GT, Kiers A, Foudraine NA, Portaels F, Kolk AH, Kremer K, van Embden JD. Diagnosis of Mycobacterium microti infections among humans by using novel genetic markers. J Clin Microbiol. 1998 Jul;36(7):1840–5.

8. Miltgen J, Morillon M, Koeck J-L, Varnerot A, Briant J-F, Nguyen G, Verrot D, Bonnet D, Vincent V. Two cases of pulmonary tuberculosis caused by Mycobacterium tuberculosis subsp canetti. Emerg Infect Dis. 2002 Nov;8(11):1350–2.

9. Panteix G, Gutierrez MC, Boschiroli ML, Rouviere M, Plaidy A, Pressac D, Porcheret H, Chyderiotis G, Ponsada M, Van Oortegem K, Salloum S, Cabuzel S, Bañuls AL, Van de Perre P, Godreuil S. Pulmonary tuberculosis due to Mycobacterium microti: a study of six recent cases in France. J Med Microbiol. 2010 Aug;59(Pt 8):984–9.

10. Evans JT, Smith EG, Banerjee A, Smith RMM, Dale J, Innes JA, Hunt D, Tweddell A, Wood A, Anderson C, Hewinson RG, Smith NH, Hawkey PM, Sonnenberg P. Cluster of human tuberculosis caused by Mycobacterium bovis: evidence for person-to-person transmission in the UK. Lancet Lond Engl. 2007 Apr 14;369(9569):1270–6.

11. Gagneux S. Host-pathogen coevolution in human tuberculosis. Philos Trans R Soc Lond B Biol Sci. 2012 Mar 19;367(1590):850–9.

12. Comas I, Coscolla M, Luo T, Borrell S, Holt KE, Kato-Maeda M, Parkhill J, Malla B, Berg S, Thwaites G, Yeboah-Manu D, Bothamley G, Mei J, Wei L, Bentley S, Harris SR, Niemann S, Diel R, Aseffa A, Gao Q, Young D, Gagneux S. Out-of-Africa migration and Neolithic coexpansion of Mycobacterium tuberculosis with modern humans. Nat Genet. 2013 Oct;45(10):1176–82.

13. Chisholm RH, Trauer JM, Curnoe D, Tanaka MM. Controlled fire use in early humans might have triggered the evolutionary emergence of tuberculosis. Proc Natl Acad Sci U S A. 2016 Jul 25;

14. Bocquet-Appel J-P. When the world's population took off: the springboard of the Neolithic Demographic Transition. Science. 2011 Jul 29;333(6042):560–1.

15. Coscolla M, Gagneux S. Consequences of genomic diversity in Mycobacterium tuberculosis. Semin Immunol. 2014 Dec;26(6):431–44.

16. Riley RL. Airborne infection. Am J Med. 1974 Sep;57(3):466–75.

17. Riley RL. Aerial dissemination of pulmonary tuberculosis. Am Rev Tuberc. 1957 Dec;76(6):931–41.

18. Yates TA, Khan PY, Knight GM, Taylor JG, McHugh TD, Lipman M, White RG, Cohen T, Cobelens FG, Wood R, Moore DAJ, Abubakar I. The transmission of Mycobacterium tuberculosis in high burden settings. Lancet Infect Dis. 2016 Feb;16(2):227–38.

19. Jones-López EC, Namugga O, Mumbowa F, Ssebidandi M, Mbabazi O, Moine S, Mboowa G, Fox MP, Reilly N, Ayakaka I, Kim S, Okwera A, Joloba M, Fennelly KP. Cough aerosols of Mycobacterium tuberculosis predict new infection: a household contact study. Am J Respir Crit Care Med. 2013 May 1;187(9):1007–15.

20. Dewan P, Gomber S, Das S. Congenital tuberculosis: a rare manifestation of a common disease. Paediatr Int Child Health. 2014 Feb;34(1):60–2.

21. Young DB, Gideon HP, Wilkinson RJ. Eliminating latent tuberculosis. Trends Microbiol. 2009 May;17(5):183–8.

22. Barry CE, Boshoff HI, Dartois V, Dick T, Ehrt S, Flynn J, Schnappinger D, Wilkinson RJ, Young D. The spectrum of latent tuberculosis: rethinking the biology and intervention strategies. Nat Rev Microbiol. 2009 Dec;7(12):845–55.

23. Dutta NK, Karakousis PC. Latent tuberculosis infection: myths, models, and molecular mechanisms. Microbiol Mol Biol Rev MMBR. 2014 Sep;78(3):343–71.

24. Esmail H, Barry CE, Young DB, Wilkinson RJ. The ongoing challenge of latent tuberculosis. Philos Trans R Soc Lond B Biol Sci. 2014;369(1645):20130437.

25. WHO | Guidelines on the management of latent tuberculosis infection. 2015 [Internet]. WHO. [cited 2016 Jul 14]. Available from: http://www.who.int/tb/publications/latent-tuberculosis-infection/en/

26. Koul A, Arnoult E, Lounis N, Guillemont J, Andries K. The challenge of new drug discovery for tuberculosis. Nature. 2011 Jan 27;469(7331):483–90.

27. Pai M, Behr MA, Dowdy D, Dheda K, Divangahi M, Boehme CC, Ginsberg A, Swaminathan S, Spigelman M, Getahun H, Menzies D, Raviglione M. Tuberculosis. Nat Rev Dis Primer. 2016 Oct 27;2:16076.

28. Solovic I, Sester M, Gomez-Reino JJ, Rieder HL, Ehlers S, Milburn HJ, Kampmann B, Hellmich B, Groves R, Schreiber S, Wallis RS, Sotgiu G, Schölvinck EH, Goletti D, Zellweger JP, Diel R, Carmona L, Bartalesi F, Ravn P, Bossink A, Duarte R, Erkens C, Clark J, Migliori GB, Lange C. The risk of tuberculosis related to tumour necrosis factor antagonist therapies: a TBNET consensus statement. Eur Respir J. 2010 Nov;36(5):1185–206.

29. Sharma SK, Mohan A. Extrapulmonary tuberculosis. Indian J Med Res. 2004 Oct;120(4):316–53.

30. Verrall AJ, Netea MG, Alisjahbana B, Hill PC, van Crevel R. Early clearance of Mycobacterium tuberculosis: a new frontier in prevention. Immunology. 2014 Apr;141(4):506–13.

31. Ewer K, Millington KA, Deeks JJ, Alvarez L, Bryant G, Lalvani A. Dynamic antigen-specific T-cell responses after point-source exposure to Mycobacterium tuberculosis. Am J Respir Crit Care Med. 2006 Oct 1;174(7):831–9.

32. Paulson T. Epidemiology: A mortal foe. Nature. 2013 Oct 10;502(7470):S2-3.

33. WHO | Global tuberculosis report 2016 [Internet]. [cited 2016 Nov 28]. Available from: http://www.who.int/tb/publications/global_report/en/

34. Marais BJ, Graham SM. Childhood tuberculosis: A roadmap towards zero deaths. J Paediatr Child Health. 2016 Mar;52(3):258–61.

35. Jenkins HE, Yuen CM, Rodriguez CA, Nathavitharana RR, McLaughlin MM, Donald P, Marais BJ, Becerra MC. Mortality in children diagnosed with tuberculosis: a systematic review and meta-analysis. Lancet Infect Dis. 2016 Dec 7;

36. Marais BJ, Gie RP, Schaaf HS, Beyers N, Donald PR, Starke JR. Childhood pulmonary tuberculosis: old wisdom and new challenges. Am J Respir Crit Care Med. 2006 May 15;173(10):1078–90.

37. Swaminathan S, Rekha B. Pediatric tuberculosis: global overview and challenges. Clin Infect Dis Off Publ Infect Dis Soc Am. 2010 May 15;50 Suppl 3:S184-194.

38. Cuevas LE, Browning R, Bossuyt P, Casenghi M, Cotton MF, Cruz AT, Dodd LE, Drobniewski F, Gale M, Graham SM, Grzemska M, Heinrich N, Hesseling AC, Huebner R, Jean-Philippe P, Kabra SK, Kampmann B, Lewinsohn D, Li M, Lienhardt C, Mandalakas AM, Marais BJ, Menzies HJ, Montepiedra G, Mwansambo C, Oberhelman R, Palumbo P, Russek-Cohen E, Shapiro DE, Smith B, Soto-Castellares G, Starke JR, Swaminathan S, Wingfield C, Worrell C. Evaluation of tuberculosis diagnostics in children: 2. Methodological issues for conducting and reporting research evaluations of tuberculosis diagnostics for intrathoracic tuberculosis in children. Consensus from an expert panel. J Infect Dis. 2012 May 15;205 Suppl 2:S209-215.

39. Chan ED, Iseman MD. Multidrug-resistant and extensively drug-resistant tuberculosis: a review. Curr Opin Infect Dis. 2008 Dec;21(6):587–95.

40. Obuku EA, Meynell C, Kiboss-Kyeyune J, Blankley S, Atuhairwe C, Nabankema E, Lab M, Jeffrey N, Ndungutse D. Socio-demographic determinants and prevalence of Tuberculosis knowledge in three slum populations of Uganda. BMC Public Health. 2012 Jul 23;12:536.

41. Janssens J-P, Rieder HL. An ecological analysis of incidence of tuberculosis and per capita gross domestic product. Eur Respir J. 2008 Nov;32(5):1415–6.

42. Dye C, Lonnroth K, Jaramillo E, Williams B, Raviglione M. Trends in tuberculosis incidence and their determinants in 134 countries. Bull World Health Organ. 2009 Sep;87(9):683–91.

43. Davies RPO null, Tocque K, Bellis MA, Remmington T, Davies PDO null. Historical declines in tuberculosis in England and Wales: improving social conditions or natural selection? Vesalius Acta Int Hist Med. 1999 Jun;5(1):25–9.

44. Holloway KL, Staub K, Rühli F, Henneberg M. Lessons from history of socioeconomic improvements: a new approach to treating multi-drug-resistant tuberculosis. J Biosoc Sci. 2014 Sep;46(5):600–20.

45. Zürcher K, Ballif M, Zwahlen M, Rieder HL, Egger M, Fenner L. Tuberculosis Mortality and Living Conditions in Bern, Switzerland, 1856-1950. PloS One. 2016;11(2):e0149195.

46. Ukwaja KN, Modebe O, Igwenyi C, Alobu I. The economic burden of tuberculosis care for patients and households in Africa: a systematic review. Int J Tuberc Lung Dis Off J Int Union Tuberc Lung Dis. 2012 Jun;16(6):733–9.

47. Russell S. The economic burden of illness for households in developing countries: a review of studies focusing on malaria, tuberculosis, and human immunodeficiency virus/acquired immunodeficiency syndrome. Am J Trop Med Hyg. 2004 Aug;71(2 Suppl):147–55.

48. Kirigia JM, Muthuri RDK. Productivity losses associated with tuberculosis deaths in the World Health Organization African region. Infect Dis Poverty. 2016;5(1):43.

49. WHO | Fact Sheet No 104. Geneva, Switzerland: WHO, 2002. [Internet]. [cited 2016 Jun 23]. Available from: http://www.who.int/mediacentre/factsheets/who104/en/print.html

50. United Nations. Millennium Development Goals. [Internet]. [cited 2016 Jun 24]. Available from: http://www.un.org/millenniumgoals/reports.shtml

51. WHO | Global tuberculosis report 2015 [Internet]. [cited 2016 Jan 31]. Available from: http://www.who.int/tb/publications/global_report/en/

52. United Nations. Department of Economic and Social Affairs. Sustainable Development Knowledge Platform. Goal 3.3 [Internet]. [cited 2016 Jun 23]. Available from: https://sustainabledevelopment.un.org/sdg3

53. WHO | WHO End TB Strategy [Internet]. [cited 2016 Jun 23]. Available from: http://www.who.int/tb/post2015_strategy/en/

54. Stop TB Partnership. The Global Plan to End TB. The Global Plan to Stop TB 2016 - 2020 [Internet]. [cited 2016 Jun 23]. Available from: http://www.stoptb.org/global/plan/plan2/

55. Abu-Raddad LJ, Sabatelli L, Achterberg JT, Sugimoto JD, Longini IM, Dye C, Halloran ME. Epidemiological benefits of more-effective tuberculosis vaccines, drugs, and diagnostics. Proc Natl Acad Sci U S A. 2009 Aug 18;106(33):13980–5.

56. Lienhardt C, Kraigsley AM, Sizemore CF. Driving the Way to Tuberculosis Elimination: The Essential Role of Fundamental Research. Clin Infect Dis Off Publ Infect Dis Soc Am. 2016 Jun 5;

57. Fletcher HA, Schrager L. TB vaccine development and the End TB Strategy: importance and current status. Trans R Soc Trop Med Hyg. 2016 Apr;110(4):212–8.

58. Lönnroth K, Raviglione M. The WHO's new End TB Strategy in the post-2015 era of the Sustainable Development Goals. Trans R Soc Trop Med Hyg. 2016 Mar;110(3):148–50.

59. Schito M, Hanna D, Zumla A. Tuberculosis Eradication versus Control. Int J Infect Dis IJID Off Publ Int Soc Infect Dis. 2016 Nov 18;

60. Rasanathan K, Sivasankara Kurup A, Jaramillo E, Lönnroth K. The social determinants of health: key to global tuberculosis control. Int J Tuberc Lung Dis Off J Int Union Tuberc Lung Dis. 2011 Jun;15 Suppl 2:S30-36.

61. Gomes MGM, Barreto ML, Glaziou P, Medley GF, Rodrigues LC, Wallinga J, Squire SB. End TB strategy: the need to reduce risk inequalities. BMC Infect Dis. 2016;16(1):132.

62. Zumla A, Davies PDO. Progress towards achieving global tuberculosis control: so near, yet so far. Int J Tuberc Lung Dis Off J Int Union Tuberc Lung Dis. 2016 Mar;20(3):285–6.

63. Suthar AB, Zachariah R, Harries AD. Ending tuberculosis by 2030: can we do it? Int J Tuberc Lung Dis Off J Int Union Tuberc Lung Dis. 2016 Sep;20(9):1148–54.

64. Prasad A, Ross A, Rosenberg P, Dye C. A world of cities and the end of TB. Trans R Soc Trop Med Hyg. 2016 Mar;110(3):151–2.

65. Lienhardt C, Lönnroth K, Menzies D, Balasegaram M, Chakaya J, Cobelens F, Cohn J, Denkinger CM, Evans TG, Källenius G, Kaplan G, Kumar AMV, Matthiessen L, Mgone CS, Mizrahi V, Mukadi Y, Nguyen VN, Nordström A, Sizemore CF, Spigelman M, Squire SB, Swaminathan S, Helden PDV, Zumla A, Weyer K, Weil D, Raviglione M. Translational Research for Tuberculosis Elimination: Priorities, Challenges, and Actions. PLOS Med. 2016 Mar 2;13(3):e1001965.

66. Perkins MD, Cunningham J. Facing the crisis: improving the diagnosis of tuberculosis in the HIV era. J Infect Dis. 2007 Aug 15;196 Suppl 1:S15-27.

67. Pai M, Nicol MP, Boehme CC. Tuberculosis Diagnostics: State of the Art and Future Directions. Microbiol Spectr. 2016 Oct;4(5).

68. WHO | High-priority target product profiles for new tuberculosis diagnostics: report of a consensus meeting. In: Programme GT, ed. Geneva: World Health Organization, 2014.

69. Keeler E, Perkins MD, Small P, Hanson C, Reed S, Cunningham J, Aledort JE, Hillborne L, Rafael ME, Girosi F, Dye C. Reducing the global burden of tuberculosis: the contribution of improved diagnostics. Nature. 2006 Nov 23;444 Suppl 1:49–57.

70. Gandhi NR, Moll A, Sturm AW, Pawinski R, Govender T, Lalloo U, Zeller K, Andrews J, Friedland G. Extensively drug-resistant tuberculosis as a cause of death in patients co-infected with tuberculosis and HIV in a rural area of South Africa. Lancet Lond Engl. 2006 Nov 4;368(9547):1575–80.

71. TDR Diagnostics Evaluation Expert Panel, Banoo S, Bell D, Bossuyt P, Herring A, Mabey D, Poole F, Smith PG, Sriram N, Wongsrichanalai C, Linke R, O'Brien R, Perkins M, Cunningham J, Matsoso P, Nathanson CM, Olliaro P, Peeling RW, Ramsay A. Evaluation of diagnostic tests for infectious diseases: general principles. Nat Rev Microbiol. 2010 Dec;8(12 Suppl):S17-29.

72. Whiting PF, Weswood ME, Rutjes AWS, Reitsma JB, Bossuyt PNM, Kleijnen J. Evaluation of QUADAS, a tool for the quality assessment of diagnostic accuracy studies. BMC Med Res Methodol. 2006 Mar 6;6:9.

73. Bossuyt PM, Reitsma JB, Bruns DE, Gatsonis CA, Glasziou PP, Irwig LM, Moher D, Rennie D, de Vet HCW, Lijmer JG. The STARD statement for reporting studies of diagnostic accuracy: explanation and elaboration. Clin Chem. 2003 Jan;49(1):7–18.

74. Bossuyt PM, Cohen JF, Gatsonis CA, Korevaar DA, STARD group. STARD 2015: updated reporting guidelines for all diagnostic accuracy studies. Ann Transl Med. 2016 Feb;4(4):85.

75. Rutjes AWS, Reitsma JB, Coomarasamy A, Khan KS, Bossuyt PMM. Evaluation of diagnostic tests when there is no gold standard. A review of methods. Health Technol Assess Winch Engl. 2007 Dec;11(50):iii, ix-51.

76. Naaktgeboren CA, Bertens LCM, van Smeden M, de Groot JAH, Moons KGM, Reitsma JB. Value of composite reference standards in diagnostic research. BMJ. 2013 Oct 25;347:f5605.

77. WHO | Stop TB Partnership's New Diagnostics Working Group and World Health Organization. Pathways to better diagnostics for tuberculosis: a blueprint for the development of TB diagnostics. Geneva: World Health Organization; 2009.

78. WHO | Moving research findings into new WHO policies [Internet]. WHO. [cited 2016 Aug 12]. Available from: http://www.who.int/tb/advisory_bodies/research_to_policy/en/

79. Alonso-Coello P, Schünemann HJ, Moberg J, Brignardello-Petersen R, Akl EA, Davoli M, Treweek S, Mustafa RA, Rada G, Rosenbaum S, Morelli A, Guyatt GH, Oxman AD, GRADE Working Group. GRADE Evidence to Decision (EtD) frameworks: a systematic and transparent approach to making well informed healthcare choices. 1: Introduction. BMJ. 2016 Jun 28;353:i2016.

80. Alonso-Coello P, Oxman AD, Moberg J, Brignardello-Petersen R, Akl EA, Davoli M, Treweek S, Mustafa RA, Vandvik PO, Meerpohl J, Guyatt GH, Schünemann HJ, GRADE Working Group. GRADE Evidence to Decision (EtD) frameworks: a systematic and transparent approach to making well informed healthcare choices. 2: Clinical practice guidelines. BMJ. 2016 Jun 30;353:i2089.

81. Schünemann HJ, Mustafa R, Brozek J, Santesso N, Alonso-Coello P, Guyatt G, Scholten R, Langendam M, Leeflang MM, Akl EA, Singh JA, Meerpohl J, Hultcrantz M, Bossuyt P, Oxman AD, GRADE Working Group. GRADE Guidelines: 16. GRADE evidence to decision frameworks for tests in clinical practice and public health. J Clin Epidemiol. 2016 Aug;76:89–98.

82. Cobelens F, van den Hof S, Pai M, Squire SB, Ramsay A, Kimerling ME, Evidence for Scale-up Group. Which new diagnostics for tuberculosis, and when? J Infect Dis. 2012 May 15;205 Suppl 2:S191-198.

83. R Koch. Die Äetiologie der Tuberkulose. Berl Klin Wochenschr. 1882;15:221–30.

84. Sakula A. Robert Koch: centenary of the discovery of the tubercle bacillus, 1882. Thorax. 1982 Apr;37(4):246–51.

85. Blevins SM, Bronze MS. Robert Koch and the "golden age" of bacteriology. Int J Infect Dis IJID Off Publ Int Soc Infect Dis. 2010 Sep;14(9):e744-751.

86. Steingart KR, Ramsay A, Pai M. Optimizing sputum smear microscopy for the diagnosis of pulmonary tuberculosis. Expert Rev Anti Infect Ther. 2007 Jun;5(3):327–31.

87. Gilpin C, Kim SJ, Lumb R, Rieder HL, Van Deun A, Working Group on Sputum Smear Microscopy. Critical appraisal of current recommendations and practices for tuberculosis sputum smear microscopy. Int J Tuberc Lung Dis Off J Int Union Tuberc Lung Dis. 2007 Sep;11(9):946–52.

88. P. K. H. Hagemann. Fluoreszenzmikroskopische Untersuchungen über Virus und andere Mikroben. Zentralblatt Für Bakteriol Parasitenkd Infekt Hyg. 1937;(Band 140):184.

89. Boehme CC, Saacks S, O'Brien RJ. The changing landscape of diagnostic services for tuberculosis. Semin Respir Crit Care Med. 2013 Feb;34(1):17–31.

90. Davis JL, Cattamanchi A, Cuevas LE, Hopewell PC, Steingart KR. Diagnostic accuracy of same-day microscopy versus standard microscopy for pulmonary tuberculosis: a systematic review and meta-analysis. Lancet Infect Dis. 2013 Feb;13(2):147–54.

91. Cattamanchi A, Dowdy DW, Davis JL, Worodria W, Yoo S, Joloba M, Matovu J, Hopewell PC, Huang L. Sensitivity of direct versus concentrated sputum smear microscopy in HIV-infected patients suspected of having pulmonary tuberculosis. BMC Infect Dis. 2009;9:53.

92. Steingart KR, Ng V, Henry M, Hopewell PC, Ramsay A, Cunningham J, Urbanczik R, Perkins MD, Aziz MA, Pai M. Sputum processing methods to improve the sensitivity of smear microscopy for tuberculosis: a systematic review. Lancet Infect Dis. 2006 Oct;6(10):664–74.

93. Steingart KR, Henry M, Ng V, Hopewell PC, Ramsay A, Cunningham J, Urbanczik R, Perkins M, Aziz MA, Pai M. Fluorescence versus conventional sputum smear microscopy for tuberculosis: a systematic review. Lancet Infect Dis. 2006 Sep;6(9):570–81.

94. Lipsky BA, Gates J, Tenover FC, Plorde JJ. Factors affecting the clinical value of microscopy for acid-fast bacilli. Rev Infect Dis. 1984 Apr;6(2):214–22.

95. Perkins MD, Roscigno G, Zumla A. Progress towards improved tuberculosis diagnostics for developing countries. Lancet Lond Engl. 2006 Mar 18;367(9514):942–3.

96. Singhal R, Myneedu VP. Microscopy as a diagnostic tool in pulmonary tuberculosis. Int J Mycobacteriology. 2015 Mar;4(1):1–6.

97. Taylor GM, Stewart GR, Cooke M, Chaplin S, Ladva S, Kirkup J, Palmer S, Young DB. Koch's bacillus - a look at the first isolate of Mycobacterium tuberculosis from a modern perspective. Microbiol Read Engl. 2003 Nov;149(Pt 11):3213–20.

98. Schön T, Miotto P, Köser CU, Viveiros M, Böttger E, Cambau E. Mycobacterium tuberculosis drug-resistance testing: challenges, recent developments and perspectives. Clin Microbiol Infect Off Publ Eur Soc Clin Microbiol Infect Dis. 2016 Nov 1;

99. Lu D, Heeren B, Dunne WM. Comparison of the Automated Mycobacteria Growth Indicator Tube System (BACTEC 960/MGIT) with Löwenstein-Jensen medium for recovery of mycobacteria from clinical specimens. Am J Clin Pathol. 2002 Oct;118(4):542–5.

100. van Zyl-Smit RN, Binder A, Meldau R, Mishra H, Semple PL, Theron G, Peter J, Whitelaw A, Sharma SK, Warren R, Bateman ED, Dheda K. Comparison of quantitative techniques including Xpert MTB/RIF to evaluate mycobacterial burden. PloS One. 2011;6(12):e28815.

101. Beste DJV, Espasa M, Bonde B, Kierzek AM, Stewart GR, McFadden J. The genetic requirements for fast and slow growth in mycobacteria. PloS One. 2009;4(4):e5349.

102. Stop TB Partnership - Global Laboratory Initiative (GLI): Mycobacteriology Laboratory Manual [Internet]. [cited 2016 Sep 22]. Available from: http://www.stoptb.org/wg/gli/documents.asp?xpand=3

103. WHO | Policy statement: Liquid media for culture and DST | Use of liquid TB culture and drug susceptibility testing (DST) in low- and medium-income settings. Summary report of the Expert Group Meeting on the Use of Liquid Culture Media, Geneva, 26 March 2007. Geneva, Switzerland: WHO, 2007. [Internet]. WHO. [cited 2016 Sep 22]. Available from: http://www.who.int/tb/laboratory/policy_liquid_medium_for_culture_dst/en/

104. Hepple P, Novoa-Cain J, Cheruiyot C, Richter E, Ritmeijer K. Implementation of liquid culture for tuberculosis diagnosis in a remote setting: lessons learned. Int J Tuberc Lung Dis Off J Int Union Tuberc Lung Dis. 2011 Mar;15(3):405–7.

105. Lerner BH. The perils of "X-ray vision": How radiographic images have historically influenced perception. Perspect Biol Med. 1992;35(3):382–97.

106. Bakari M, Arbeit RD, Mtei L, Lyimo J, Waddell R, Matee M, Cole BF, Tvaroha S, Horsburgh CR, Soini H, Pallangyo K, von Reyn CF. Basis for treatment of tuberculosis among HIV-infected patients in Tanzania: the role of chest x-ray and sputum culture. BMC Infect Dis. 2008 Mar 6;8:32.

107. Schalekamp S, van Ginneken B, Karssemeijer N, Schaefer-Prokop CM. Chest radiography: new technological developments and their applications. Semin Respir Crit Care Med. 2014 Feb;35(1):3–16.

108. GlobalDiagnostiX | A consortium developing a robust, high-tech & low-cost medical x-ray system for low and middle-income countries [Internet]. [cited 2016 Nov 29]. Available from: http://www.globaldiagnostix.org/

109. Pande T, Pai M, Khan FA, Denkinger CM. Use of chest radiography in the 22 highest tuberculosis burden countries. Eur Respir J. 2015 Dec;46(6):1816–9.

110. van't Hoog AH, Langendam M, Mitchell E, Cobelens FG, Sinclair D, Leeflang MMG, et al. A systematic review of the sensitivity and specificity of symptom- and chest-radiography screening for active pulmonary tuberculosis in HIV-negative persons and persons with unknown HIV status [Internet]

World Health Organization; 2013. Available from: http://www.who.int/tb/Review2Accuracyofscreeningtests.pdf?ua=1.

111. van't Hoog AH, Meme HK, Laserson KF, Agaya JA, Muchiri BG, Githui WA, Odeny LO, Marston BJ, Borgdorff MW. Screening strategies for tuberculosis prevalence surveys: the value of chest radiography and symptoms. PloS One. 2012;7(7):e38691.

112. Hoog AHV, Meme HK, van Deutekom H, Mithika AM, Olunga C, Onyino F, Borgdorff MW. High sensitivity of chest radiograph reading by clinical officers in a tuberculosis prevalence survey. Int J Tuberc Lung Dis Off J Int Union Tuberc Lung Dis. 2011 Oct;15(10):1308–14.

113. van Cleeff MRA, Kivihya-Ndugga L, Githui W, Nganga L, Odhiambo J, Klatser PR. A comprehensive study of the efficiency of the routine pulmonary tuberculosis diagnostic process in Nairobi. Int J Tuberc Lung Dis Off J Int Union Tuberc Lung Dis. 2003 Feb;7(2):186–9.

114. Davis JL, Worodria W, Kisembo H, Metcalfe JZ, Cattamanchi A, Kawooya M, Kyeyune R, den Boon S, Powell K, Okello R, Yoo S, Huang L. Clinical and radiographic factors do not accurately diagnose smear-negative tuberculosis in HIV-infected inpatients in Uganda: a cross-sectional study. PloS One. 2010 Mar 26;5(3):e9859.

115. Balabanova Y, Coker R, Fedorin I, Zakharova S, Plavinskij S, Krukov N, Atun R, Drobniewski F. Variability in interpretation of chest radiographs among Russian clinicians and implications for screening programmes: observational study. BMJ. 2005 Aug 13;331(7513):379–82.

116. Zellweger JP, Heinzer R, Touray M, Vidondo B, Altpeter E. Intra-observer and overall agreement in the radiological assessment of tuberculosis. Int J Tuberc Lung Dis Off J Int Union Tuberc Lung Dis. 2006 Oct;10(10):1123–6.

117. Sakurada S, Hang NTL, Ishizuka N, Toyota E, Hung LD, Chuc PT, Lien LT, Thuong PH, Bich PTN, Keicho N, Kobayashi N. Inter-rater agreement in the assessment of abnormal chest X-ray findings for tuberculosis between two Asian countries. BMC Infect Dis. 2012 Feb 1;12:31.

118. Coulborn RM, Panunzi I, Spijker S, Brant WE, Duran LT, Kosack CS, Murowa MM. Feasibility of using teleradiology to improve tuberculosis screening and case management in a district hospital in Malawi. Bull World Health Organ. 2012 Sep 1;90(9):705–11.

119. Melendez J, van Ginneken B, Maduskar P, Philipsen RHHM, Reither K, Breuninger M, Adetifa IMO, Maane R, Ayles H, Sanchez CI. A novel multiple-instance learning-based approach to computer-aided detection of tuberculosis on chest x-rays. IEEE Trans Med Imaging. 2015 Jan;34(1):179–92.

120. Hogeweg L, Sanchez C, Maduskar P, Philipsen R, Story A, Dawson R, Theron G, Dheda K, Peters-Bax L, van Ginneken B. Automatic detection of tuberculosis in chest radiographs using a combination of textural, focal, and shape abnormality analysis. IEEE Trans Med Imaging. 2015 Feb 19;

121. Maduskar P. Automated analysis of tuberculosis in chest radiographs Development and Validation. Doctoral thesis. Diagnostic Image Analysis Group, Radboud University Medical Center (Nijmegen, the Netherlands); 2015.

122. WHO | Automated real-time nucleic acid amplification technology for rapid and simultaneous detection of tuberculosis and rifampicin resistance: Xpert MTB/RIF system. Policy statement. Geneva, World Health Organization, 2011 (http://whqlibdoc.who.int/publications/2011/9789241501545_).

123. WHO | Ground-breaking new test for rapid diagnosis of tuberculosis and multidrug-resistant tuberculosis [Internet]. 2010 [cited 2016 Dec 2]. Available from: http://www.euro.who.int/en/health-topics/communicable-diseases/tuberculosis/news/news/2010/12/ground-breaking-new-test-for-rapid-diagnosis-of-tuberculosis-and-multidrug-resistant-tuberculosis

124. WHO | Automated Real-Time Nucleic Acid Amplification Technology for Rapid and Simultaneous Detection of Tuberculosis and Rifampicin Resistance: Xpert MTB/RIF Assay for the Diagnosis of Pulmonary and Extrapulmonary TB in Adults and Children: Policy Update [Internet]. Geneva: World Health Organization; 2013 [cited 2015 Jun 17]. (WHO Guidelines Approved by the Guidelines Review Committee). Available from: http://www.ncbi.nlm.nih.gov/books/NBK258608/

125. Tyagi S, Kramer FR. Molecular beacons: probes that fluoresce upon hybridization. Nat Biotechnol. 1996 Mar;14(3):303–8.

126. Helb D, Jones M, Story E, Boehme C, Wallace E, Ho K, Kop J, Owens MR, Rodgers R, Banada P, Safi H, Blakemore R, Lan NTN, Jones-López EC, Levi M, Burday M, Ayakaka I, Mugerwa RD, McMillan B, Winn-Deen E, Christel L, Dailey P, Perkins MD, Persing DH, Alland D. Rapid detection of Mycobacterium tuberculosis and rifampin resistance by use of on-demand, near-patient technology. J Clin Microbiol. 2010 Jan;48(1):229–37.

127. Lawn SD, Nicol MP. Xpert® MTB/RIF assay: development, evaluation and implementation of a new rapid molecular diagnostic for tuberculosis and rifampicin resistance. Future Microbiol. 2011 Sep;6(9):1067–82.

128. Telenti A, Imboden P, Marchesi F, Lowrie D, Cole S, Colston MJ, Matter L, Schopfer K, Bodmer T. Detection of rifampicin-resistance mutations in Mycobacterium tuberculosis. Lancet Lond Engl. 1993 Mar 13;341(8846):647–50.

129. Blakemore R, Story E, Helb D, Kop J, Banada P, Owens MR, Chakravorty S, Jones M, Alland D. Evaluation of the analytical performance of the Xpert MTB/RIF assay. J Clin Microbiol. 2010 Jul;48(7):2495–501.

130. Lawn SD, Mwaba P, Bates M, Piatek A, Alexander H, Marais BJ, Cuevas LE, McHugh TD, Zijenah L, Kapata N, Abubakar I, McNerney R, Hoelscher M, Memish ZA, Migliori GB, Kim P, Maeurer M, Schito M, Zumla A. Advances in tuberculosis diagnostics: the Xpert MTB/RIF assay and future prospects for a point-of-care test. Lancet Infect Dis. 2013 Apr;13(4):349–61.

131. Banada PP, Sivasubramani SK, Blakemore R, Boehme C, Perkins MD, Fennelly K, Alland D. Containment of bioaerosol infection risk by the Xpert MTB/RIF assay and its applicability to point-of-care settings. J Clin Microbiol. 2010 Oct;48(10):3551–7.

132. Steingart KR, Sohn H, Schiller I, Kloda LA, Boehme CC, Pai M, Dendukuri N. Xpert® MTB/RIF assay for pulmonary tuberculosis and rifampicin resistance in adults. Cochrane Database Syst Rev. 2013;1:CD009593.

133. Detjen AK, DiNardo AR, Leyden J, Steingart KR, Menzies D, Schiller I, Dendukuri N, Mandalakas AM. Xpert MTB/RIF assay for the diagnosis of pulmonary tuberculosis in children: a systematic review and meta-analysis. Lancet Respir Med. 2015 Jun;3(6):451–61.

134. Coetzee L, Nicol MP, Jacobson R, Schubert PT, van Helden PD, Warren RM, Wright CA. Rapid diagnosis of pediatric mycobacterial lymphadenitis using fine needle aspiration biopsy. Pediatr Infect Dis J. 2014 Sep;33(9):893–6.

135. Bholla M, Kapalata N, Masika E, Chande H, Jugheli L, Sasamalo M, Glass TR, Beck H-P, Reither K. Evaluation of Xpert® MTB/RIF and Ustar EasyNAT™ TB IAD for diagnosis of tuberculous lymphadenitis of children in Tanzania: a prospective descriptive study. BMC Infect Dis. 2016 Jun 6;16:246.

136. Pohl C, Rutaihwa LK, Haraka F, Nsubuga M, Aloi F, Ntinginya NE, Mapamba D, Heinrich N, Hoelscher M, Marais BJ, Jugheli L, Reither K. Limited value of whole blood Xpert(®) MTB/RIF for diagnosing tuberculosis in children. J Infect. 2016 Oct;73(4):326–35.

137. Friedrich SO, Rachow A, Saathoff E, Singh K, Mangu CD, Dawson R, Phillips PP, Venter A, Bateson A, Boehme CC, Heinrich N, Hunt RD, Boeree MJ, Zumla A, McHugh TD, Gillespie SH, Diacon AH, Hoelscher M, Pan African Consortium for the Evaluation of Anti-tuberculosis Antibiotics (PanACEA). Assessment of the sensitivity and specificity of Xpert MTB/RIF assay as an early sputum biomarker of response to tuberculosis treatment. Lancet Respir Med. 2013 Aug;1(6):462–70.

138. Albert H, Nathavitharana RR, Isaacs C, Pai M, Denkinger CM, Boehme CC. Development, roll-out and impact of Xpert MTB/RIF for tuberculosis: what lessons have we learnt and how can we do better? Eur Respir J. 2016 Aug;48(2):516–25.

139. Creswell J, Codlin AJ, Andre E, Micek MA, Bedru A, Carter EJ, Yadav R-P, Mosneaga A, Rai B, Banu S, Brouwer M, Blok L, Sahu S, Ditiu L. Results from early programmatic implementation of Xpert MTB/RIF testing in nine countries. BMC Infect Dis. 2014 Jan 2;14:2.

140. Raizada N, Sachdeva KS, Sreenivas A, Vadera B, Gupta RS, Parmar M, Kulsange S, Babre A, Thakur R, Gray C, Ramachandran R, Alavadi U, Ghedia M, Vollepore B, Dewan P, Boehme C, Paramsivan CN. Feasibility of decentralised deployment of Xpert MTB/RIF test at lower level of health system in India. PloS One. 2014;9(2):e89301.

141. Srivastava S, Ernst JD, Desvignes L. Beyond macrophages: the diversity of mononuclear cells in tuberculosis. Immunol Rev. 2014 Nov;262(1):179–92.

142. Ernst JD. The immunological life cycle of tuberculosis. Nat Rev Immunol. 2012 Aug;12(8):581–91.

143. Marino S, Pawar S, Fuller CL, Reinhart TA, Flynn JL, Kirschner DE. Dendritic cell trafficking and antigen presentation in the human immune response to Mycobacterium tuberculosis. J Immunol Baltim Md 1950. 2004 Jul 1;173(1):494–506.

144. Mihret A. The role of dendritic cells in Mycobacterium tuberculosis infection. Virulence. 2012 Nov 15;3(7):654–9.

145. Srivastava S, Ernst JD. Cell-to-cell transfer of M. tuberculosis antigens optimizes CD4 T cell priming. Cell Host Microbe. 2014 Jun 11;15(6):741–52.

146. Prezzemolo T, Guggino G, La Manna MP, Di Liberto D, Dieli F, Caccamo N. Functional signatures of human CD4 and CD8 T cell responses to Mycobacterium tuberculosis. Microb Immunol. 2014;5:180.

147. Flynn JL, Chan J, Triebold KJ, Dalton DK, Stewart TA, Bloom BR. An essential role for interferon gamma in resistance to Mycobacterium tuberculosis infection. J Exp Med. 1993 Dec 1;178(6):2249–54.

148. Flynn JL, Goldstein MM, Chan J, Triebold KJ, Pfeffer K, Lowenstein CJ, Schreiber R, Mak TW, Bloom BR. Tumor necrosis factor-alpha is required in the protective immune response against Mycobacterium tuberculosis in mice. Immunity. 1995 Jun;2(6):561–72.

149. Sutherland JS, Adetifa IM, Hill PC, Adegbola RA, Ota MOC. Pattern and diversity of cytokine production differentiates between Mycobacterium tuberculosis infection and disease. Eur J Immunol. 2009 Mar;39(3):723–9.

150. Herrera MT, Torres M, Nevels D, Perez-Redondo CN, Ellner JJ, Sada E, Schwander SK. Compartmentalized bronchoalveolar IFN-gamma and IL-12 response in human pulmonary tuberculosis. Tuberc Edinb Scotl. 2009 Jan;89(1):38–47.

151. Perreau M, Rozot V, Welles HC, Belluti-Enders F, Vigano S, Maillard M, Dorta G, Mazza-Stalder J, Bart P-A, Roger T, Calandra T, Nicod L, Harari A. Lack of Mycobacterium tuberculosis-specific interleukin-17A-producing CD4+ T cells in active disease. Eur J Immunol. 2013 Apr;43(4):939–48.

152. Jurado JO, Pasquinelli V, Alvarez IB, Peña D, Rovetta AI, Tateosian NL, Romeo HE, Musella RM, Palmero D, Chuluyán HE, García VE. IL-17 and IFN-γ expression in lymphocytes from patients with active tuberculosis correlates with the severity of the disease. J Leukoc Biol. 2012 Jun;91(6):991–1002.

153. Lanzavecchia A, Sallusto F. Understanding the generation and function of memory T cell subsets. Curr Opin Immunol. 2005 Jun;17(3):326–32.

154. Petruccioli E, Petrone L, Vanini V, Sampaolesi A, Gualano G, Girardi E, Palmieri F, Goletti D. IFNγ/TNFα specific-cells and effector memory phenotype associate with active tuberculosis. J Infect. 2013 Jun;66(6):475–86.

155. Sester U, Fousse M, Dirks J, Mack U, Prasse A, Singh M, Lalvani A, Sester M. Whole-blood flow-cytometric analysis of antigen-specific CD4 T-cell cytokine profiles distinguishes active tuberculosis from non-active states. PloS One. 2011;6(3):e17813.

156. Reece ST, Kaufmann SHE. Floating between the poles of pathology and protection: can we pin down the granuloma in tuberculosis? Curr Opin Microbiol. 2012 Feb;15(1):63–70.

157. Ulrichs T, Kaufmann SHE. New insights into the function of granulomas in human tuberculosis. J Pathol. 2006 Jan;208(2):261–9.

158. Dannenberg AM. Immune mechanisms in the pathogenesis of pulmonary tuberculosis. Rev Infect Dis. 1989 Apr;11 Suppl 2:S369-378.

159. Sester M, Sotgiu G, Lange C, Giehl C, Girardi E, Migliori GB, Bossink A, Dheda K, Diel R, Dominguez J, Lipman M, Nemeth J, Ravn P, Winkler S, Huitric E, Sandgren A, Manissero D. Interferon-γ release assays for the diagnosis of active tuberculosis: a systematic review and meta-analysis. Eur Respir J. 2011 Jan;37(1):100–11.

160. Rangaka MX, Wilkinson KA, Glynn JR, Ling D, Menzies D, Mwansa-Kambafwile J, Fielding K, Wilkinson RJ, Pai M. Predictive value of interferon-γ release assays for incident active tuberculosis: a systematic review and meta-analysis. Lancet Infect Dis. 2012 Jan;12(1):45–55.

161. WHO | New WHO recommendations on use of commercial TB Interferon-Gamma Release Assays (IGRAs) in low- and middle-income countries [Internet]. WHO. [cited 2014 Jan 10]. Available from: http://www.who.int/tb/features_archive/igra_policy24oct/en/

162. Riou C, Gray CM, Lugongolo M, Gwala T, Kiravu A, Deniso P, Stewart-Isherwood L, Omar SV, Grobusch MP, Coetzee G, Conradie F, Ismail N, Kaplan G, Fallows D. A subset of circulating blood mycobacteria-specific CD4 T cells can predict the time to Mycobacterium tuberculosis sputum culture conversion. PloS One. 2014;9(7):e102178.

163. Adekambi T, Ibegbu CC, Cagle S, Kalokhe AS, Wang YF, Hu Y, Day CL, Ray SM, Rengarajan J. Biomarkers on patient T cells diagnose active tuberculosis and monitor treatment response. J Clin Invest. 2015 Sep;125(9):3723.

164. Jiang J, Wang X, Wang X, Cao Z, Liu Y, Dong M, Tong A, Cheng X. Reduced CD27 expression on antigen-specific CD4+ T cells correlates with persistent active tuberculosis. J Clin Immunol. 2010 Jul;30(4):566–73.

165. Streitz M, Tesfa L, Yildirim V, Yahyazadeh A, Ulrichs T, Lenkei R, Quassem A, Liebetrau G, Nomura L, Maecker H, Volk H-D, Kern F. Loss of receptor on tuberculin-reactive T-cells marks active pulmonary tuberculosis. PloS One. 2007;2(1):e735.

166. Bijland LR, Bomers MK, Smulders YM. Smelling the diagnosis: a review on the use of scent in diagnosing disease. Neth J Med. 2013 Aug;71(6):300–7.

167. Shirasu M, Touhara K. The scent of disease: volatile organic compounds of the human body related to disease and disorder. J Biochem (Tokyo). 2011 Sep;150(3):257–66.

168. Gardner J W, Bartlett P N. A brief history of electronic noses. Sens Actuat B: Chem. 1994;(18):211–20.

169. Kolk A, Hoelscher M, Maboko L, Jung J, Kuijper S, Cauchi M, Bessant C, van Beers S, Dutta R, Gibson T, Reither K. Electronic-nose technology using sputum samples in diagnosis of patients with tuberculosis. J Clin Microbiol. 2010 Nov;48(11):4235–8.

170. Bruins M, Rahim Z, Bos A, van de Sande WWJ, Endtz HP, van Belkum A. Diagnosis of active tuberculosis by e-nose analysis of exhaled air. Tuberc Edinb Scotl. 2013 Mar;93(2):232–8.

171. Phillips M, Cataneo RN, Condos R, Ring Erickson GA, Greenberg J, La Bombardi V, Munawar MI, Tietje O. Volatile biomarkers of pulmonary tuberculosis in the breath. Tuberc Edinb Scotl. 2007 Jan;87(1):44–52.

172. Phillips M, Basa-Dalay V, Bothamley G, Cataneo RN, Lam PK, Natividad MPR, Schmitt P, Wai J. Breath biomarkers of active pulmonary tuberculosis. Tuberc Edinb Scotl. 2010 Mar;90(2):145–51.

173. Phillips M, Basa-Dalay V, Blais J, Bothamley G, Chaturvedi A, Modi KD, Pandya M, Natividad MPR, Patel U, Ramraje NN, Schmitt P, Udwadia ZF. Point-of-care breath test for biomarkers of active pulmonary tuberculosis. Tuberc Edinb Scotl. 2012 Jul;92(4):314–20.

174. Teodoro-Morrison T, Diamandis EP, Rifai N, Weetjens BJC, Pennazza G, de Boer NK, Bomers MK. Animal olfactory detection of disease: promises and pitfalls. Clin Chem. 2014 Dec;60(12):1473–9.

175. Mgode GF, Weetjens BJ, Nawrath T, Cox C, Jubitana M, Machang'u RS, Cohen-Bacrie S, Bedotto M, Drancourt M, Schulz S, Kaufmann SHE. Diagnosis of tuberculosis by trained African giant pouched rats and confounding impact of pathogens and microflora of the respiratory tract. J Clin Microbiol. 2012 Feb;50(2):274–80.

176. Weetjens BJC, Mgode GF, Machang'u RS, Kazwala R, Mfinanga G, Lwilla F, Cox C, Jubitana M, Kanyagha H, Mtandu R, Kahwa A, Mwessongo J, Makingi G, Mfaume S, Van Steenberge J, Beyene NW, Billet M, Verhagen R. African pouched rats for the detection of pulmonary tuberculosis in sputum samples. Int J Tuberc Lung Dis Off J Int Union Tuberc Lung Dis. 2009 Jun;13(6):737–43.

177. Marais BJ, Gie RP, Hesseling AC, Schaaf HS, Lombard C, Enarson DA, Beyers N. A refined symptom-based approach to diagnose pulmonary tuberculosis in children. Pediatrics. 2006 Nov;118(5):e1350-1359.

178. Graham SM, Ahmed T, Amanullah F, Browning R, Cardenas V, Casenghi M, Cuevas LE, Gale M, Gie RP, Grzemska M, Handelsman E, Hatherill M, Hesseling AC, Jean-Philippe P, Kampmann B, Kabra SK, Lienhardt C, Lighter-Fisher J, Madhi S, Makhene M, Marais BJ, McNeeley DF, Menzies H, Mitchell C, Modi S, Mofenson L, Musoke P, Nachman S, Powell C, Rigaud M, Rouzier V, Starke JR, Swaminathan S, Wingfield C. Evaluation of tuberculosis diagnostics in children: 1. Proposed clinical case definitions for classification of intrathoracic tuberculosis disease. Consensus from an expert panel. J Infect Dis. 2012 May 15;205 Suppl 2:S199-208.

179. Graham SM, Cuevas LE, Jean-Philippe P, Browning R, Casenghi M, Detjen AK, Gnanashanmugam D, Hesseling AC, Kampmann B, Mandalakas A, Marais BJ, Schito M, Spiegel HML, Starke JR, Worrell C, Zar HJ. Clinical Case Definitions for Classification of Intrathoracic Tuberculosis in Children: An Update. Clin Infect Dis Off Publ Infect Dis Soc Am. 2015 Oct 15;61Suppl 3:S179-187.

180. Marais BJ, Gie RP, Schaaf HS, Hesseling AC, Obihara CC, Starke JJ, Enarson DA, Donald PR, Beyers N. The natural history of childhood intra-thoracic tuberculosis: a critical review of literature from the pre-chemotherapy era. Int J Tuberc Lung Dis Off J Int Union Tuberc Lung Dis. 2004 Apr;8(4):392–402.

181. WHO | Global tuberculosis report 2014 [Internet]. WHO. [cited 2014 Nov 4]. Available from: http://www.who.int/tb/publications/global_report/en/

182. Ehmann R, Boedeker E, Friedrich U, Sagert J, Dippon J, Friedel G, Walles T. Canine scent detection in the diagnosis of lung cancer: revisiting a puzzling phenomenon. Eur Respir J. 2012 Mar;39(3):669–76.

183. Bomers MK, van Agtmael MA, Luik H, van Veen MC, Vandenbroucke-Grauls CMJE, Smulders YM. Using a dog's superior olfactory sensitivity to identify Clostridium difficile in stools and patients: proof of principle study. BMJ. 2012;345:e7396.

184. Niimura Y, Nei M. Extensive gains and losses of olfactory receptor genes in mammalian evolution. PloS One. 2007;2(8):e708.

185. Weetjens BJ, Mgode GF, Davis WB, Cox C, Beyene NW. "African giant rats for tuberculosis detection: a novel diagnostic technology," in Global Forum Update on Research for Health, C. A. Gardner, S. Jupp, S. A. Matlin, and C. Mauroux, Eds., vol. 6 of Innovating for the Health of All, Pro-Book, Woolbridge, UK, 2009. In.

186. Poling A, Weetjens BJ, Cox C, Mgode G, Jubitana M, Kazwala R, Mfinanga GS, Huis In 't Veld D. Using giant African pouched rats to detect tuberculosis in human sputum samples: 2009 findings. Am J Trop Med Hyg. 2010 Dec;83(6):1308–10.

187. Mahoney AM, Weetjens BJ, Cox C, Beyene N, Mgode G, Jubitana M, Kuipers D, Kazwala R, Mfinanga GS, Durgin A, Poling A. Using giant African pouched rats to detect tuberculosis in human sputum samples: 2010 findings. Pan Afr Med J. 2011;9:28.

188. Mgode GF, Weetjens BJ, Cox C, Jubitana M, Machang'u RS, Lazar D, Weiner J, Van Geertruyden J-P, Kaufmann SHE. Ability of Cricetomys rats to detect Mycobacterium tuberculosis and discriminate it from other microorganisms. Tuberc Edinb Scotl. 2012 Mar;92(2):182–6.

189. Mahoney A, Weetjens BJ, Cox C, Beyene N, Reither K, Makingi G, Jubitana M, Kazwala R, Mfinanga GS, Kahwa A, Durgin A, Poling A. Pouched rats' detection of tuberculosis in human sputum: comparison to culturing and polymerase chain reaction. Tuberc Res Treat. 2012;2012:716989.

190. Mgode GF, Cohen-Bacrie S, Bedotto M, Weetjens BJ, Cox C, Jubitana M, Kuipers D, Machang'u RS, Kazwala R, Mfinanga SG, Kaufmann SHE, Drancourt M. Mycobacterium Genotypes in Pulmonary Tuberculosis Infections and Their Detection by Trained African Giant Pouched Rats. Curr Microbiol. 2014 Oct 2;

191. Pai M, O'Brien R. Tuberculosis diagnostics trials: do they lack methodological rigor? Expert Rev Mol Diagn. 2006 Jul;6(4):509–14.

192. Pai M, Ramsay A, O'Brien R. Evidence-based tuberculosis diagnosis. PLoS Med. 2008 Jul 22;5(7):e156.

193. Leth van. First Tuberculosis Prevalence Survey in the United Republic of Tanzania (2013). Final Report. Ministry of Health and Social Welfare. United Republic of Tanzania.

194. Buderer NM. Statistical methodology: I. Incorporating the prevalence of disease into the sample size calculation for sensitivity and specificity. Acad Emerg Med Off J Soc Acad Emerg Med. 1996 Sep;3(9):895–900.

195. Diagnostic Standards and Classification of Tuberculosis in Adults and Children. This official statement of the American Thoracic Society and the Centers for Disease Control and Prevention was adopted by the ATS Board of Directors, July 1999. This statement was endorsed by the Council of the Infectious Disease Society of America, September 1999. Am J Respir Crit Care Med. 2000 Apr;161(4 Pt 1):1376–95.

196. Poling A, Weetjens B, Cox C, Beyene N, Durgin A, Mahoney A. Tuberculosis detection by giant african pouched rats. Behav Anal MABA. 2011;34(1):47–54.

197. Banoo S, Bell D, Bossuyt P, Herring A, Mabey D, Poole F, Smith PG, Sriram N, Wongsrichanalai C, Linke R, O'Brien R, Perkins M, Cunningham J, Matsoso P, Nathanson CM, Olliaro P, Peeling RW, Ramsay A. Evaluation of diagnostic tests for infectious diseases: general principles. Nat Rev Microbiol. 2010 Dec;8(12 Suppl):S17-29.

198. Peeling RW, Smith PG, Bossuyt PMM. A guide for diagnostic evaluations. Nat Rev Microbiol. 2010 Dec;8(12 Suppl):S2-6.

199. ICH Official web site : ICH [Internet]. [cited 2013 Jan 13]. Available from: http://www.ich.org/

200. World Medical Association Declaration of Helsinki. Ethical principles for medical research involving human subjects. Bull World Health Organ. 2001;79(4):373–4.

201. Joloba ML, Johnson JL, Namale A, Morrissey A, Assegghai AE, Mugerwa RD, Ellner JJ, Eisenach KD. Quantitative sputum bacillary load during rifampin-containing short course chemotherapy in human immunodeficiency virus-infected and non-infected adults with pulmonary tuberculosis. Int J Tuberc Lung Dis Off J Int Union Tuberc Lung Dis. 2000 Jun;4(6):528–36.

202. Chamie G, Luetkemeyer A, Walusimbi-Nanteza M, Okwera A, Whalen CC, Mugerwa RD, Havlir DV, Charlebois ED. Significant variation in presentation of pulmonary tuberculosis across a high resolution of CD4 strata. Int J Tuberc Lung Dis Off J Int Union Tuberc Lung Dis. 2010 Oct;14(10):1295–302.

203. Huang L, Crothers K. HIV-associated opportunistic pneumonias. Respirol Carlton Vic. 2009 May;14(4):474–85.

204. Hegde MC, Kumar A, Bhat G, Sreedharan S. Oral Microflora: A Comparative Study in HIV and Normal Patients. Indian J Otolaryngol Head Neck Surg Off Publ Assoc Otolaryngol India. 2014 Jan;66(Suppl 1):126–32.

205. Mukherjee PK, Chandra J, Retuerto M, Sikaroodi M, Brown RE, Jurevic R, Salata RA, Lederman MM, Gillevet PM, Ghannoum MA. Oral mycobiome analysis of HIV-infected patients: identification of Pichia as an antagonist of opportunistic fungi. PLoS Pathog. 2014 Mar;10(3):e1003996.

206. Nawrath T, Mgode GF, Weetjens B, Kaufmann SHE, Schulz S. The volatiles of pathogenic and nonpathogenic mycobacteria and related bacteria. Beilstein J Org Chem. 2012;8:290–9.

207. Dang NA, Mourão M, Kuijper S, Walters E, Janssen H-G, Kolk AHJ. Direct detection of Mycobacterium tuberculosis in sputum using combined solid phase extraction-gas chromatography-mass spectrometry. J Chromatogr B Analyt Technol Biomed Life Sci. 2015 Feb 9;986–987C:115–22.

208. WHO | Systematic screening for active tuberculosis: principles and recommendations [Internet]. WHO. [cited 2015 Jun 10]. Available from: http://www.who.int/tb/tbscreening/en/

209. Denkinger CM, Kik SV, Cirillo DM, Casenghi M, Shinnick T, Weyer K, Gilpin C, Boehme CC, Schito M, Kimerling M, Pai M. Defining the Needs for Next Generation Assays for Tuberculosis. J Infect Dis. 2015 Apr 1;211(suppl 2):S29–38.

210. Story A, Aldridge RW, Abubakar I, Stagg HR, Lipman M, Watson JM, Hayward AC. Active case finding for pulmonary tuberculosis using mobile digital chest radiography: an observational study. Int J Tuberc Lung Dis Off J Int Union Tuberc Lung Dis. 2012 Nov;16(11):1461–7.

211. den Boon S, White NW, van Lill SWP, Borgdorff MW, Verver S, Lombard CJ, Bateman ED, Irusen E, Enarson DA, Beyers N. An evaluation of symptom and chest radiographic screening in tuberculosis prevalence surveys. Int J Tuberc Lung Dis Off J Int Union Tuberc Lung Dis. 2006 Aug;10(8):876–82.

212. van Cleeff MRA, Kivihya-Ndugga LE, Meme H, Odhiambo JA, Klatser PR. The role and performance of chest X-ray for the diagnosis of tuberculosis: a cost-effectiveness analysis in Nairobi, Kenya. BMC Infect Dis. 2005;5:111.

213. Graham S, Das GK, Hidvegi RJ, Hanson R, Kosiuk J, Al ZK, Menzies D. Chest radiograph abnormalities associated with tuberculosis: reproducibility and yield of active cases. Int J Tuberc Lung Dis Off J Int Union Tuberc Lung Dis. 2002 Feb;6(2):137–42.

214. Den Boon S, Bateman ED, Enarson DA, Borgdorff MW, Verver S, Lombard CJ, Irusen E, Beyers N, White NW. Development and evaluation of a new chest radiograph reading and recording system for epidemiological surveys of tuberculosis and lung disease. Int J Tuberc Lung Dis Off J Int Union Tuberc Lung Dis. 2005 Oct;9(10):1088–96.

215. Balabanova Y, Coker R, Fedorin I, Zakharova S, Plavinskij S, Krukov N, Atun R, Drobniewski F. Variability in interpretation of chest radiographs among Russian clinicians and implications for screening programmes: observational study. BMJ. 2005 Aug 13;331(7513):379–82.

216. Conners R., Harlow C., Dwyer III S. Radiographic image analysis: Past and present, in Proc. 6th. Int. Conf. Pattern Recognition, Munich, Germany, 1982, pp. 1152–1168. In.

217. Samulski M, Hupse R, Boetes C, Mus RDM, den Heeten GJ, Karssemeijer N. Using computer-aided detection in mammography as a decision support. Eur Radiol. 2010 Oct;20(10):2323–30.

218. Maduskar P, Muyoyeta M, Ayles H, Hogeweg L, Peters-Bax L, van Ginneken B. Detection of tuberculosis using digital chest radiography: automated reading vs. interpretation by clinical officers. Int J Tuberc Lung Dis Off J Int Union Tuberc Lung Dis. 2013 Dec;17(12):1613–20.

219. Hogeweg L, Story A, Hayward A, Aldridge I, Abubakar I, Maduskar P, van Ginneken B. Computer-aided detection of tuberculosis among high risk groups: potential for automated triage, in: Annual Meeting of the Radiological Society of North America, 2011.

220. Jaeger S, Karargyris A, Candemir S, Siegelman J, Folio L, Antani S, Thoma G. Automatic screening for tuberculosis in chest radiographs: a survey. Quant Imaging Med Surg. 2013 Apr;3(2):89–99.

221. Hogeweg L, Mol C, de Jong PA, Dawson R, Ayles H, van Ginneken B. Fusion of local and global detection systems to detect tuberculosis in chest radiographs. Med Image Comput Comput-Assist Interv MICCAI Int Conf Med Image Comput Comput-Assist Interv. 2010;13(Pt 3):650–7.

222. van Ginneken B, Schaefer-Prokop CM, Prokop M. Computer-aided diagnosis: how to move from the laboratory to the clinic. Radiology. 2011 Dec;261(3):719–32.

223. ICAP Columbia University (2014) Resources. Available: http://icap.columbia. edu/resources/. Accessed 2014 May 9.

224. DeLong ER, DeLong DM, Clarke-Pearson DL. Comparing the areas under two or more correlated receiver operating characteristic curves: a nonparametric approach. Biometrics. 1988 Sep;44(3):837–45.

225. R Core Team (2013) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. Available: http:// www.r-project.org/.

226. Robin X, Turck N, Hainard A, Tiberti N, Lisacek F, Sanchez J-C, Müller M. pROC: an open-source package for R and S+ to analyze and compare ROC curves. BMC Bioinformatics. 2011;12:77.

227. Stevenson M, Nunes T, Sanchez J, Thornton R, Reiczigel J, Robison-Cox J, Sebastiani P. epiR - An R package for the analysis of epidemiological data. R package version 0.9-43. 2013;

228. Wickham H. ggplot2: Elegant Graphics for Data Analysis [Internet]. New York, NY: Springer New York; 2009 [cited 2016 Jul 22]. Available from: http://link.springer.com/10.1007/978-0-387-98141-3

229. Wickham H. Reshaping Data with the reshape Package. J Stat Softw. 2007;21:1–20.

230. Lemon J. Plotrix: a package in the red light district of R. R-News. 2006;6:8–12.

231. Siddiqi K, Lambert M-L, Walley J. Clinical diagnosis of smear-negative pulmonary tuberculosis in low-income countries: the current evidence. Lancet Infect Dis. 2003 May;3(5):288–96.

232. Fourie PB, Gatner EM, Glatthaar E, Kleeberg HH. Follow-up tuberculosis prevalence survey of Transkei. Tubercle. 1980 Jun;61(2):71–9.

233. Buijtels PCAM, van-der-Sande MAB, de-Graaff CS, Parkinson S, Verbrugh HA, Petit PLC, van-Soolingen D. Nontuberculous mycobacteria, zambia. Emerg Infect Dis. 2009 Feb;15(2):242–9.

234. Aliyu G, El-Kamary SS, Abimiku A 'le, Brown C, Tracy K, Hungerford L, Blattner W. Prevalence of non-tuberculous mycobacterial infections among tuberculosis suspects in Nigeria. PloS One. 2013;8(5):e63170.

235. Griffith DE, Aksamit T, Brown-Elliott BA, Catanzaro A, Daley C, Gordin F, Holland SM, Horsburgh R, Huitt G, Iademarco MF, Iseman M, Olivier K, Ruoss S, von Reyn CF, Wallace RJ, Winthrop K, ATS Mycobacterial Diseases Subcommittee, American Thoracic Society, Infectious Disease Society of America. An official ATS/IDSA statement: diagnosis, treatment, and prevention of nontuberculous mycobacterial diseases. Am J Respir Crit Care Med. 2007 Feb 15;175(4):367–416.

236. van Ginneken B, Hogeweg L, Maduskar P, Peters-Bax L, Dawson R, Dheda K, Ayles H, Melendez J, Sanchez C. Performance of Inexperienced and Experienced Observers in Detection of Active Tuberculosis on Digital Chest Radiographs With and Without the Use of Computer-aided Diagnosis. at: Annual Meeting of the Radiological Society of North America, 2012. Available from: http://archive.rsna.org/2012/12029649.html

237. Ministry of Health and Social Welfare (2006) Manual of the National Tuberculosis and Leprosy Programme in Tanzania, 5th Edition.

238. Division of Leprosy Tuberculosis and Lung Disease. Ministry of Public Health and Sanitation Kenya. (2009) Guidelines on management of Leprosy and Tuberculosis.

239. The National Tuberculosis and Leprosy Programme Zambia (2008) TB Manual.

240. Ministry of Health Uganda (2010) Manual of the National Tuberculosis and Leprosy Programme, 2nd Edition.

241. National Department of Health South Africa (2008) National Tuberculosis Management Guidelines.

242. Ministry of Health and Social Services Namibia (2011) National Guidelines for the Management of Tuberculosis, 3rd Edition.

243. Tuberculosis Leprosy and TB/HIV Prevention and Control Programme (2008) Federal Ministry of Health Ethiopia Manual, 4th Edition.

244. Zimbabwe National Tuberculosis Control Programme (2010) National TB Guidelines.

245. WHO | Improving the diagnosis and treatment of smear-negative pulmonary and extra-pulmonary tuberculosis among adults and adolescents [Internet]. WHO. [cited 2016 Aug 9]. Available from: http://www.who.int/hiv/pub/tb/pulmonary/en/

246. Kranzer K, Afnan-Holmes H, Tomlin K, Golub JE, Shapiro AE, Schaap A, Corbett EL, Lönnroth K, Glynn JR. The benefits to communities and individuals of screening for active tuberculosis disease: a systematic review. Int J Tuberc Lung Dis Off J Int Union Tuberc Lung Dis. 2013 Apr;17(4):432–46.

247. Uplekar M, Creswell J, Ottmani S-E, Weil D, Sahu S, Lönnroth K. Programmatic approaches to screening for active tuberculosis. Int J Tuberc Lung Dis Off J Int Union Tuberc Lung Dis. 2013 Oct;17(10):1248–56.

248. WHO | Workshop to scale up the implementation of collaborative TB/HIV activities in Africa [Internet]. WHO. [cited 2016 Aug 9]. Available from: http://www.who.int/hiv/pub/meetingreports/maputo_access/en/

249. Zennaro F, Oliveira Gomes JA, Casalino A, Lonardi M, Starc M, Paoletti P, Gobbo D, Giusto C, Not T, Lazzerini M. Digital radiology to improve the quality of care in countries with limited resources: a feasibility study from Angola. PloS One. 2013;8(9):e73939.

250. Newton SM, Brent AJ, Anderson S, Whittaker E, Kampmann B. Paediatric tuberculosis. Lancet Infect Dis. 2008 Aug;8(8):498–510.

251. Marais BJ, Graham SM, Maeurer M, Zumla A. Progress and challenges in childhood tuberculosis. Lancet Infect Dis. 2013 Apr;13(4):287–9.

252. Starke JR. Pediatric tuberculosis: time for a new approach. Tuberc Edinb Scotl. 2003;83(1–3):208–12.

253. Zar HJ, Hanslo D, Apolles P, Swingler G, Hussey G. Induced sputum versus gastric lavage for microbiological confirmation of pulmonary tuberculosis in infants and young children: a prospective study. Lancet. 2005 Jan 8;365(9454):130–4.

254. Lighter J, Rigaud M. Diagnosing childhood tuberculosis: traditional and innovative modalities. Curr Probl Pediatr Adolesc Health Care. 2009 Mar;39(3):61–88.

255. Marais BJ, Gie RP, Schaaf HS, Hesseling AC, Enarson DA, Beyers N. The spectrum of disease in children treated for tuberculosis in a highly endemic area. Int J Tuberc Lung Dis Off J Int Union Tuberc Lung Dis. 2006 Jul;10(7):732–8.

256. Graham SM. The use of diagnostic systems for tuberculosis in children. Indian J Pediatr. 2011 Mar;78(3):334–9.

257. WHO | WHO monitoring of Xpert MTB/RIF roll-out [Internet]. WHO. [cited 2013 Nov 6]. Available from: http://who.int/tb/laboratory/mtbrifrollout/en/

258. WHO | Xpert MTB/RIF: WHO Policy update and Implementation manual [Internet]. [cited 2014 May 7]. Available from: http://www.who.int/tb/laboratory/xpert_launchupdate/en/

259. Boehme CC, Nicol MP, Nabeta P, Michael JS, Gotuzzo E, Tahirli R, Gler MT, Blakemore R, Worodria W, Gray C, Huang L, Caceres T, Mehdiyev R, Raymond L, Whitelaw A, Sagadevan K, Alexander H, Albert H, Cobelens F, Cox H, Alland D, Perkins MD. Feasibility, diagnostic accuracy, and effectiveness of decentralised use of the Xpert MTB/RIF test for diagnosis of tuberculosis and multidrug resistance: a multicentre implementation study. Lancet [Internet]. 2011 Apr 18 [cited 2011 Apr 26]; Available from: http://www.ncbi.nlm.nih.gov/pubmed/21507477

260. Nicol MP, Workman L, Isaacs W, Munro J, Black F, Eley B, Boehme CC, Zemanay W, Zar HJ. Accuracy of the Xpert MTB/RIF test for the diagnosis of pulmonary tuberculosis in children admitted to hospital in Cape Town, South Africa: a descriptive study. Lancet Infect Dis. 2011 Nov;11(11):819–24.

261. Rachow A, Clowes P, Saathoff E, Mtafya B, Michael E, Ntinginya EN, Kowour D, Rojas-Ponce G, Kroidl A, Maboko L, Heinrich N, Reither K, Hoelscher M. Increased and expedited case detection by Xpert MTB/RIF assay in childhood tuberculosis: a prospective cohort study. Clin Infect Dis Off Publ Infect Dis Soc Am. 2012 May;54(10):1388–96.

262. Sekadde MP, Wobudeya E, Joloba ML, Ssengooba W, Kisembo H, Bakeera-Kitaka S, Musoke P. Evaluation of the Xpert MTB/RIF test for the diagnosis of childhood pulmonary tuberculosis in Uganda: a cross-sectional diagnostic study. BMC Infect Dis. 2013;13:133.

263. Nhu NTQ, Ha DTM, Anh ND, Thu DDA, Duong TN, Quang ND, Lan NTN, Quyet TV, Tuyen NTB, Ha VT, Giang DC, Dung NH, Wolbers M, Farrar J, Caws M. Evaluation of Xpert MTB/RIF and MODS assay for the diagnosis of pediatric tuberculosis. BMC Infect Dis. 2013;13:31.

264. Zar HJ, Workman L, Isaacs W, Dheda K, Zemanay W, Nicol MP. Rapid diagnosis of pulmonary tuberculosis in African children in a primary care setting by use of Xpert MTB/RIF on respiratory specimens: a prospective study. Lancet Glob Health. 2013 Aug;1(2):e97–104.

265. WHO | The WHO Child Growth Standards [Internet]. [cited 2013 Nov 11]. Available from: http://www.who.int/childgrowth/en/

266. 1994 Revised Classification System for Human Immunodeficiency Virus Infection in Children Less Than 13 Years of Age [Internet]. [cited 2013 Nov 7]. Available from: http://www.cdc.gov/mmwr/preview/mmwrhtml/00032890.htm

267. R Development Core Team (2013). R: A language and environment forstatistical computing. R Foundation for Statistical Com.

268. Lunn D, Spiegelhalter D, Thomas A, Best N. The BUGS project: Evolution, critique and future directions. Stat Med. 2009 Nov 10;28(25):3049–67.

269. Simel DL, Samsa GP, Matchar DB. Likelihood ratios with confidence: sample size estimation for diagnostic test studies. J Clin Epidemiol. 1991;44(8):763–70.

270. Jenkins HE, Tolman AW, Yuen CM, Parr JB, Keshavjee S, Pérez-Vélez CM, Pagano M, Becerra MC, Cohen T. Incidence of multidrug-resistant tuberculosis disease in children: systematic review and global estimates. Lancet. 2014 Mar 21;

271. Perez-Velez CM, Marais BJ. Tuberculosis in children. N Engl J Med. 2012 Jul 26;367(4):348–61.

272. Cuevas LE, Petrucci R, Swaminathan S. Tuberculosis diagnostics for children in high-burden countries: what is available and what is needed. Paediatr Int Child Health. 2012 Nov;32 Suppl 2:S30-37.

273. Marais BJ, Pai M. New approaches and emerging technologies in the diagnosis of childhood tuberculosis. Paediatr Respir Rev. 2007 Jun;8(2):124–33.

274. Zar HJ, Connell TG, Nicol M. Diagnosis of pulmonary tuberculosis in children: new advances. Expert Rev Anti Infect Ther. 2010 Mar;8(3):277–88.

275. Steingart KR, Schiller I, Horne DJ, Pai M, Boehme CC, Dendukuri N. Xpert® MTB/RIF assay for pulmonary tuberculosis and rifampicin resistance in adults. In: Cochrane Database of Systematic Reviews [Internet]. John Wiley & Sons, Ltd; 1996 [cited 2014 Feb 12]. Available from: http://onlinelibrary.wiley.com/doi/10.1002/14651858.CD009593.pub3/abstract

276. Pai M, Denkinger CM, Kik SV, Rangaka MX, Zwerling A, Oxlade O, Metcalfe JZ, Cattamanchi A, Dowdy DW, Dheda K, Banaei N. Gamma interferon release assays for detection of Mycobacterium tuberculosis infection. Clin Microbiol Rev. 2014 Jan;27(1):3–20.

277. Nikitina IY, Kondratuk NA, Kosmiadi GA, Amansahedov RB, Vasilyeva IA, Ganusov VV, Lyadova IV. Mtb-specific CD27low CD4 T cells as markers of lung tissue destruction during pulmonary tuberculosis in humans. PloS One. 2012;7(8):e43733.

278. Geldmacher C, Ngwenyama N, Schuetz A, Petrovas C, Reither K, Heeregrave EJ, Casazza JP, Ambrozak DR, Louder M, Ampofo W, Pollakis G, Hill B, Sanga E, Saathoff E, Maboko L, Roederer M, Paxton WA, Hoelscher M, Koup RA. Preferential infection and depletion of Mycobacterium tuberculosis-specific CD4 T cells after HIV-1 infection. J Exp Med. 2010 Dec 20;207(13):2869–81.

279. Schuetz A, Haule A, Reither K, Ngwenyama N, Rachow A, Meyerhans A, Maboko L, Koup RA, Hoelscher M, Geldmacher C. Monitoring CD27 expression to evaluate Mycobacterium tuberculosis activity in HIV-1 infected individuals in vivo. PloS One. 2011;6(11):e27284.

280. De Jong R, Brouwer M, Hooibrink B, Van der Pouw-Kraan T, Miedema F, Van Lier RA. The CD27- subset of peripheral blood memory CD4+ lymphocytes contains functionally differentiated T lymphocytes that develop by persistent antigenic stimulation in vivo. Eur J Immunol. 1992 Apr;22(4):993–9.

281. Kapina MA, Shepelkova GS, Mischenko VV, Sayles P, Bogacheva P, Winslow G, Apt AS, Lyadova IV. CD27low CD4 T lymphocytes that accumulate in the mouse lungs during mycobacterial infection differentiate from CD27high precursors in situ, produce IFN-gamma, and protect the host against tuberculosis infection. J Immunol Baltim Md 1950. 2007 Jan 15;178(2):976–85.

282. Li L, Qiao D, Li Q, Zhang X, Lao S, Wu C. Distinct polyfunctional CD4+ T cell responses to BCG, ESAT-6 and CFP-10 in tuberculous pleurisy. Tuberc Edinb Scotl. 2012 Jan;92(1):63–71.

283. Zar HJ, Tannenbaum E, Apolles P, Roux P, Hanslo D, Hussey G. Sputum induction for the diagnosis of pulmonary tuberculosis in infants and young children in an urban setting in South Africa. Arch Dis Child. 2000 Apr;82(4):305–8.

284. Wright CA, Hesseling AC, Bamford C, Burgess SM, Warren R, Marais BJ. Fine-needle aspiration biopsy: a first-line diagnostic procedure in paediatric tuberculosis suspects with peripheral lymphadenopathy? Int J Tuberc Lung Dis Off J Int Union Tuberc Lung Dis. 2009 Nov;13(11):1373–9.

285. Geldmacher C, Schuetz A, Ngwenyama N, Casazza JP, Sanga E, Saathoff E, Boehme C, Geis S, Maboko L, Singh M, Minja F, Meyerhans A, Koup RA, Hoelscher M. Early depletion of Mycobacterium tuberculosis-specific T helper 1 cell responses after HIV-1 infection. J Infect Dis. 2008 Dec 1;198(11):1590–8.

286. WHO | WHO case definitions of HIV for surveillance and revised clinical staging and immunological classification of HIV-related disease in adults and children [Internet]. WHO. [cited 2014 Jun 20]. Available from: http://www.who.int/hiv/pub/guidelines/hivstaging/en/

287. Newton SM, Brent AJ, Anderson S, Whittaker E, Kampmann B. Paediatric tuberculosis. Lancet Infect Dis. 2008 Aug;8(8):498–510.

288. Graham SM, Ahmed T, Amanullah F, Browning R, Cardenas V, Casenghi M, Cuevas LE, Gale M, Gie RP, Grzemska M, Handelsman E, Hatherill M, Hesseling AC, Jean-Philippe P, Kampmann B, Kabra SK, Lienhardt C, Lighter-Fisher J, Madhi S, Makhene M, Marais BJ, McNeeley DF, Menzies H, Mitchell C, Modi S, Mofenson L, Musoke P, Nachman S, Powell C, Rigaud M, Rouzier V, Starke JR, Swaminathan S, Wingfield C. Evaluation of tuberculosis diagnostics in children: 1. Proposed clinical case definitions for classification of intrathoracic tuberculosis disease. Consensus from an expert panel. J Infect Dis. 2012 May 15;205 Suppl 2:S199-208.

289. Nicol MP, Workman L, Isaacs W, Munro J, Black F, Eley B, Boehme CC, Zemanay W, Zar HJ. Accuracy of the Xpert MTB/RIF test for the diagnosis of pulmonary tuberculosis in children admitted to hospital in Cape Town, South Africa: a descriptive study. Lancet Infect Dis [Internet]. 2011 Jul 15 [cited 2011 Sep 4]; Available from: http://www.ncbi.nlm.nih.gov/pubmed/21764384

290. Rachow A, Clowes P, Saathoff E, Mtafya B, Michael E, Ntinginya EN, Kowour D, Rojas-Ponce G, Kroidl A, Maboko L, Heinrich N, Reither K, Hoelscher M. Increased and Expedited Case Detection by Xpert MTB/RIF Assay in Childhood Tuberculosis: A Prospective Cohort Study. Clin Infect Dis Off Publ Infect Dis Soc Am [Internet]. 2012 Apr 3 [cited 2012 Apr 9]; Available from: http://www.ncbi.nlm.nih.gov/pubmed/22474220

291. Zar HJ, Workman L, Isaacs W, Munro J, Black F, Eley B, Allen V, Boehme CC, Zemanay W, Nicol MP. Rapid molecular diagnosis of pulmonary tuberculosis in children using nasopharyngeal specimens. Clin Infect Dis Off Publ Infect Dis Soc Am. 2012 Oct;55(8):1088–95.

292. Bull M, Lee D, Stucky J, Chiu Y-L, Rubin A, Horton H, McElrath MJ. Defining blood processing parameters for optimal detection of cryopreserved antigen-specific responses for HIV vaccine trials. J Immunol Methods. 2007 Apr 30;322(1–2):57–69.

293. Ferrara G, Losi M, D'Amico R, Roversi P, Piro R, Meacci M, Meccugni B, Dori IM, Andreani A, Bergamini BM, Mussini C, Rumpianesi F, Fabbri LM, Richeldi L. Use in routine clinical practice of two commercial blood tests for diagnosis of infection with Mycobacterium tuberculosis: a prospective study. Lancet. 2006 Apr 22;367(9519):1328–34.

294. Thomas TA, Mondal D, Noor Z, Liu L, Alam M, Haque R, Banu S, Sun H, Peterson KM. Malnutrition and helminth infection affect performance of an interferon gamma-release assay. Pediatrics. 2010 Dec;126(6):e1522-1529.

295. Fritsch RD, Shen X, Sims GP, Hathcock KS, Hodes RJ, Lipsky PE. Stepwise differentiation of CD4 memory T cells defined by expression of CCR7 and CD27. J Immunol Baltim Md 1950. 2005 Nov 15;175(10):6489–97.

296. Appay V, van Lier RAW, Sallusto F, Roederer M. Phenotype and function of human T lymphocyte subsets: consensus and issues. Cytom Part J Int Soc Anal Cytol. 2008 Nov;73(11):975–83.

297. Kalsdorf B, Scriba TJ, Wood K, Day CL, Dheda K, Dawson R, Hanekom WA, Lange C, Wilkinson RJ. HIV-1 infection impairs the bronchoalveolar T-cell response to mycobacteria. Am J Respir Crit Care Med. 2009 Dec 15;180(12):1262–70.

298. Wells WA, Boehme CC, Cobelens FGJ, Daniels C, Dowdy D, Gardiner E, Gheuens J, Kim P, Kimerling ME, Kreiswirth B, Lienhardt C, Mdluli K, Pai M, Perkins MD, Peter T, Zignol M, Zumla A, Schito M. Alignment of new tuberculosis drug regimens and drug susceptibility testing: a framework for action. Lancet Infect Dis. 2013 May;13(5):449–58.

299. Trauner A, Borrell S, Reither K, Gagneux S. Evolution of drug resistance in tuberculosis: recent progress and implications for diagnosis and therapy. Drugs. 2014 Jul;74(10):1063–72.

300. Kik SV, Denkinger CM, Casenghi M, Vadnais C, Pai M. Tuberculosis diagnostics: which target product profiles should be prioritised? Eur Respir J. 2014 Aug;44(2):537–40.

301. Stop TB Partnership - New Diagnostics Working Group. Task Force on LTBI and test of progression. [Internet]. [cited 2017 Jan 18]. Available from: http://www.stoptb.org/wg/new_diagnostics/ltbi.asp

302. 2014 23 May. Giant rats that can sniff out TB [Internet]. [cited 2016 Dec 22]. Available from: http://www.bbc.com/future/story/20140523-giant-rats-that-can-sniff-out-tb

303. The rats who sniff out tuberculosis | Science | The Guardian [Internet]. [cited 2016 Dec 22]. Available from: https://www.theguardian.com/science/2016/feb/23/rats-who-sniff-out-tubersulosis

304. Reither K, Jugheli L, Glass TR, Sasamalo M, Mhimbira FA, Weetjens BJ, Cox C, Edwards TL, Mulder C, Beyene NW, Mahoney A. Evaluation of Giant African Pouched Rats for Detection of Pulmonary Tuberculosis in Patients from a High-Endemic Setting. PloS One. 2015;10(10):e0135877.

305. Poling A, Valverde E, Beyene N, Mulder C, Cox C, Mgode G, Edwards TL. Active tuberculosis detection by pouched rats in 2014: More than 2,000 new patients found in two countries. J Appl Behav Anal. 2016 Oct 8;

306. Poling A. Using pouched rats to help people: Notes from the field. Am Psychol. 2016 Nov;71(8):835–42.

307. CAD4TB CE Certified! [Internet]. [cited 2016 Dec 22]. Available from: http://www.delftimagingsystems.com/cad4tb-ce-certified!.html

308. Breuninger M, van Ginneken B, Philipsen RHHM, Mhimbira F, Hella JJ, Lwilla F, van den Hombergh J, Ross A, Jugheli L, Wagner D, Reither K. Diagnostic accuracy of computer-aided detection of pulmonary tuberculosis in chest radiographs: a validation study from sub-Saharan Africa. PloS One. 2014;9(9):e106381.

309. Pande T, Cohen C, Pai M, Ahmad Khan F. Computer-aided detection of pulmonary tuberculosis on digital chest radiographs: a systematic review. Int J Tuberc Lung Dis Off J Int Union Tuberc Lung Dis. 2016 Sep;20(9):1226–30.

310. WHO | Chest radiography in tuberculosis detection: summary of current WHO recommendations and guidance on programmatic approaches. [Internet]. World Health Organization; 2016 [cited 2016 Dec 21]. Available from: http://www.who.int/iris/handle/10665/252424

311. Drain PK, Mayeza L, Bartman P, Hurtado R, Moodley P, Varghese S, Maartens G, Alvarez GG, Wilson D. Diagnostic accuracy and clinical role of rapid C-reactive protein testing in HIV-infected individuals with presumed tuberculosis in South Africa. Int J Tuberc Lung Dis Off J Int Union Tuberc Lung Dis. 2014 Jan;18(1):20–6.

312. Huang S-L, Lee H-C, Yu C-W, Chen H-C, Wang C-C, Wu J-Y, Lee C-C. Value of procalcitonin in differentiating pulmonary tuberculosis from other pulmonary infections: a meta-analysis. Int J Tuberc Lung Dis Off J Int Union Tuberc Lung Dis. 2014 Apr;18(4):470–7.

313. Agranoff D, Fernandez-Reyes D, Papadopoulos MC, Rojas SA, Herbster M, Loosemore A, Tarelli E, Sheldon J, Schwenk A, Pollok R, Rayner CFJ, Krishna S. Identification of diagnostic markers for tuberculosis by proteomic fingerprinting of serum. Lancet Lond Engl. 2006 Sep 16;368(9540):1012–21.

314. Petrone L, Cannas A, Vanini V, Cuzzi G, Aloi F, Nsubuga M, Sserunkuma J, Nazziwa RA, Jugheli L, Lukindo T, Girardi E, Antinori A, Pucci L, Reither K, Goletti D. Blood and urine inducible protein 10 as potential markers of disease activity. Int J Tuberc Lung Dis Off J Int Union Tuberc Lung Dis. 2016 Nov;20(11):1554–61.

315. van't Hoog AH, Cobelens F, Vassall A, van Kampen S, Dorman SE, Alland D, Ellner J. Optimal Triage Test Characteristics to Improve the Cost-Effectiveness of the Xpert MTB/RIF Assay for TB Diagnosis: A Decision Analysis. PLoS ONE [Internet]. 2013 Dec 18 [cited 2015 Feb 13];8(12). Available from: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3867409/

316. Dowdy DW, Basu S, Andrews JR. Is passive diagnosis enough? The impact of subclinical disease on diagnostic strategies for tuberculosis. Am J Respir Crit Care Med. 2013 Mar 1;187(5):543–51.

317. Van't Hoog AH, Onozaki I, Lonnroth K. Choosing algorithms for TB screening: a modelling study to compare yield, predictive value and diagnostic burden. BMC Infect Dis. 2014;14:532.

318. García-Basteiro AL, Cobelens F. Triage tests: a new priority for tuberculosis diagnostics. Lancet Respir Med. 2015 Mar;3(3):177–8.

319. Philipsen RHHM, Sánchez CI, Maduskar P, Melendez J, Peters-Bax L, Peter JG, Dawson R, Theron G, Dheda K, van Ginneken B. Automated chest-radiography as a triage for Xpert testing in resource-constrained settings: a prospective study of diagnostic accuracy and costs. Sci Rep. 2015 Jul 27;5:12215.

320. Murray M, Cattamanchi A, Denkinger C, van't Hoog A, Pai M, Dowdy D. Cost-effectiveness of triage testing for facility-based systematic screening of tuberculosis among Ugandan adults. BMJ Glob Health [Internet]. 2016 Sep 28;1(2). Available from: http://gh.bmj.com/content/1/2/e000064.abstract

321. Muyoyeta M, Maduskar P, Moyo M, Kasese N, Milimo D, Spooner R, Kapata N, Hogeweg L, van Ginneken B, Ayles H. The sensitivity and specificity of using a computer aided diagnosis program for automatically scoring chest X-rays of presumptive TB patients compared with Xpert MTB/RIF in Lusaka Zambia. PloS One. 2014;9(4):e93757.

322. Gie RP. Diagnostic atlas of intrathoracic tuberculosis in children: a guide for low income countries. IUATLD; 2003.

323. Reither K, Manyama C, Clowes P, Rachow A, Mapamba D, Steiner A, Ross A, Mfinanga E, Sasamalo M, Nsubuga M, Aloi F, Cirillo D, Jugheli L, Lwilla F. Xpert MTB/RIF assay for diagnosis of pulmonary tuberculosis in children: A prospective, multi-centre evaluation. J Infect. 2015 Apr;70(4):392–9.

324. Detjen AK, Walters E. Editorial Commentary: Improving Children's Access to New Tuberculosis Diagnostic Tools Starts With the Collection of Appropriate Specimens. Clin Infect Dis Off Publ Infect Dis Soc Am. 2016 May 1;62(9):1169–71.

325. DiNardo AR, Detjen A, Ustero P, Ngo K, Bacha J, Mandalakas AM. Culture is an imperfect and heterogeneous reference standard in pediatric tuberculosis. Tuberc Edinb Scotl. 2016 Sep 28;

326. Engelbrecht AL, Marais BJ, Donald PR, Schaaf HS. A critical look at the diagnostic value of culture-confirmation in childhood tuberculosis. J Infect. 2006 Dec;53(6):364–9.

327. David Alland, Mazhgan Rowneki, Laura Smith, Jamie Ryan, Mitchell Chancellor, Ann Marie Simmons, David Persing, Robert Kwiatkowski, Martin Jones, Soumitesh Chakravorty. Xpert MTB/RIF Ultra: A New Near-Patient TB Test With Sensitivity Equal to Culture | CROI Conference. [Internet]. 2015 [cited 2016 Dec 22]. Available from: http://www.croiconference.org/sessions/xpert-mtbrif-ultra-new-near-patient-tb-test-sensitivity-equal-culture

328. Portevin D, Moukambi F, Clowes P, Bauer A, Chachage M, Ntinginya NE, Mfinanga E, Said K, Haraka F, Rachow A, Saathoff E, Mpina M, Jugheli L, Lwilla F, Marais BJ, Hoelscher M, Daubenberger C, Reither K, Geldmacher C. Assessment of the novel T-cell activation marker-tuberculosis assay for diagnosis of active tuberculosis in children: a prospective proof-of-concept study. Lancet Infect Dis. 2014 Oct;14(10):931–8.

329. Nicol MP, Gnanashanmugam D, Browning R, Click ES, Cuevas LE, Detjen A, Graham SM, Levin M, Makhene M, Nahid P, Perez-Velez CM, Reither K, Song R, Spiegel HML, Worrell C, Zar HJ, Walzl G. A Blueprint to Address Research Gaps in the Development of Biomarkers for Pediatric Tuberculosis. Clin Infect Dis Off Publ Infect Dis Soc Am. 2015 Oct 15;61Suppl 3:S164-172.

330. Pollock KM, Whitworth HS, Montamat-Sicotte DJ, Grass L, Cooke GS, Kapembwa MS, Kon OM, Sampson RD, Taylor GP, Lalvani A. T-cell immunophenotyping distinguishes active from latent tuberculosis. J Infect Dis. 2013 Sep;208(6):952–68.

331. Vir P, Arrigucci R, Lakehal K, Davidow AL, Pine R, Tyagi S, Bushkin Y, Lardizabal A, Gennaro ML. Single-Cell Cytokine Gene Expression in Peripheral Blood Cells Correlates with Latent Tuberculosis Status. PloS One. 2015;10(12):e0144904.

332. Bushkin Y, Radford F, Pine R, Lardizabal A, Mangura BT, Gennaro ML, Tyagi S. Profiling T cell activation using single-molecule fluorescence in situ hybridization and flow cytometry. J Immunol Baltim Md 1950. 2015 Jan 15;194(2):836–41.

333. Tuberculosis diagnostics technology and market landscape - 4th edition. UNITAID. October 2015 [Internet]. [cited 2016 Dec 8]. Available from: http://www.unitaid.eu/en/resources/publications/technical-reports#tb

334. Bossuyt PMM, Reitsma JB, Linnet K, Moons KGM. Beyond diagnostic accuracy: the clinical utility of diagnostic tests. Clin Chem. 2012 Dec;58(12):1636–43.

335. Schünemann HJ, Schünemann AHJ, Oxman AD, Brozek J, Glasziou P, Jaeschke R, Vist GE, Williams JW, Kunz R, Craig J, Montori VM, Bossuyt P, Guyatt GH, GRADE Working Group. Grading quality of evidence and strength of recommendations for diagnostic tests and strategies. BMJ. 2008 May 17;336(7653):1106–10.

336. Ferrante di Ruffano L, Hyde CJ, McCaffery KJ, Bossuyt PMM, Deeks JJ. Assessing the value of diagnostic tests: a framework for designing and evaluating trials. BMJ. 2012 Feb 21;344:e686.

337. Bossuyt PM, Lijmer JG, Mol BW. Randomised comparisons of medical tests: sometimes invalid, not always efficient. Lancet Lond Engl. 2000 Nov 25;356(9244):1844–7.

338. Mann G, Squire SB, Bissell K, Eliseev P, Du Toit E, Hesseling A, Nicol M, Detjen A, Kritski A. Beyond accuracy: creating a comprehensive evidence base for TB diagnostic tools. Int J Tuberc Lung Dis Off J Int Union Tuberc Lung Dis. 2010 Dec;14(12):1518–24.

339. Pai MP, Brunet L, Minion J, Steingart K, Ramsay ARC, Lienhardt C. Mapping the landscape and quality of TB diagnostic research. Report to Stop TB Partnership 2009; : 1–24.

340. Menzies NA, Cohen T, Lin H-H, Murray M, Salomon JA. Population health impact and cost-effectiveness of tuberculosis diagnosis with Xpert MTB/RIF: a dynamic simulation and economic evaluation. PLoS Med. 2012;9(11):e1001347.

341. Langley I, Lin H-H, Egwaga S, Doulla B, Ku C-C, Murray M, Cohen T, Squire SB. Assessment of the patient, health system, and population effects of Xpert MTB/RIF and alternative diagnostics for tuberculosis in Tanzania: an integrated modelling approach. Lancet Glob Health. 2014 Oct;2(10):e581-591.

342. Yoon C, Cattamanchi A, Davis JL, Worodria W, den Boon S, Kalema N, Katagira W, Kaswabuli S, Miller C, Andama A, Albert H, Nabeta P, Gray C, Ayakaka I, Huang L. Impact of Xpert MTB/RIF Testing on Tuberculosis Management and Outcomes in Hospitalized Patients in Uganda. PloS One. 2012;7(11):e48599.

343. Theron G, Zijenah L, Chanda D, Clowes P, Rachow A, Lesosky M, Bara W, Mungofa S, Pai M, Hoelscher M, Dowdy D, Pym A, Mwaba P, Mason P, Peter J, Dheda K, TB-NEAT team. Feasibility, accuracy, and clinical effect of point-of-care Xpert MTB/RIF testing for tuberculosis in primary-care settings in Africa: a multicentre, randomised, controlled trial. Lancet Lond Engl. 2014 Feb 1;383(9915):424–35.

344. Cox HS, Mbhele S, Mohess N, Whitelaw A, Muller O, Zemanay W, Little F, Azevedo V, Simpson J, Boehme CC, Nicol MP. Impact of Xpert MTB/RIF for TB diagnosis in a primary care clinic with high TB and HIV prevalence in South Africa: a pragmatic randomised trial. PLoS Med. 2014 Nov;11(11):e1001760.

345. Mupfumi L, Makamure B, Chirehwa M, Sagonda T, Zinyowera S, Mason P, Metcalfe JZ, Mutetwa R. Impact of Xpert MTB/RIF on Antiretroviral Therapy-Associated Tuberculosis and Mortality: A Pragmatic Randomized Controlled Trial. Open Forum Infect Dis. 2014 Mar;1(1):ofu038.

346. Durovni B, Saraceni V, van den Hof S, Trajman A, Cordeiro-Santos M, Cavalcante S, Menezes A, Cobelens F. Impact of replacing smear microscopy with Xpert MTB/RIF for diagnosing tuberculosis in Brazil: a stepped-wedge cluster-randomized trial. PLoS Med. 2014 Dec;11(12):e1001766.

347. Churchyard GJ, Stevens WS, Mametja LD, McCarthy KM, Chihota V, Nicol MP, Erasmus LK, Ndjeka NO, Mvusi L, Vassall A, Sinanovic E, Cox HS, Dye C, Grant AD, Fielding KL. Xpert MTB/RIF versus sputum microscopy as the initial diagnostic test for tuberculosis: a cluster-randomised trial embedded in South African roll-out of Xpert MTB/RIF. Lancet Glob Health. 2015 Aug;3(8):e450-457.

348. Creswell J, Rai B, Wali R, Sudrungrot S, Adhikari LM, Pant R, Pyakurel S, Uranw D, Codlin AJ. Introducing new tuberculosis diagnostics: the impact of Xpert(®) MTB/RIF testing on case notifications in Nepal. Int J Tuberc Lung Dis Off J Int Union Tuberc Lung Dis. 2015 May;19(5):545–51.

349. Trajman A, Durovni B, Saraceni V, Menezes A, Cordeiro-Santos M, Cobelens F, Van den Hof S. Impact on Patients' Treatment Outcomes of XpertMTB/RIF Implementation for the Diagnosis of Tuberculosis: Follow-Up of a Stepped-Wedge Randomized Clinical Trial. PloS One. 2015;10(4):e0123252.

350. Mbonze NB, Tabala M, Wenzi LK, Bakoko B, Brouwer M, Creswell J, Van Rie A, Behets F, Yotebieng M. Xpert(®) MTB/RIF for smear-negative presumptive TB: impact on case notification in DR Congo. Int J Tuberc Lung Dis Off J Int Union Tuberc Lung Dis. 2016 Feb;20(2):240–6.

351. Auld AF, Fielding KL, Gupta-Wright A, Lawn SD. Xpert MTB/RIF - why the lack of morbidity and mortality impact in intervention trials? Trans R Soc Trop Med Hyg. 2016 Aug;110(8):432–44.

352. Theron G, Peter J, Dowdy D, Langley I, Squire SB, Dheda K. Do high rates of empirical treatment undermine the potential effect of new diagnostic tests for tuberculosis in high-burden settings? Lancet Infect Dis. 2014 Jun;14(6):527–32.

353. Hsiang E, Little KM, Haguma P, Hanrahan CF, Katamba A, Cattamanchi A, Davis JL, Vassall A, Dowdy D. Higher cost of implementing Xpert(®) MTB/RIF in Ugandan peripheral settings: implications for cost-effectiveness. Int J Tuberc Lung Dis Off J Int Union Tuberc Lung Dis. 2016 Sep;20(9):1212–8.

354. Pantoja A, Fitzpatrick C, Vassall A, Weyer K, Floyd K. Xpert MTB/RIF for diagnosis of tuberculosis and drug-resistant tuberculosis: a cost and affordability analysis. Eur Respir J. 2013 Sep;42(3):708–20.

355. Schnippel K, Meyer-Rath G, Long L, MacLeod W, Sanne I, Stevens WS, Rosen S. Scaling up Xpert MTB/RIF technology: the costs of laboratory- vs. clinic-based roll-out in South Africa. Trop Med Int Health TM IH. 2012 Sep;17(9):1142–51.

356. Lessells RJ, Cooke GS, Newell M-L, Godfrey-Faussett P. Evaluation of tuberculosis diagnostics: establishing an evidence base around the public health impact. J Infect Dis. 2011 Nov 15;204 Suppl 4:S1187-1195.

357. Alsdurf H, Hill PC, Matteelli A, Getahun H, Menzies D. The cascade of care in diagnosis and treatment of latent tuberculosis infection: a systematic review and meta-analysis. Lancet Infect Dis. 2016 Nov;16(11):1269–78.

358. Cazabon D, Alsdurf H, Satyanarayana S, Nathavitharana R, Subbaraman R, Daftary A, Pai M. Quality of tuberculosis care in high burden countries: the urgent need to address gaps in the care cascade. Int J Infect Dis IJID Off Publ Int Soc Infect Dis. 2016 Oct 26;

359. Ramsay A, Steingart KR, Pai M. Assessing the impact of new diagnostics on tuberculosis control. Int J Tuberc Lung Dis Off J Int Union Tuberc Lung Dis. 2010 Dec;14(12):1506–7.

360. Zumla A, Nahid P, Cole ST. Advances in the development of new tuberculosis drugs and treatment regimens. Nat Rev Drug Discov. 2013 May;12(5):388–404.

361. Guyatt GH, Tugwell PX, Feeny DH, Haynes RB, Drummond M. A framework for clinical evaluation of diagnostic technologies. CMAJ Can Med Assoc J J Assoc Medicale Can. 1986 Mar 15;134(6):587–94.

362. Taylor CR, Elmore JG, Sun K, Inouye SK. Technology assessment in diagnostic imaging. A proposal for a phased approach to evaluating radiology research. Invest Radiol. 1993 Feb;28(2):155–61.

363. Lijmer JG, Leeflang M, Bossuyt PM. Proposals for a Phased Evaluation of Medical Tests. In: Medical Tests-White Paper Series [Internet]. Rockville (MD): Agency for Healthcare Research and Quality (US); 2009 [cited 2016 Dec 15]. (AHRQ Methods for Effective Health Care). Available from: http://www.ncbi.nlm.nih.gov/books/NBK49467/

364. Sackett DL, Haynes RB. The architecture of diagnostic research. BMJ. 2002 Mar 2;324(7336):539–41.

365. Van den Bruel A, Cleemput I, Aertgeerts B, Ramaekers D, Buntinx F. The evaluation of diagnostic tests: evidence on technical and diagnostic accuracy, impact on patient outcome and cost-effectiveness is needed. J Clin Epidemiol. 2007 Nov;60(11):1116–22.

366. Bossuyt PM, Irwig L, Craig J, Glasziou P. Comparative accuracy: assessing new tests against existing diagnostic pathways. BMJ. 2006 May 6;332(7549):1089–92.

367. Schumacher SG, Sohn H, Qin ZZ, Gore G, Davis JL, Denkinger CM, Pai M. Impact of Molecular Diagnostics for Tuberculosis on Patient-Important Outcomes: A Systematic Review of Study Methodologies. PloS One. 2016;11(3):e0151073.

368. ISO 14155:2011 - Clinical investigation of medical devices for human subjects -- Good clinical practice [Internet]. ISO. [cited 2017 Jan 12]. Available from: http://www.iso.org/iso/catalogue_detail?csnumber=45557

369. Acosta CJ, Galindo CM, Ochiai RL, Danovaro-Holliday MC, Laure-Page A, Thiem VD, Jin Y, Khan MI, Sahito SM, Hamza HB, Park JK, Lee H, Bock H, Elyazeed RA, Albert MJ, Ascaso C, Robles TQ, Ali M, Ngai P, Puri MK, Koo YM, Agtini MD, Soeharno R, Bai-qing D, Kohl D, Xu Z-Y, Ivanoff B, Jodar L, Pang T, Bhutta Z, Clemens JD. Implementation of good clinical practice guidelines in vaccine trials in developing countries. Vaccine. 2007 Apr 12;25(15):2852–7.

370. Vischer N, Pfeiffer C, Joller A, Klingmann I, Ka A, Kpormegbe SK, Burri C. The Good Clinical Practice guideline and its interpretation - perceptions of clinical trial teams in sub-Saharan Africa. Trop Med Int Health TM IH. 2016 Aug;21(8):1040–8.

371. Rugera SP, McNerney R, Poon AK, Akimana G, Mariki RF, Kajumbula H, Kamau E, Mpawenimana S, Said SY, Toroitich A, Ronoh W, Sollis KA, Sonoiya S, Peeling RW. Regulation of medical diagnostics and medical devices in the East African community partner states. BMC Health Serv Res. 2014 Oct 31;14:524.

372. Fontela PS, Pant Pai N, Schiller I, Dendukuri N, Ramsay A, Pai M. Quality and reporting of diagnostic accuracy studies in TB, HIV and malaria: evaluation using QUADAS and STARD standards. PloS One. 2009;4(11):e7753.

373. Hopewell S, Altman DG, Moher D, Schulz KF. Endorsement of the CONSORT Statement by high impact factor medical journals: a survey of journal editors and journal "Instructions to Authors." Trials. 2008 Apr 18;9:20.

374. Rylance J, Pai M, Lienhardt C, Garner P. Priorities for tuberculosis research: a systematic review. Lancet Infect Dis. 2010 Dec;10(12):886–92.

375. Lienhardt C, Espinal M, Pai M, Maher D, Raviglione MC. What research is needed to stop TB? Introducing the TB Research Movement. PLoS Med. 2011 Nov;8(11):e1001135.

Curriculum Vitae

DR. MED. KLAUS REITHER

Head of Clinical Research Unit |Group Leader Clinical TB Research
Swiss Tropical and Public Health Institute, 4002 Basel, Switzerland
+41 612848967 | klaus.reither@unibas.ch

EXPERIENCE

Clinical Research in resource-poor countries

since 6/2014	Head of Clinical Research Unit, Swiss Tropical and Public Health Institute
since 7/2013	Group Leader Clinical TB Research, Swiss Tropical and Public Health Institute
6/2009-6/2013	TB Team Leader / Senior Scientist. Bagamoyo Research and Training Centre / Ifakara Health Institute. Employed by the Swiss Tropical and Public Health Institute.
6/2006-5/2009	TB research coordinator / Scientist. Mbeya Medical Research Programme (MMRP), Mbeya, Tanzania. Employed by the Department of Infectious Diseases & Tropical Medicine, LMU Munich, Germany.
2/2005- 2/2006	Study coordinator / Scientist. Northern Region Malaria Project (NORMAP), Tamale, Ghana. Employed by the Institute of Tropical Medicine, Charité – Humboldt University Berlin, Germany

Diagnostic Radiology and Internal Medicine

09/2001 - 01/2005	Consultant Radiologist. Radiology Clinic, Dr. Neumann, Eberswalde and locum tenens at Radiology Clinics, Germany.
08/1996 - 08/2001	Speciality Registrar. Benjamin Franklin University Hospital, Radiology Department, Free University of Berlin, Germany.
05/1995 - 06/1996	Senior House Officer. Radiology Clinic, Drs. Bredenbeck / Boewer, Berlin, Germany.
10-11/1994	Short-term research fellowship. Section of Lipid Metabolism, University Hospital Rudolf-Virchow, Berlin, Germany.
02/1993 - 08/1994	House Officer. Hospital of Neukölln, Department of Internal Medicine / Cardiology, Berlin, Germany.

EDUCATION/QUALIFICATION

2006	Master of Science in International Health, Humboldt University Berlin, Germany
2001	Specialist in Diagnostic Radiology, Berlin, Germany
1994	Medical License (Ärztliche Approbation), Berlin, Germany
1993	Medical thesis (Dr. med.), German Heart Centre, Berlin, Germany,
	(Title: Clinical implications of quantitative coronarangiography; magna cum laude)
1989-90	Medical School, Free University of Berlin, Germany
1983-89	Medical School, RWTH Aachen, Germany

SCHOLARSHIPS

2005-2006	Scholarship German Academic Exchange Service / German Malaria Initiative
1991-1993	Scholarship Maria-Sonnenfeld-Gedächtnisstiftung for MD thesis

PUBLICATIONS

1. Mhimbira F, Hella J, Said K, Kamwela L, Sasamalo M, Maroa T, Chiryamkubi M, Mhalu G, Schindler C, **Reither K**, Knopp S, Utzinger J, Gagneux S, Fenner L. Prevalence and clinical relevance of helminth co-infections among tuberculosis patients in urban Tanzania. PLoS Negl Trop Dis. 2017 Feb 8;11(2)
2. Boeree MJ, Heinrich N, Aarnoutse R, Diacon AH, Dawson R, Rehal S, Kibiki GS, Churchyard G, Sanne I, Ntinginya NE, Minja LT, Hunt RD, Charalambous S, Hanekom M, Semvua HH, Mpagama SG, Manyama C, Mtafya B, **Reither K**, Wallis RS, Venter A, Narunsky K, Mekota A, Henne S, Colbers A, van Balen GP, Gillespie SH, Phillips PP, Hoelscher M; PanACEA consortium.. High-dose rifampicin, moxifloxacin, and SQ109 for treating tuberculosis: a multi-arm, multi-stage randomised controlled trial. Lancet Infect Dis. 2017 Jan;17(1):39-49.
3. Boillat-Blanco N, Bovet P, Ramaiya KL, Mganga M, Minja LT, Saleh L, Imboden M, Schindler C, Gagneux S, Daubenberger C, **Reither K**, Probst-Hensch N. Association between tuberculosis, diabetes and 25 hydroxyvitamin D in Tanzania: a longitudinal case control study. BMC Infect Dis. 2016 Nov 3;16(1):626.
4. Petrone L, Cannas A, Vanini V, Cuzzi G, Aloi F, Nsubuga M, Sserunkuma J, Nazziwa RA, Jugheli L, Lukindo T, Girardi E, Antinori A, Pucci L, **Reither K**, Goletti D. Blood and urine inducible protein 10 as potential markers of disease activity. Int J Tuberc Lung Dis. 2016 Nov;20(11):1554-1561.
5. Sikalengo G, Ramirez A, Faini D, Mwamelo K, Battegay M, Jugheli L, Hatz C, **Reither K**, Letang E. Tuberculous spondylitis diagnosed through Xpert MTB/RIF assay in urine: a case report. BMC Infect Dis. 2016 Sep 26;16(1):514.
6. Mhimbira F, Hella J, Maroa T, Kisandu S, Chiryamkubi M, Said K, Mhalu G, Mkopi A, Mutayoba B, **Reither K**, Gagneux S, Fenner L. Home-Based and Facility-Based Directly Observed Therapy of Tuberculosis Treatment under Programmatic Conditions in Urban Tanzania. PLoS One. 2016 Aug 11;11(8):e0161171.
7. Pohl C, Rutaihwa LK, Haraka F, Nsubuga M, Aloi F, Ntinginya NE, Mapamba D, Heinrich N, Hoelscher M, Marais BJ, Jugheli L, **Reither K**. Limited value of whole blood Xpert(®) MTB/RIF for diagnosing tuberculosis in children. J Infect. 2016 Oct;73(4):326-35.
8. Bholla M, Kapalata N, Masika E, Chande H, Jugheli L, Sasamalo M, Glass TR, Beck HP, **Reither K**. Evaluation of Xpert® MTB/RIF and Ustar EasyNAT™ TB IAD for diagnosis of tuberculous lymphadenitis of children in Tanzania: a prospective descriptive study. BMC Infect Dis. 2016 Jun 6;16(1):246.
9. Zumla A, Rao M, Wallis RS, Kaufmann SH, Rustomjee R, Mwaba P, Vilaplana C, Yeboah-Manu D, Chakaya J, Ippolito G, Azhar E, Hoelscher M, Maeurer M; Host-Directed Therapies Network consortium. Host-directed therapies for infectious diseases: current status, recent progress, and future prospects. Lancet Infect Dis. 2016 Apr;16(4):e47-63.(consortium member)
10. Steiner, A, Mangu, C, van den Hombergh, J, van Deutekom, H, van Ginneken, B, Clowes, P, Mhimbira, F, Mfinanga, S, Rachow, A., **Reither, K**, Hoelscher, M. Screening for pulmonary tuberculosis in a Tanzanian prison and computer-aided interpretation of chest X-rays. Public Health Action, Volume 5, Number 4, pp. 249-254(6)

11. Boillat-Blanco N, Ramaiya KL, Mganga M, Minja LT, Bovet P, Schindler C, Von Eckardstein A, Gagneux S, Daubenberger C, **Reither K**, Probst-Hensch N. Transient Hyperglycemia in Patients With Tuberculosis in Tanzania: Implications for Diabetes Screening Algorithms. J Infect Dis. 2015 Nov 25.
12. **Reither K**, Jugheli L, Glass TR, Sasamalo M, Mhimbira FA, Weetjens BJ, Cox C, Edwards TL, Mulder C, Beyene NW, Mahoney A. Evaluation of Giant African Pouched Rats for Detection of Pulmonary Tuberculosis in Patients from a High-Endemic Setting. PLoS One. 2015 Oct 7;10(10):e0135877.
13. Nicol MP, Gnanashanmugam D, Browning R, Click ES, Cuevas LE, Detjen A, Graham SM, Levin M, Makhene M, Nahid P, Perez-Velez CM, **Reither K**, Song R, Spiegel HM, Worrell C, Zar HJ, Walzl G. A Blueprint to Address Research Gaps in the Development of Biomarkers for Pediatric Tuberculosis. Clin Infect Dis. 2015 Oct 15;61Suppl 3:S164-72.
14. Petrone L, Cannas A, Aloi F, Nsubuga M, Sserumkuma J, Nazziwa RA, Jugheli L, Lukindo T, Girardi E, **Reither K**, Goletti D. Blood or Urine IP-10 Cannot Discriminate between Active Tuberculosis and Respiratory Diseases Different from Tuberculosis in Children. Biomed Res Int. 2015;2015:589471.
15. Cabibbe AM, Miotto P, Moure R, Alcaide F, Feuerriegel S, Pozzi G, Nikolayevskyy V, Drobniewski F, Niemann S, **Reither K**, Cirillo DM; TM-REST Consortium; TB-CHILD Consortium. A lab-on-chip based platform for fast molecular diagnosis of multi-drug resistant tuberculosis. J Clin Microbiol. 2015 Aug 5.
16. Zumla A, Maeurer M; Host-Directed Therapies Network (HDT-NET) Consortium.Host-Directed Therapies for Tackling Multi-Drug Resistant Tuberculosis: Learning From the Pasteur-Bechamp Debates. Clin Infect Dis. 2015 Nov 1;61(9):1432-8. (consortium member)
17. Zumla A, Maeurer M; Host-Directed Therapies Network., Chakaya J, Hoelscher M, Ntoumi F, Rustomjee R, Vilaplana C, Yeboah-Manu D, Rasolof V, Munderi P, Singh N, Aklillu E, Padayatchi N, Macete E, Kapata N, Mulenga M, Kibiki G, Mfinanga S, Nyirenda T, Maboko L, Garcia-Basteiro A, Rakotosamimanana N, Bates M, Mwaba P, **Reither K**, Gagneux S, Edwards S, Mfinanga E, Abdulla S, Cardona PJ, Russell JB, Gant V, Noursadeghi M, Elkington P, Bonnet M, Menendez C, Dieye TN, Diarra B, Maiga A, Aseffa A, Parida S, Wejse C, Petersen E, Kaleebu P, Oliver M, Craig G, Corrah T, Tientcheu L, Antonio M, Rao M, McHugh TD, Sheikh A, Ippolito G, Ramjee G, Kaufmann SH, Churchyard G, Steyn A, Grobusch M, Sanne I, Martinson N, Madansein R, Wilkinson RJ, Mayosi B, Schito M, Wallis RS. Towards host-directed therapies for tuberculosis. Nat Rev Drug Discov. 2015 Aug;14(8):511-2.
18. Kroidl I, Clowes P, **Reither K**, Mtafya B, Rojas-Ponce G, Ntinginya EN, Kalomo M, Minja LT, Kowuor D, Saathoff E, Kroidl A, Heinrich N, Maboko L, Bates M, O'Grady J, Zumla A, Hoelscher M, Rachow A. Performance of urine lipoarabinomannan assays for paediatric tuberculosis in Tanzania. Eur Respir J. 2015 Sep;46(3):761-70.
19. Portevin D, Moukambi F, Mpina M, Bauer A, Haraka F, Chachage M, Metzger P, Saathoff E, Clowes P, Ntinginya NE, Rachow A, Hoelscher M, **Reither K**, Daubenberger CA, Geldmacher C. Maturation and Mip-1β Production of Cytomegalovirus-Specific T Cell Responses in Tanzanian Children, Adolescents and Adults: Impact by HIV and Mycobacterium tuberculosis Co-Infections. PLoS One. 2015 May 14
20. Lenz N, Schindler T, Kagina BM, Zhang JD, Lukindo T, Mpina M, Bang P, Kromann I, Hoff ST, Andersen P, **Reither K**, Churchyard GJ, Certa U, Daubenberger CA. Antiviral innate immune activation in HIV infected adults negatively affects H1/IC31® induced vaccine-specific memory CD4+ T cells. Clin Vaccine Immunol. 2015 Apr 29.
21. Dawson R, Diacon AH, Everitt D, van Niekerk C, Donald PR, Burger DA, Schall R, Spigelman M, Conradie A, Eisenach K, Venter A, Ive P, Page-Shipp L, Variava E, **Reither K**, Ntinginya NE, Pym A, von Groote-Bidlingmaier F, Mendel CM. Efficiency and safety of the combination of moxifloxacin, pretomanid (PA-824), and pyrazinamide during the first 8 weeks of antituberculosis treatment: a phase 2b, open-label, partly randomised trial in patients with drug-susceptible or drug-resistant pulmonary tuberculosis. Lancet. 2015 Mar 17.
22. Heinrich N, Dawson R, du Bois J, Narunsky K, Horwith G, Phipps AJ, Nacy CA, Aarnoutse RE, Boeree MJ, Gillespie SH, Venter A, Henne S, Rachow A, Phillips PP, Hoelscher M, Diacon AH; Pan African Consortium for the Evaluation of Antituberculosis Antibiotics (PanACEA); Pan African Consortium for the Evaluation of Antituberculosis Antibiotics PanACEA. Early phase evaluation of SQ109 alone and in combination with rifampicin in pulmonary TB patients. J Antimicrob Chemother. 2015 May;70(5):1558-66. (collaborator)
23. Mhimbira FA, Bholla M, Sasamalo M, Mukurasi W, Hella JJ, Jugheli L, **Reither K**. Detection of Mycobacterium tuberculosis by EasyNAT Diagnostic Kit in Sputum Samples from Tanzania. J Clin Microbiol. 2015 Apr;53(4):1342-4.
24. Olaru ID, Rachow A, Lange C, Ntinginya NE, **Reither K**, Hoelscher M, Vollrath O, Niemann S. Ascertaining in vivo virulence of Mycobacterium tuberculosis lineages in patients in Mbeya, Tanzania. Int J Tuberc Lung Dis. 2015 Jan;19(1):70-3.
25. **Reither K**, Katsoulis L, Beattie T, Gardiner N, Lenz N, Said K, Mfinanga E, Pohl C, Fielding KL, Jeffery H, Kagina BM, Hughes EJ, Scriba TJ, Hanekom WA, Hoff ST, Bang P, Kromann I, Daubenberger C, Andersen P, Churchyard GJ. Safety and Immunogenicity of H1/IC31®, an Adjuvanted TB Subunit Vaccine, in HIV-Infected Adults with CD4+ Lymphocyte Counts Greater than 350 cells/mm3: A Phase II, Multi-Centre, Double-Blind, Randomized, Placebo-Controlled Trial. PLoS One. 2014 Dec 9;9(12):e114602.
26. **Reither K**, Manyama C, Clowes P, Rachow A, Mapamba D, Steiner A, Ross A, Mfinanga E, Sasamalo M, Nsubuga M, Aloi F, Cirillo D, Jugheli L, Lwilla F. Xpert MTB/RIF assay for diagnosis of pulmonary tuberculosis in children: A prospective, multi-centre evaluation. J Infect. 2014 Oct 13.
27. Breuninger M, van Ginneken B, Philipsen RH, Mhimbira F, Hella JJ, Lwilla F, van den Hombergh J, Ross A, Jugheli L, Wagner D, **Reither K**. Diagnostic accuracy of computer-aided detection of pulmonary tuberculosis in chest radiographs: a validation study from sub-Saharan Africa. PLoS One. 2014 Sep 5;9(9):e106381.

28. Portevin D, Moukambi F, Clowes P, Bauer A, Chachage M, Ntinginya NE, Mfinanga E, Said K, Haraka F, Rachow A, Saathoff E, Mpina M, Jugheli L, Lwilla F, Marais BJ, Hoelscher M, Daubenberger C, **Reither K***, Geldmacher C*. Assessment of the novel T-cell activation marker-tuberculosis assay for diagnosis of active tuberculosis in children: a prospective proof-of-concept study. Lancet Infect Dis. 2014 Oct;14(10):931-8.(*contributed equally)
29. Melendez J, van Ginneken B, Maduskar P, Philipsen RH, **Reither K**, Breuninger M, Adetifa IM, Maane R, Ayles H, Sanchez CI. A novel multiple-instance learning-based approach to computer-aided detection of tuberculosis on chest x-rays. IEEE Trans Med Imaging. 2015 Jan;34(1):179-92.
30. Trauner A, Borrell S, **Reither K**, Gagneux S. Evolution of drug resistance in tuberculosis: recent progress and implications for diagnosis and therapy. Drugs. 2014 Jul;74(10):1063-72.
31. Honeyborne I, Mtafya B, Phillips PP, Hoelscher M, Ntinginya EN, Kohlenberg A, Rachow A, Rojas-Ponce G, McHugh TD, Heinrich N; Pan African Consortium for the Evaluation of Anti-tuberculosis Antibiotics. The molecular bacterial load assay replaces solid culture for measuring early bactericidal response to antituberculosis treatment. J Clin Microbiol. 2014 Aug;52(8):3064-7.(consortium member)
32. Said K, Mkopi A, Verver S, Lwilla F, Churchyard G, Shekalaghe S, Battegay M, **Reither K**. Improved services to enrollees into an HIV rural care and treatment center in Tanzania. Pan Afr Med J. 2013 Sep 30;16:34.
33. Friedrich SO, Rachow A, Saathoff E, Singh K, Mangu CD, Dawson R, Phillips PP, Venter A, Bateson A, Boehme CC, Heinrich N, Hunt RD, Boeree MJ, Zumla A, McHugh TD, Gillespie SH, Diacon AH, Hoelscher M; Pan African Consortium for the Evaluation of Anti-tuberculosis Antibiotics (PanACEA). Assessment of the sensitivity and specificity of Xpert MTB/RIF assay as an early sputum biomarker of response to tuberculosis treatment. Lancet Respir Med. 2013 Aug;1(6):462-70.(collaborator)
34. Miotto P, Mwangoka G, Valente IC, Norbis L, Sotgiu G, Bosu R, Ambrosi A, Codecasa LR, Goletti D, Matteelli A, Ntinginya EN, Aloi F, Heinrich N, **Reither K**, Cirillo DM. miRNA Signatures in Sera of Patients with Active Pulmonary Tuberculosis. PLoS One. 2013 Nov 21;8(11)
35. Kroidl I, Clowes P, Mwakyelu J, Maboko L, Kiangi A, Rachow A, **Reither K**, Jung J, Nsojo A, Saathoff E, Hoelscher M. Reasons for false-positive lipoarabinomannan ELISA results in a Tanzanian population. Scand J Infect Dis. 2013 Nov 25.
36. Pohl C, Jugheli L, Haraka F, Mfinanga E, Said K, **Reither K**. Pulmonary aspergilloma: a treatment challenge in sub-saharan Africa. PLoS Negl Trop Dis. 2013 Oct 24;7(10):e2352.
37. Khadija Said, Abdallah Mkopi, Suzanne Verver, Fred Lwilla, Gavin Churchyard, Seif Shekalaghe, Manuel Battegay, **Klaus Reither**. Improved services to enrollees into an HIV rural care and treatment center in Tanzania. The Pan African Medical Journal. 2013;16:34
38. Kayigire XA, Friedrich SO, Venter A, Dawson R, Gillespie SH, Boeree MJ, Heinrich N, Hoelscher M, Diacon AH; Pan African Consortium for the Evaluation of Anti-tuberculosis Antibiotics. Direct comparison of Xpert MTB/RIF assay with liquid and solid mycobacterial culture for quantification of early bactericidal activity. J Clin Microbiol. 2013 Jun;51(6):1894-8. (collaborator)
39. Hella JJ, Hiza HC, Mfinanga E, **Reither K**. Paraspinal abscess secondary to tuberculous spondylitis diagnosed by Xpert MTB/RIF assay in rural Tanzania. BMJ Case Rep. 2013 Apr 10; 2013
40. Ntinginya EN, Squire SB, Millington KA, Mtafya B, Saathoff E, Heinrich N, Rojas-Ponce G, Kowuor D, Maboko L, **Reither K**, Clowes P, Hoelscher M, Rachow A. Performance of the Xpert(®) MTB/RIF assay in an active case-finding strategy: a pilot study from Tanzania. Int J Tuberc Lung Dis. 2012 Sep.
41. Beyene N, Mahoney A, Cox C, Weetjens B, Makingi G, Mgode G, Durgin A, Kuipers D, Jubitana M, Egwaga S, Kamara D, Lwilla F, Mfinanga SG, Kahwa A, Machang'u R, Kazwala R, **Reither K**, Kaufmann SHE, Poling A (2012) APOPO's tuberculosis research agenda: achievements, challenges and prospects. Tanzan J Health Res 14, 1-13.
42. Haraka F, Rutaihwa LK, Battegay M, **Reither K**. Mycobacterium intracellulare infection in non-HIV infected patient in a region with a high burden of tuberculosis. BMJ Case Rep. 2012 May 8; 2012.
43. Rachow A, Clowes P, Saathoff E, Mtafya B, Michael E, Ntinginya EN, Kowour D, Rojas-Ponce G, Kroidl A, Maboko L, Heinrich N, **Reither K**, Hoelscher M. Increased and expedited case detection by Xpert MTB/RIF assay in childhood tuberculosis: a prospective cohort study. Clin Infect Dis. 2012 May
44. Schuetz A, Dirks J, Sester U, Haule A, Elias N, Geldmacher C, Sanga E, Maboko L, **Reither K**, Hoelscher M, Meyerhans A, Sester M. Pathogen prevalence may determine maintenance of antigen-specific T-cell responses in HIV-infected individuals. AIDS. 2012 Feb 1.
45. Schuetz A, Haule A, **Reither K**, Ngwenyama N, Rachow A, Meyerhans A, Maboko L, Koup RA, Hoelscher M, Geldmacher C. Monitoring CD27 Expression to Evaluate Mycobacterium Tuberculosis Activity in HIV-1 Infected Individuals In Vivo. PLoS One. 2011;6(11):e27284. Epub 2011 Nov 7.
46. Rachow A, Zumla A, Heinrich N, Rojas-Ponce G, Mtafya B, **Reither K**, Ntinginya EN, O'Grady J, Huggett J, Dheda K, Boehme C, Perkins M, Saathoff E, Hoelscher M. Rapid and Accurate Detection of Mycobacterium tuberculosis in Sputum Samples by Cepheid Xpert MTB/RIF Assay-A Clinical Validation Study. PLoS One. 2011;6(6):e20458. Epub 2011 Jun 29.
47. Geldmacher C, Ngwenyama N, Schuetz A, Petrovas C, **Reither K**, Heeregrave EJ, Casazza J, Ambrozak D, Marc Louder M, Ampofo W, Pollakis G, Hill B, Sanga E, Saathoff E, Maboko L, Roederer M, Paxton WA, Hoelscher M and Koup RA. Preferential infection and depletion of Mycobacterium tuberculosis (MTB)-specific CD4 T cells after HIV-1 infection. J Exp Med. 2010 Nov 29. Epub 2010 Nov 29.
48. Buchholz U, Kobbe R, Danquah I, Zanger P, **Reither K**, Abruquah HH, Grobusch MP, Ziniel P, May J, Mockenhaupt FP. Multiplicity of Plasmodium falciparum infection following intermittent preventive treatment in infants. Malar J. 2010 Aug 26;9(1):244. [Epub ahead of print]

49. Kolk A, Hoelscher M, Maboko L, Jung J, Kuijper S, Cauchi M, Bessant C, van Beers S, Dutta R, Gibson T, **Reither K**. Electronic-nose technology in diagnosis of TB patients using sputum samples. J Clin Microbiol. 2010 Aug 18. Epub 2010 Aug 18.
50. **Reither K**, Saathoff E, Jung J, Minja LT, Machibya H, Maboko L, Perkins MD, Hoelscher M, and Boehme CC. Evaluation of Diagnos TB AG, a novel flow-through immunoassay for rapid detection of pulmonary tuberculosis. Int J Tuberc Lung Dis. 2010 Feb;14(2):238-40
51. Cannas A, Kalunga G, Green C, Calvo L, Katemangwe P, **Reither K**, Perkins MD, Maboko L, Hoelscher M, Talbot EA, Mwaba P, Zumla AI, Girardi E, Huggett JF. Implications of storing urinary DNA from different populations for molecular analyses. PLoS ONE 2009;4(9):e6985.
52. **Reither K**, Saathoff E, Jung J, Minja LT, Kroidl I, Saad E, Huggett JF, Ntinginya EN, Maganga L, Maboko L, Hoelscher M. Low sensitivity of a urine LAM-ELISA in the diagnosis of pulmonary tuberculosis. BMC Infect. Dis 2009;9:141.
53. Green C, Huggett JF, Talbot E, Mwaba P, **Reither K**, Zumla AI. Rapid diagnosis of tuberculosis through the detection of mycobacterial DNA in urine by nucleic acid amplification methods. Lancet Infect Dis 2009 Aug;9(8):505-511.
54. Danquah I, Dietz E, Zanger P, **Reither K**, Anemana SD, Bienzle U, Mockenhaupt FP. Reduced efficacy of intermittent preventive treatment of malaria in malnourished children. Antimicrob Agents Chemother. 2009 Feb 17. Epub 2009 Feb 17.
55. Silva PA, Stark K, Mockenhaupt FP, **Reither K**, Weitzel T, Ignatius R, Saad E, Seidu-Korkor A, Bienzle U, Schreier E. Molecular Characterization of Enteric Viral Agents from Children in the Northern Region of Ghana. J Med Virol. 2008 Oct;80(10):1790-8
56. Djie-Maletz A, **Reither K**, Danour S, Anyidoho L, Saad E, Danikuu F, Ziniel P, Weitzel T, Wagner J, Bienzle U, Stark K, Seidu-Korkor A, Mockenhaupt FP, Ignatius, R. High rate of resistance to locally used antibiotics among enteric bacteria from children in Northern Ghana. J Antimicrob Chemother. 2008 Mar 19. Epub 2008 Mar 19.
57. **Reither K**, Ignatius R, Weitzel T, Seidu-Korkor A, Anyidoho L, Saad E, Djie-Maletz A, Ziniel P, Amoo-Sakyi F, Danikuu F, Danour S, Otchwemah RN, Schreier E, Bienzle U, Stark K, Mockenhaupt FP. Acute childhood diarrhoea in northern Ghana: epidemiological, clinical and microbiological characteristics. BMC Infect Dis. 2007 Sep 6;7:104.
58. Mockenhaupt FP, **Reither K**, Zanger P, Roepcke F, Danquah I, Saad E, Ziniel P, Dzisi SY, Frempong M, Agana-Nsiire P, Amoo-Sakyi F, Otchwemah R, Cramer JP, Anemana SD, Dietz E, Bienzle U. Intermittent preventive treatment in infants as a means of malaria control: a randomized, double-blind, placebo-controlled trial in northern Ghana. Antimicrob Agents Chemother. 2007 Sep;51(9):3273-81. Epub 2007 Jul 16.
59. Weitzel T, **Reither K**, Mockenhaupt FP, Stark K, Ignatius R, Saad E, Seidu-Korkor A, Bienzle U, Schreier E. Field evaluation of a rota- and adenovirus immunochromatographic assay using stool samples from children with acute diarrhea in Ghana. J Clin Microbiol. 2007 Aug;45(8):2695-7. Epub 2007 Jun 27.
60. Kopitzko A, Cornely D, **Reither K**, Wolf KJ, Albrecht T. Low contrast dose voiding urosonography in children with phase inversion imaging. Eur Radiol. 2004 Dec;14(12):2290-6. Epub 2004 Jun 30.
61. Wacker FK, **Reither K**, Ebert W, Wendt M, Lewin JS, Wolf KJ. MR image-guided endovascular procedures with the ultrasmall superparamagnetic iron oxide SH U 555 C as an intravascular contrast agent: study in pigs. Radiology. (2003) Feb;226(2):459-64
62. Wacker FK, **Reither K**, Ritz JP, Roggan A, Germer CT, Wolf KJ. MR-guided interstitial laser-induced thermotherapy of hepatic metastasis combined with arterial blood flow reduction: First results in an open MR system. J Magn Reson Imaging. (2001) Jan;13(1):31-6
63. **Reither K**, Wacker F, Ritz JP, Isbert C, Germer CT, Roggan A, Wendt M, Wolf KJ. Laser-induced thermotherapy (LITT) for liver metastasis in an open 0.2T MRI. Rofo 172 (2000), 175-178
64. Wacker FK, Faiss S, **Reither K**, Zimmer T, Wendt M, Wolf KJ. MR imaging-guided biliary drainage in an open low-field system: first clinical experiences. Rofo 172(9) (2000), 744-7
65. Wacker FK, **Reither K**, Branding G, Wendt M, Wolf KJ. MR guided Vascular Catheterization: Feasibility Using a Passive Tracking Technique at 0.2 T in a Pig Model. J Magn Reson Imaging 10 (1999), 841-844
66. Müller HP, Godde P, Czerski K, Agrawal R, Feilcke G, **Reither K**, Wolf KJ, Oeff M. Localization of a ventricular tachycardia-focus with multichannel magnetocardiography and three-dimensional current density reconstruction. J Med Eng Technol (1999) May-Jun, 23(3), 108-115
67. C. Bossaller, **K. Reither**, C. Hehlert-Friedrich, W. Auch-Schwelk, K. Graf, M. Gräfe, E. Fleck. In vivo measurement of endothelium-dependent vasodilation with substance P in man. Herz (1992), 17, 284-29