# Challenges in diagnosis and treatment of extrapulmonary tuberculosis

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Thesis for the degree of Philosophiae Doctor (PhD)
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# Contents

# List of abbreviations

AFB – acid fast bacilli
AIDS – acquired immune deficiency syndrome
BCG – bacille Calmette-Guérin
CD4 – cluster of differentitation 4
DNA – deoxyribonucleic acid
DOTS – Direct Observed Treatment, Short-Course
ELISA – enzyme-linked immunosorbent assay
FNA – fine-needle aspirate
HIV – human immunodeficiency virus
ICC – immunocytochemistry
IHC – immunohistochemistry
IP-10 – interferon-γ inducible protein 10
MPT64 test -MPT64 antigen detection test
NTM – non-tuberculosis mycobacteria
PCR – polymerase chain reaction
RNA – ribonucleic acid
TB – tuberculosis
Ultra – Xpert MTB/RIF Ultra assay
WHO – World Health Organization

## Scientific environment

The present work was conducted between 2015-2020 at Centre for International Health, Department of Global Public Health and Primary Care, Faculty of Medicine, University of Bergen, Norway, as a collaboration between the Centre for International Health, Department of Clinical Science and Department of Clinical Medicine. Laboratory training, experiments and analyses were performed at Department of Clinical Science and Department of Clinical Medicine at the Faculty of Medicine and at Department of Biological Sciences at the Faculty of Mathematics and Natural Sciences, University of Bergen. The study constituting the fourth paper was conducted in collaboration with researchers at Statens Serum Institut, Copenhagen, Denmark.

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# Abstract

The infectious disease tuberculosis (TB) is a global health problem. Diagnosis of extrapulmonary TB is often challenging due to non-specific symptoms and findings, and the lack of sensitive diagnostic tests. This leads to both under- and overdiagnosis and extensive use of empirical TB treatment, which increases the risk of placing individuals without TB or individuals with multi-drug resistant TB on prolonged, incorrect treatment. New tools for diagnosis and assessment of treatment response of extrapulmonary TB are needed to improve TB care.

The overall aim of this thesis was to investigate new methods for diagnosis and treatment monitoring of extrapulmonary TB. Specific aims included to assess the performance of the MPT64 antigen detection test (MPT64 test) and the Xpert Ultra assay for the diagnosis of extrapulmonary TB in a low TB prevalent setting. Further, to reproduce an anti-MPT64 antibody for large-scale use of the MPT64 test. Finally, to study the potential of IP-10 measured in dried plasma or blood spots as a biomarker for treatment response in patients with extrapulmonary TB.

Extrapulmonary samples received for TB diagnostics at the Microbiology and/or Pathology laboratories in a clinical setting in Norway were collected and subjected to the MPT64 test and/or Xpert Ultra. The performance of the new tests was compared to results of routine TB diagnostics. New anti-MPT64 antibodies were developed in mice and rabbits by use of different antigen-adjuvant combinations and screening in immunohistochemistry. The best performing individual antibodies were pooled, and the performance of the final pooled anti-MPT64 antibody was evaluated in patients samples. IP-10 dynamics during TB treatment were studied in extrapulmonary TB patients at a tertiary care hospital on Zanzibar, Tanzania. Plasma, dried plasma spots and dried blood spots were collected at baseline, 2 months of treatment and end of treatment. IP-10 levels were measured by ELISA and compared to clinical improvement.

In paper I, we found that the sensitivity of the MPT64 test for diagnosing extrapulmonary TB was lower compared to results obtained in previous studies

conducted in TB endemic low-resource settings. The best test performance in our setting was demonstrated in formalin-fixed biopsies, where the MPT64 test showed an excellent specificity and a sensitivity in the same range as PCR-based tests, but lower than culture. The results of paper II indicate that it is possible to reproduce a functional polyclonal anti-MPT64 antibody for large-scale use of the MPT64 test, but that careful selection of the antigen-adjuvant combination for immunisation and comprehensive screening strategies are nessecary to obtain high-performance antibodies. In paper III, we found that the overall sensitivity and specificty of Xpert Ultra for diagnosing extrapulmonary TB in low-TB prevalent high-income setting were high and close to the performance of culture. In paper IV, we report that a significant decline in IP-10 levels in plasma, dried plasma spots and dried blood spots during treatment was observed in extrapulmonary TB patients, and the decline was significant already after two months in HIV-negative patients. The correlation between IP-10 measured in plasma and dried plasma spots or dried blood spots was high.

To conclude, potential new tools for improved diagnosis and treatment monitoring of extrapulmonary TB have been identified. Our results indicate that Xpert Ultra can contribute to a more rapid diagnosis of extrapulmonary TB in our setting, and that IP-10 measured in dried blood spots is a promising marker for response to treatment in extrapulmonary TB patients. However, further studies with larger sample sizes that include relevant negative controls and longer follow-up of patients to evaluate the clinical impact of these new tools are needed. The utility of the MPT64 test is likely limited in settings with well-functioning culture facilities, but the test can contribute to strengthen the TB diagnosis in the event that samples have not been subjected to culture.

# List of Publications

Paper 1: Hoel, I. M., Sviland, L., Syre, H., Dyrhol-Riise, A. M., Skarstein, I., Jebsen, P., Jørstad, M. D., Wiker, H., & Mustafa, T. (2020). Diagnosis of extrapulmonary tuberculosis using the MPT64 antigen detection test in a high-income low tuberculosis prevalence setting. *BMC infectious diseases*, *20*(1), 130. https://doi.org/10.1186/s12879-020-4852-z

Paper 2: Hoel, I. M., Mohammed Ali, I. A., Ishtiaq, S., Sviland, L., Wiker, H., Mustafa, T. Immunochemistry-based diagnosis of extrapulmonary tuberculosis; A strategy for large-scale production of MPT64-antibodies for use in the MPT64 antigen detection test. (submitted manuscript)

Paper 3: Hoel, I. M., Syre, H., Skarstein, I., & Mustafa, T. (2020). Xpert MTB/RIF ultra for rapid diagnosis of extrapulmonary tuberculosis in a high-income low-tuberculosis prevalence setting. *Scientific reports*, *10*(1), 13959. https://doi.org/10.1038/s41598-020-70613-x

Paper 4: Hoel, I. M., Jørstad, M. D., Marijani, M., Ruhwald, M., Mustafa, T., & Dyrhol-Riise, A. M. (2019). IP-10 dried blood spots assay monitoring treatment efficacy in extrapulmonary tuberculosis in a low-resource setting. *Scientific reports*, *9*(1), 3871. https://doi.org/10.1038/s41598-019-40458-0

# 1. Introduction

Tuberculosis (TB) is an ancient infectious disease that has plagued humans throughout history [1,2]. Today, TB is still one of the top 10 causes of death worldwide, even though it is both treatable and curable [3]. The disease is caused by the bacillus *Mycobacterium tuberculosis* and other species belonging to the *M. tuberculosis* complex. It primarily affects the lungs (pulmonary TB) but can also affect other sites of the body (extrapulmonary TB). With falling TB incidence in the early 20th century and the discovery of anti-TB chemotherapy in the 1940s, the war on TB was considered winnable and the attention to TB gradually decreased [4]. However, along with downscaling of TB control programs, neglected TB epidemics in resource-poor countries, globalisation and the emergence of the HIV/AIDS pandemic, an unexpected resurgence of TB occurred in the 1980s and 1990s [5]. As a result, TB was declared a global emergency by the World Health Organization (WHO) in 1993 [5,6], and an internationally recommended TB control strategy, later named DOTS (Directly Observed Treatment, Short-Course) [7], was launched in 1994. The DOTS strategy, and the following Stop TB Strategy that built on DOTS, have contributed to improved national TB control programmes and progress in TB control. Still, these efforts have so far only led to gradual reductions in global TB incidence. The new End TB Strategy aims at a 90% reduction in TB incidence and a 95% reduction in TB deaths by 2035 [8]. In order to reach these ambitious targets, and to finally end the TB epidemic, new diagnostic, treatment and prevention tools are needed.

## 1.1 Global tuberculosis epidemiology

Recent estimates suggest that a quarter of the world's population is infected with *M. tuberculosis* without showing signs or symptoms of active TB disease (latent TB infection [9]) [10,11]. This number is based on several uncertain assumptions and may be overestimated [10-12], but still indicates that a large reservoir of *M. tuberculosis* exists in the human population, and that exposure to TB and TB latency is widespread. Among

people infected with *M. tuberculosis*, approximately 5-10% will develop active TB disease within their lifespan [3,13,14]. In 2019, there were an estimated 10.0 million new cases of active TB disease and 1.4 million deaths caused by TB [3]. There are large variations in the incidence of TB disease both between countries and different populations within countries. Figure 1 shows the geographical distribution of TB incidence in 2019. The majority of new TB cases occurred in low- and lower middle-income countries in the South-East Asian region (44%), followed by the African (25%) and Western Pacific (18%) regions [3]. India alone accounted for 26% of the global total, whereas the highest numbers of incident TB cases relative to population size were found in southern African countries, which also had the highest proportion of TB cases co-infected with HIV. Within-country variation in TB incidence is associated with factors that increase the risk of TB disease and transmission in subgroups of the population. Increased exposure to TB bacilli due to limited access to health care and crowded and poorly ventilated home and work environments is seen in poor communities and among migrants, prisoners and other socially marginalised people. Additionally, factors that weaken the immune system, including HIV, diabetes, under-nutrition, alcoholism and tobacco smoke are overrepresented in these vulnerable groups and also increase the risk of developing TB.

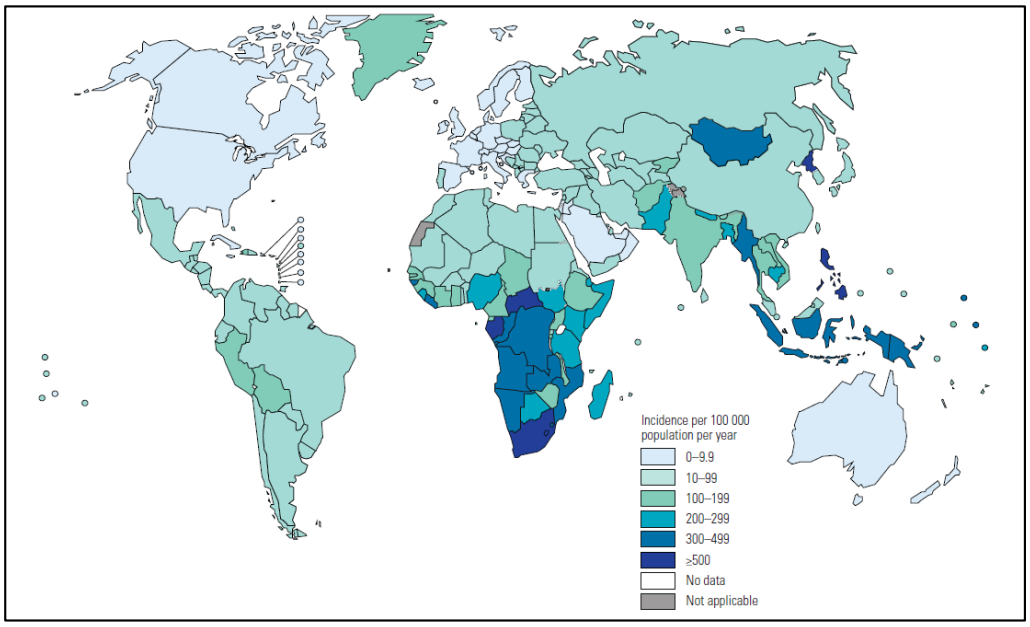



**Figure 1. Estimated TB incidence rates, 2019.** Adapted from: Global tuberculosis report 2020. Geneva: World Health Organization; 2020. Licence: CC BY-NC-SA 3.0 IGO.

Global TB incidence rates have been declining in the past decades due to efforts of national TB programmes and wide implementation of the cost-effective DOTS strategy [15]. However, the decline in TB incidence of 1,7% per year from 2000 to 2018 [3] is considered unacceptable low and is not sufficient to achieve the WHO End TB strategy milestone of a 20% reduction between 2015 and 2020. Further, the emergence of drug-resistant TB has worsened the TB epidemic in many countries due to longer and more expensive treatment regimens, more adverse drug side effects and low treatment adherence. The low treatment success rate of multidrug- and rifampicin-resistant TB threatens global TB control [3].

## 1.2 Tuberculosis in Norway

In Norway, TB mortality peaked around 1900 and TB disease was endemic until the 1950s [16]. With the exception of an increase in TB incidence from the mid-1990s to 2013, which occurred in parallel with increased immigration from TB endemic areas,

the TB incidence rate in Norway has been continuously declining during the last century. Norway currently has one of the lowest TB incidence rates in the world with 4/100 000 per year in 2018 [17]. Most of the notified TB cases in Norway are found among people infected abroad, of which the majority come from the Horn of Africa and the Philippines. Like in many other high-income countries, a very low TB rate has been achieved in the general non-foreign-born population in Norway. In 2018, the TB incidence was 0.7/100 000 in this population, which is at the pre-elimination level (<10 notified cases per million population), as defined by WHO [18]. Still, occasional outbreaks and high TB-prevalence in subgroups of the population, including migrants from TB endemic areas, makes TB a persistent public health problem that must be addressed.

## 1.3 Mycobacteria

Mycobacteria are aerobic, rod shaped, non-motile bacteria belonging to the genus *Mycobacterium* of the *Actinobacteria* [19]. The bacilli are characterised by a unique, thick and protective cell wall rich in mycolic acids and other lipids, which gives the bacilli their acid-fastness and contributes to the toughness of the genus [20]. The species that cause tuberculosis in humans and animals are collectively referred to as the *M. tuberculosis* complex and comprises the exclusively human pathogens *M. tuberculosis* (the major cause of human TB), *M. africanum* and *M. canettii*, and several animal pathogens with a wide host spectrum, including *M. bovis* [21]. Non-tuberculous mycobacteria (NTM) include all mycobacteria other than the *M. tuberculosis* complex species and *M. leprae* [22,23]. Albeit most NTM are non-pathogenic, fast-growing, environmental saprophytes, a few NTM are opportunistic pathogens that can cause human disease at a variety of different body sites. Immunocompromised, children and people with severe chronic lunge disease are at increased risk of NTM infection, particularly with the clinically important *M. avium* complex species, *M. kansasii* and *M. abscessus* [24-26]. Cervical lymphadenitis caused by NTM is relatively common in children [27], and poses diagnostic challenges because

the signs and symptoms are similar to TB lymphadenitis. As the treatment of NTM disease differs from treatment of TB, it is important to separate these conditions [28].

## 1.4 Pathogenesis of tuberculosis

The success of *M. tuberculosis* as a human pathogen is based on its extraordinary hardiness and capacity to adapt to environmental changes during infection [29], as well as the ability to manipulate and take advantage of the host immune system and transit into a stage of dormancy [30,31]. The disease is airborne and spreads via respiratory aerosols produced by an infected host. A new host inhales the aerosols deep into the lungs where the bacilli encounter and infect macrophages and other antigen-presenting cells of the innate immune system [32,33]. The bacilli further expand by recruiting and infecting additional macrophages, and a localised inflammatory lesion consisting of immature granulomas, the primary focus, occurs [34]. Actively secreted mycobacterial proteins are believed to play an important role in the modulation of host immune responses to secure intracellular survival and persistance in this early phase of primary TB infection [35]. Bacilli from the primary focus are also carried by the lymphatic system to the draining regional lymph node during primary infection [36], or they may enter the bloodstream and spread to other tissues and organs. Infected and activated macrophages present *M. tuberculosis* antigens to T cells in the draining lymph node, thereby inducing the adaptive immune system [37,38]. Antigen-specific CD4+ T cells are activated and differentiate into effector T-cells that participate in the formation of mature primary granulomas [39] and into circulating memory T-cells [37,40]. If the adaptive immune response fails to contain the infection, progression to early TB disease (primary TB disease) occurs. Children and individuals with weakened immune systems are at higher risk of primary TB disease, and the disease spectrum ranges from disseminated TB, TB meningitis, miliary TB, to limited disease involving lungs or extrapulmonary organs [41-43]. However, in the majority of infected individuals the adaptive immune response will contain infection, and more than 90% will not develop manifest TB disease after infection [13,14]. Still, the immune system is not able to eliminate *M. tuberculosis*, and viable bacilli persist in the body in a non-

replicating or low-replicating state without causing signs or symptoms of TB disease (latent TB infection) [44,45]. Little is known about the location or physiological state of the bacilli during latency [46-48], or the factors that drive infection towards active TB disease [49,50], but in approximately 5% of infected individuals, dormant bacilli will reactivate to cause post-primary TB disease [13,14]. The risk of reactivation is highest within the first two years after infection, and is very low after five years [13,14], albeit it can occur even decades later [51,52]. Post-primary TB can also be caused by exogenous reinfection, which makes an important contribution to TB disease in high endemic areas [53]. Pulmonary post-primary TB is characterised by caseous pneumonia and formation of cavitary lung lesions that results in efficient spread of bacilli through coughing [3,43]. It accounts for the majority of TB cases worldwide and nearly all TB transmission [3]. If left untreated, the mortality rate from TB disease is high. Studies performed in the pre-chemotherapy era found that 70% of patients with smear positive pulmonary TB and 20% of patients with smear negative, culture positive pulmonary TB died within 10 years after diagnosis [54].

## 1.5 Extrapulmonary tuberculosis

Extrapulmonary TB is defined by WHO as active TB disease at any other site than the lungs [3]. In 2019, 16 % of all notified TB cases worldwide were extrapulmonary [3]. However, the proportion of notified extrapulmonary TB cases varies greatly among countries, from 25-35% in most low TB incidence high income countries (and up to as much as 40% in the Netherlands, UK and Norway), to 10-20%, or lower, in most high TB burden countries [3]. In several high-income countries, the relative proportion of extrapulmonary TB cases has been continuously increasing over the last decades, and the increase has mainly been seen in foreign-born individuals [55-58]. The observed variation in extrapulmonary TB incidence between countries may reflect true differences in epidemiology as extrapulmonary TB prevalence has been linked to geography, ethnicity, *M. tuberculosis* lineage and HIV prevalence [59-64], or may reflect differences in screening, diagnosis and reporting of extrapulmonary disease. Whereas extrapulmonary TB case notification to national TB programmes can be assumed to

provide a good proxy for extrapulmonary TB incidence in most high-income countries, more underdiagnosing and underreporting of extrapulmonary TB likely occur in many low-resource high-incidence settings because identification of contagious pulmonary TB is prioritised over extrapulmonary TB case finding. This can lead to underestimated global extrapulmonary TB prevalence estimates.

The diagnosis of extrapulmonary TB can be challenging due to the non-specific and broad spectrum of clinical presentations, difficulties in obtaining specimens from various sites of infection, and low sensitivity of diagnostic tests. A significant delay in the diagnosis of extrapulmonary TB has been reported in studies from both high and low TB burden settings [65-69]. TB lymphadenitis and TB pleuritis are the most common forms of extrapulmonary disease [70,71], whereas TB meningitis is the most lethal form and has a poor prognosis [72]. In addition to ethnicity, several other host factors are associated with extrapulmonary disease. A higher incidence of extrapulmonary TB is reported in females compared to males [55,73], in young children [41,74,75], and in immunocompromised individuals due to HIV infection, use of steroids and tumor necrosis factor-α inhibitors, chronic renal failure and diabetes mellitus [76-79]. Environmental factors, including smoking, alcohol, iron and vitamin D status are also associated with extrapulmonary TB, but data is limited [59,80-82]. Although the extrapulmonary forms of TB are not contagious and therefore considered less important compared to pulmonary TB in a public health perspective, they still lead to significant morbidity and mortality, especially in vulnerable groups [83-86]. Among people living with HIV, TB remains the main cause of death, accounting for 1 in 3 HIV-related deaths [3].

## 1.6 Tuberculosis diagnostic tests

The lack of inexpensive, sensitive and rapid point-of-care tests for diagnosing TB is a major obstacle to achieve global TB control. There is still a large gap between the estimated global incidence of 10 million new TB cases and the notified 7.1 million new TB cases in 2019, which in part is caused by underdiagnosing [3]. Moreover, among the notified pulmonary TB cases in 2019, only 57% were bacteriologically

confirmed [3], and this proportion is assumed to be even lower for extrapulmonary TB cases [86,87]. The high number of clinically diagnosed TB cases indicates that both under- and overdiagnosis of TB occur, leading to delayed or unnecessary initiation of TB treatment, both with potentially serious consequences for the patient and community.

## 1.7 WHO recommended TB diagnostic tests

*M. tuberculosis* was first identified by the German scientist Robert Koch in 1882 by use of a novel staining technique which was later modified into the well-known Ziehl-Neelsen [88] and fluorescence [89] stains for detection of acid-fast bacilli (AFB) by microscopy [1]. Albeit the method was developed more than 100 years ago and lacks both sensitivity and specificity [90], AFB microscopy is still used as the initial test for diagnosing TB in many low-resource high TB burden countries [91-93]. Hence, a major WHO target to improve TB diagnosis is to increase the percentage of bacteriologically confirmed cases by scaling up the use of other recommended diagnostics that are more sensitive than AFB microscopy [90].

In addition to AFB microscopy, WHO recommends culture based methods, rapid molecular tests, and one antigen-detection based test for detection of lipoarabinomannan in urine (Alere Determine TB LAM AG Test)) (Table 1) [90]. The latter is only recommended for restricted use to assist in detection of *M. tuberculosis* in people living with HIV [94].

**Table 1. Overview of World Health Organization recommended tuberculosis diagnostic tests**

| | | | | Direct testing | | | Indirect testing | |
|---|---|---|---|---|---|---|---|---|
| | **Method** | **Level of care** | **Indication** | **Sputum specimens** | **Extrapulmonary specimens** | **Specimens from children** | **Specimen from mycobacterial culture** | **Detection of drug resistance** |
| Microscopy | Ziehl-Neelsen or Auramine staining | Microscopy centre | Diagnosis and treatment monitoring | Yes | Yes | Yes | | No |
| Culture | Løwenstein Jensen or liquid culture systems | Reference centre | Diagnosis and treatment monitoring. Reference standard. | Yes | Yes | Yes | | Yes, phenotypic drug susceptibility testing.. Reference standard |
| Xpert MTB/RIF<br><br>Xpert MTB/RIF Ultra | Automated PCR-based test | District/hospital level | Replacement for microscopy as initial test for diagnosis and detection of genotypic rifampicin resistance | Yes | Yes, conditional | Yes, conditional | | Yes, genotypic rifampicin resistance |
| TB LAMP | Manual PCR-based test | Peripheral microscopy centre level | Replacement for microscopy as initial test or as an add-on test in smear negative samples for diagnosis in settings without Xpert | Yes | No | No | | No |
| Alere Determine TB LAM AG test | Lateral flow assay | Point-of-care/community | Add-on test for diagnosis in HIV infected patients with advanced immunosuppression or presumptive pulmonary or extrapulmonary tuberculosis | No | Yes, unprocessed urine | Yes, unprocessed urine | | No |
| GenoType MTBDRplus V2 | First-line line probe assay | Reference centre. Potentially district/hospital level | Initial test for diagnosis of drug resistance in smear positive sputum or positive mycobacterial cultures | Yes, but only smear positive samples | No | Yes, but only smear positive sputum samples | Yes | Yes, genotypic isoniazid and rifampicin resistance |
| Nipro NTM+MDRTB detection kit 2 | First-line line probe assay | Reference centre. Potentially district/hospital level | Initial test for diagnosis of drug resistance in smear positive sputum and sample from culture | Yes, but only smear positive samples | No | Yes, but only smear positive sputum samples | Yes | Yes, genotypic isoniazid and rifampicin resistance |
| Genotype® MTBDRsl assay VER 2.0 | Second-line line probe assay | Reference centre. Potentially district/hospital level | Initial test in patients with confirmed rifampicin or multi-drug resistant tuberculosis for diagnosis of drug resistance to fluoroquinolones and second-line injectables | Yes | No | Yes, but only sputum samples | Yes | Yes, genotypic resistance to fluoroquinolones, aminoglycosides/cyclic peptides and ethambutol |

Culture-based methods, including solid Løwenstein-Jensen medium and automated liquid culture systems, are still regarded as the reference standard for diagnosing TB [90]. Culture has a relatively high sensitivity and a very high specificity when used in combination with antigen-detection rapid tests for species identification. Phenotypic drug susceptibility testing is also performed using culture [95,96], and culture is the only TB diagnostic test that can separate viable from dead bacilli, which makes it an important tool for evaluation of response to TB treatment. However, the reduced sensitivity of culture in paucibacillary smear-negative pulmonary TB, childhood TB and extrapulmonary TB makes it an imperfect reference standard in these patient groups [86]. Culture also requires centralised and comprehensive biosafety laboratories, and has a relatively long median turnaround time of 1-2 weeks for liquid culture and longer for solid culture [97,98], which makes it less suitable for use as the initial diagnostic test in low-resource settings.

Over the last decades, development of new technologies to improve TB diagnosis, has mainly been made in the field of rapid molecular tests [94]. These tests target specific gene sequences in the *M. tuberculosis* complex genome, by use of polymerase chain reaction (PCR) and/or DNA probes, to diagnose TB and genotypic drug resistance. WHO recommended rapid molecular tests currently include one automated (Xpert MTB/RIF and the new version Xpert MTB/RIF Ultra) and one manual PCR-based diagnostic test (TB LAMP), in addition to two line-probe assays that are primarily used for genotypic drug susceptibility testing (GenoType MTBDRplus version 1 and 2 and Nipro NTM+MDRTB detection kit 2) [99].

Additionally, data from a prospective multi-centre study coordinated by the Foundation for Innovative New Diagnostics (FIND) indicate that another novel automated PCR-based test, the TrueNat assays (Molbio Diagnostics, Goa, India) has comparable accuracy to Xpert with the advantage of being implementable at the point-of-care level [99]. The test was endorsed by WHO for initial testing of pulmonary TB and detection of rifampicin resistance in July 2020 [100,101]. Several other rapid molecular tests for use in point-of-care settings are currently being developed and evaluated [100].

Several high-throughput PCR-based test platforms for use at the reference laboratory level, including RealTime MTB (Abbott, IL, USA), Roche Cobas MTB (Roche, Switzerland), FluoroType MTBDR (Hain Lifescience, Germany) and Max MDR-TB (Beckton Dickinson, NJ, USA), are also currently routinely used for the detection of *M. tuberculosis* complex and genotypic drug resistance, especially in high-income countries. WHO is undertaking studies to evaluate the clinical validity of these tests in reference laboratories in high TB burden settings [90]. However, the tests are unsuitable for use at lower levels of care and are therefore less relevant for global TB control.

Among these rapid molecular tests, the launch of Xpert MTB/RIF (Xpert) in 2010 represents the largest global breakthrough for improved TB diagnosis so far. The fully automated cartridge-based test system simultaneously detects *M. tuberculosis* complex and genotypic rifampicin resistance within less than two hours and requires minimal operational training [102,103]. Numerous studies have shown the high sensitivity of Xpert to detect *M. tuberculosis* complex in smear positive sputum specimens, but the assay has reduced sensitivity in smear negative samples and limited ability to determine some forms of rifampicin resistance [104,105]. To overcome these limitations, a new version, the Xpert MTB/RIF Ultra (Ultra) was launched in 2017. A large, multi-centre non-inferiority study has shown that the sensitivity of Ultra in smear negative TB is significantly higher as compared to Xpert [106], and Ultra is now recommended by WHO as a replacement for Xpert in all settings [107]. The sensitivity of Ultra for diagnosing extrapulmonary TB is, however, more variable between studies and different types of sample material, and most of the research has been conducted in high-TB burden low-resource settings [108-118]. There is limited knowledge about the usefulness of Ultra in a low-TB-prevalence high-income setting. The performance of Ultra compared to routine TB diagnostic tests in high-resource setting and the potential of Ultra to contribute to a more rapid diagnosis of extrapulmonary TB was investigated in study 3.

## 1.8 Mycobacterial antigens and antigen detection tests

*M. tuberculosis* complex protein antigens have been extensively studied over the last 40 years to discover relevant antigens for use in diagnostic tools and vaccines and to better understand the TB host-pathogen interaction. Important secreted immunodominant antigens, including the Antigen 85 complex [119,120], MPT64 [120,121], 38 kDa [122], ESAT-6 [123,124], CFP-10 [125] and TB10.4 [126], were discovered in the 1980s and 1990s based on classical biochemical analyses of purified *M. tuberculosis* and *M. bovis* BCG culture filtrates, whereas the sequencing of the *M. tuberculosis* genome by Cole and colleagues [127] resulted in the identification of the latency associated antigens, reactivation-associated antigens and resuscitation-promoting factors, amongst others [128,129]. While the highly immunogenic, and often cross-reactive, antigens have been and are being investigated for use in vaccines, the discovery of antigens that are specific for *M. tuberculosis* complex [130] opened up for development of antigen-based TB diagnostic tests. Serological tests and antigen detection tests have the potential to provide rapid, simple and inexpensive TB diagnosis, and antigen-detection tests can also provide direct evidence of active TB disease. However, so far few of these tests have made it beyond the stage of development. The WHO issued a policy statement in 2011 against the use of serological tests for diagnosing TB due to substantial evidence of suboptimal test accuracy [131], and only one antigen-detection test (Alere Determine TB LAM AG Test, for detection of lipoarabinomannan in urine) is endorsed by the WHO [90]. Albeit this test provides proof-of-principle that mycobacterial antigens can be detected in easily available specimens such as urine [132], the sensitivity is low [133-135]. The best test performance is found in HIV TB co-infected people with low CD4 counts, presumably due to higher systemic antigen load in HIV-associated disseminated TB disease, and the test is currently only recommended for TB diagnosis in selected HIV-infected individuals [94]. Antigen-detection in specimens collected directly from the site of infection is being explored as a more sensitive alternative compared to detection in blood and urine, because the antigen load is likely to be higher at the site of infection, especially if the antigen of interest accumulates. Studies have shown that TB pathology seems to be

caused by large amounts of the mycobacterial antigens, despite relative low numbers of viable bacilli, causing hypersensitivity reaction and tissue destruction [43], indicating the special characteristic of the mycobacterial antigens to accumulate at the site of infection.

The *M. tuberculosis* complex-specific antigen MPT64 [121,136,137] has been detected consistently within human macrophages at the site of infection during TB disease in several studies despite the absence of viable bacilli [138-144], implying that this antigen may accumulate and persist within human macrophages [145-148]. This makes it a promising target for antigen-detection-based TB diagnostic tests. MPT64 was originally identified in *M. bovis* BCG culture filtrates in the 1980s and named based on its relative mobility of 0.64 during polyacrylamide gel electrophoresis [121,149]. The protein is immunogenic and elicits strong and TB specific delayed type hypersensitivity skin responses [121,122,150-152] and T cell interferon-γ production [153-155]. Its function is unknown, but it has been suggested to play a role in *M. tuberculosis* pathogenesis due to the accumulation within infected human macrophages [145-148] and because it is a secreted protein that is relatively abundantly expressed during early active cell growth [137,156]. Further, the deletion of the Region of Difference 2 genes, which encodes the *mpt64* gene, from some BCG substrains is associated with reduced virulence in animals and decreased vaccine lesions in humans, implying a role in virulence [157,158]. MPT64 has previously also been studied for use in other types of diagnostic tests including hypersensitivity skin tests [151,152], Interferon-γ release assays [159-161], serological assays [162-165], ELISA [166,167], immunochromatographic lateral flow assays [168,169] and PCR [170-173], and as a vaccine candidate to replace the BCG vaccine [174-177]. However, with the exception of the currently commercially available MPT64 immunochromatographic lateral flow assays for rapid speciation of positive mycobacterial cultures, none of these diagnostic tests or vaccines have reached clinical trials so far [178].

## 1.9 The MPT64 antigen-detection test

The MPT64 antigen-detection test (MPT64 test) is an immunocytochemistry/immunohistochemistry (ICC/IHC) assay for direct detection of MPT64 in extrapulmonary patient samples by use of an in-house polyclonal anti-MPT64 antibody [179]. The method was developed based on previous IHC studies of mycobacterial antigen expression in human TB disease [140,147,180] which showed that MPT64 was consistently expressed intracellularly in host macrophages with a characteristic distribution pattern of staining, while other antigens could not be detected or showed a more diffuse distribution pattern. The MPT64-staining was present in smear and culture negative samples from clinically diagnosed TB cases, indicating that the method could be useful in difficult-to-diagnose paucibacillary TB disease. Moreover, the *mpt64* gene is specific for *M. tuberculosis* complex species, with the exception of Region of Difference 2 negative BCG substrains, and MPT64 can thus diffentiate *M. tuberculosis* from NTM species [121,136,137]. These findings led to the development of the immunochemistry-based MPT64 test for diagnosing extrapulmonary TB. Table 2 shows an overview of the case-control and cohort-based diagnostic accuracy studies that have been conducted to evaluate the utility of the test in a wide range of clinical extrapulmonary specimens including biopsies, fine-needle aspirates and body fluids in different high-TB-burden settings [138-144]. The MPT64 test has performed well in the studies, being positive in all smear positive and 75-100% of culture positive samples. Against clinically or histologically/cytologically diagnosed extrapulmonary TB cases, the test had overall sensitivities of 66-100% and specificities of 81-100% [138-144], suggesting that the test may improve TB diagnosis compared to routine TB diagnostic tests. Moreover, the test method is rapid and robust, and can be performed in basic pathology laboratories without need for advanced equipment and continuous power supply, making it suitable for district/hospital level low-resource settings. These findings indicate that the MPT64 test is a promising candidate to improve the diagnosis of extrapulmonary TB. The test has, however, not been evaluated in a low-TB-burden high-income setting, and the performance of the MPT64 test in a clinical routine setting in Norway was evaluated

in study 1. Further, the polyclonal anti-MPT64 antibody that is currently used in the MPT64 test is in limited supplies. A pre-requisite for further large-scale use of the test is reproduction of a functional anti-MPT64 antibody to secure stable supplies and batch-to-batch consistency. In study 2, we investigated different strategies to develop a new anti-MPT64 antibody for the test.

| Author Year | Setting | Study design | Specimens | Negative controls | HIV status | MPT64 test accuracy against n-PCR[a] as reference standard | | | Other reference standard | MPT64 test accuracy against other reference standard | | | MPT64 test sensitivity against culture as reference standard | | Culture method (decontamination status) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | TB/non-TB cases | Sensitivity | Specificity | | TB/non-TB cases | Sensitivity | Specificity | culture pos/all cultures | Sensitivity | |
| Mustafa et al. 2006 | Tanzania and Norway | Case-control selection cross-sectional | Biopsies | OD | N/A | 40/35 | 90 (36/40) | 83 (29/35) | CRS (AFB or Hist and preTB) | 59/16 | 66 (39/59) | 81 (13/16) | 1/19 | 100 (1/1) | N/A (N/A) |
| Purohit et al. 2007 | India | Case-control selection cross-sectional | LN, abdominal biopsies | ORD | HIV neg | 136/67 | 92 (125/136) | 93 (65/67) | Hist | 153/50 | 79 (121/153) | 88 (44/50) | 31/172 | 100 (31/31) | LJ (N/A) |
| Baba et al. 2008 | South Africa | Case-control selection cross-sectional | Pleural biopsies | OD | All cases | 18/18 | 72 (13/18) | 61 (11/18) | CRS (C or AFB or Hist or preTB and RTT) | 25/11 | 80 (20/25) | 100 (11/11) | 3/14 | N/A | N/A (N/A) |
| | | | | | HIV pos | 10/6 | 90 (9/10) | 33 (2/6) | | 16/unknown | 81 (13/16) | N/A | N/A | N/A | |
| Purohit et al. 2012 | India | Cohort selection cross-sectional | LN FNAs, PF, ascites, CSF | ORD | HIV neg | 130/140 | N/A | N/A | CRS (C or AFB or n-PCR or Hist/Cyt and RTT) | 190/80 | 67 (128/190) | 95 (76/80) | 32/270 | 100 (32/32) | LJ (deconta-minated) |
| Tadele et al. 2014 | Ethiopia | Case-control selection cross-sectional | LN FNAs, PF | ORD/ OD | All cases | 42/76 IS1081 | 88 (37/42) | 91 (69/76) | CRS (C or AFB or n-PCR or Cyt) | 51/67 | 75 (38/51) | 90 (60/67) | 10/118 | 100 (10/10) | LJ (N/A) |
| | | | | | HIV pos | N/A | N/A | N/A | | 14/13 | N/A | N/A | N/A | N/A | |

| Author Year | Setting | Study design | Specimens | Negative controls | HIV status | MPT64 test accuracy against n-PCR[a] as reference standard | | | Other reference standard | MPT64 test accuracy against other reference standard | | | MPT64 test sensitivity against culture as reference standard | | Culture method (decontamination status) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | TB/non-TB cases | Sensitivity | Specificity | | TB/non-TB cases | Sensitivity | Specificity | culture pos/all cultures | Sensitivity | |
| Purohit et al. 2017 | India and Norway | Case-control selection cross-sectional | Biopsies and aspirates | ORD | N/A | 51/38 | 100 (51/51) | 97 (37/38) | CRS (C or AFB or n-PCR) | 51/38 | 100 (51/51) | 97 (37/38) | 12/40 | 100 (12/12) | LJ (N/A) |
| Jørstad et al. 2018 | Zanzibar, Tanzania | Cohort selection cross-sectional | LN FNAs, biopsies, PF, ascites, CSF, pus | ORD | All cases | N/A | N/A | N/A | CRS (C or Xpert or PreTB and Hist or Cyt or Radio or Biochem or RTT) | 69/76 samples not cases | 65 (45/69) | 96 (73/76) | 8/60 | 75 (6/8) | LJ (N/A) |
| | | | | | HIV pos | N/A | N/A | N/A | | 14/6 | 57 (8/14) | N/A | N/A | N/A | |

Abbreviations: AFB, acid-fast bacilli; Biochem, biochemistry; C, culture; CRS, composite reference standard; CSF, cerebrospinal fluid; Cyt, cytology; FNAs, fine-needle aspirates; Hist, histology; HIV, human immunodeficiency virus; LJ, Lowenstein Jensen; LN, lymph node; N/A, not available; n-PCR, nested-polymerase chain reaction; OD, other disease; ORD, other relevant disease; PF, pleural fluid; preTB, presumptive tuberculosis; Radio, radiology; RTT, response to treatment; TB, tuberculosis; Xpert, Xpert MTB/RIF assay.

[a]Nested-PCR targeting the *Mycobacterium tuberculosis* complex specific insertion element IS6110 in all studies, except in the study by Tadele and colleagues (targeting insertion element IS1081).

## 1.10 Assessment of response to anti-tuberculosis treatment

The WHO End TB Strategy targets a treatment success rate of 90% or more for national tuberculosis programs to effectively reduce TB transmission, complications and mortality [8]. Albeit the global treatment success rate has increased from 82% in 2016 to 85% in 2017 [90], the improvement is still too low. Treatment of TB is lengthy, resource-demanding and associated with potentially adverse side-effects. For new cases of pulmonary TB, and most types of extrapulmonary TB, the standard treatment regimen consists of a 2-month intensive phase with rifampicin, isoniazid, pyrazinamide and ethambutol, followed by a 4-month continuation phase with rifampicin and isoniazid [181]. Smear microscopy or culture status at two months of treatment are frequently used to assess treatment response, but have limited ability to predict treatment outcomes[182-185], and are of no use for treatment monitoring in paucibacillary, smear and culture negative forms of TB [186]. Clinical assessment is the usual way to monitor treatment response in smear and culture negative pulmonary and extrapulmonary TB patients, but this is often challenging due to non-specific symptoms and findings. Hence, new markers that can predict treatment outcomes such as failure, cure and relapse are needed, which is further emphasised by the emergence of multidrug-resistant TB [90,187]. The widespread use of empirical TB treatment in patients with clinically diagnosed extrapulmonary TB also poses a risk of overdiagnosis and overtreatment of TB, and a biomarker for early treatment response could help identify cases with alternative diagnoses to secure proper management. Biomarkers of early treatment response may also provide surrogate end-points in clinical trials for faster development of new TB drugs and shorter treatment regimens [188,189].

Numerous potential biomarkers for response to TB treatment are being investigated, as summarised in several reviews [190-192]. Pathogen specific biomarkers that have been studied for treatment monitoring include mycobacterial proteins and peptides, DNA or RNA fragments, and lipids measured in sputum or urine [193-199], but the sensitivities of these markers to predict cure and relapse have been variable and generally low.

Measurement of early bactericidal activity against *M. tuberculosis* in whole blood culture after oral dosing of anti-TB drugs appears to be a more promising biomarker for cure [198]. Early bactericidal activity correlates with culture conversion rate, has already been used during development of new anti-TB drugs [200-202], and is currently being evaluated in TB host directed therapeutics clinical trials [203]. Early bactericidal activity is, however, too resource demanding to be used to predict outcomes in routine clinical settings.

Promising host specific biomarkers to monitor treatment and predict cure have recently been reviewed [184]. These include specific T cell interferon-γ responses to stimulation with different *M. tuberculosis* complex specific antigens [204-209], altered expression of cell surface markers for activation and differentiation in subgroups of T cells during treatment [210-214], and single-markers or multiple-marker host chemokine/cytokine signatures (e.g., interferon-γ-inducible protein 10, C-reactive protein, interleukin 6, interleukin 12, interleukin 4, interleukin 10, tumor necrosis factor α and interferon γ) [215-221]. However, the low concentration in blood of most of these markers limits the type of test platforms that can be used for detection, making them less suitable as point-of-care tests like immunochromogenic lateral flow assays. An important exception is Interferon-γ-inducible protein 10 (IP-10), also known as CXCL-10. IP-10 is a pro-inflammatory chemokine produced by macrophages and other cells in response to T cell interferon-γ release. Several studies show that plasma and serum IP-10 levels decrease upon efficient treatment of TB [222-228], and the decline can be detected already after two weeks of therapy both in pulmonary and extrapulmonary TB [226]. IP-10 is a robust marker expressed at higher levels than many other candidate biomarkers [222,229], which enables use of simple test platforms for detection. Promising results have been obtained with a quantitative lateral flow assay for detection of IP-10 from antigen stimulated blood in TB patients [230,231], indicating that IP-10 based assays have the potential to be developed into point-of-care tests. IP-10 is also stable in dried plasma spots and dried blood spots applied on filter paper [229,232]. Dried plasma spots and dried blood spots are easy methods for storage and transport of blood samples at ambient temperatures from peripheral health centres to

regional level laboratories for analysis [229,233,234]. It has previously been shown that IP-10 levels in dried plasma spots decline during treatment both in pulmonary and extrapulmonary TB patients. However, the potential of IP-10 as a marker for treatment response has mostly been investigated in pulmonary TB patients, whereas the knowledge about IP-10 response in extrapulmonary TB patients is limited. In study 4, we investigated the performance of the IP-10 dried blood spot assay as a marker for response to treatment in extrapulmonary TB patients in a low-resource setting, by comparing IP-10 measured in dried blood spots and dried plasma spots to IP-10 measured directly in plasma during TB treatment.

# 2. Aims

The overall aim was to investigate new methods for diagnosis and evaluation of treatment response in patients with extrapulmonary tuberculosis.

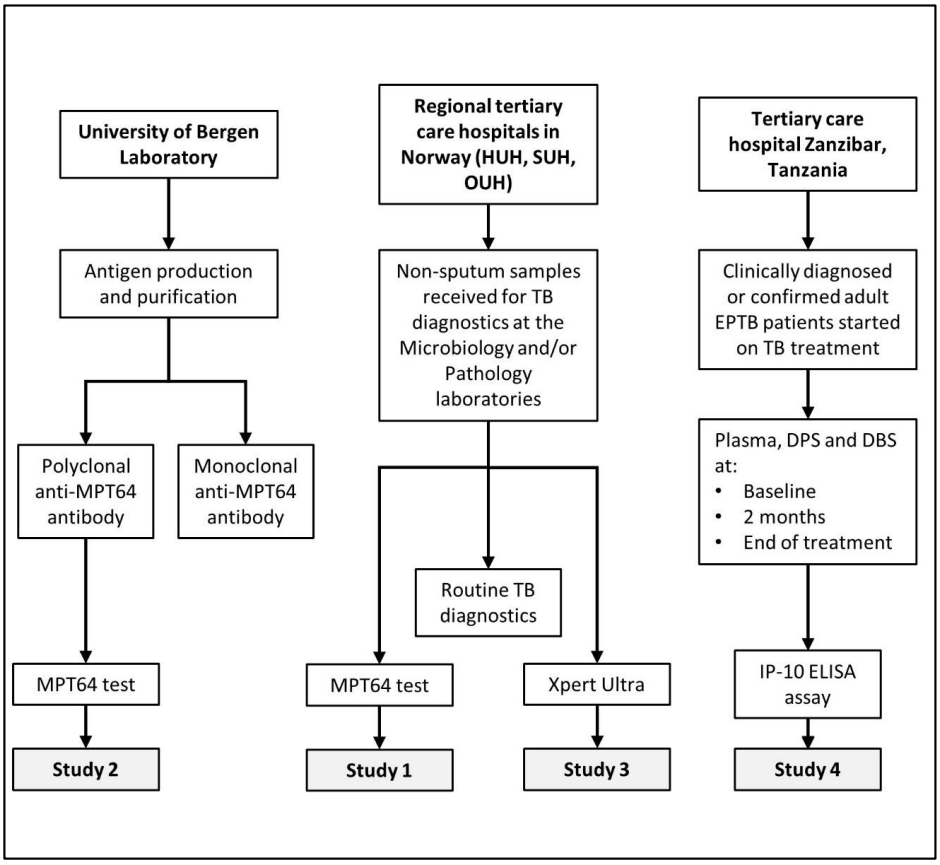
Specific aims:

- Study 1 (MPT64 Norway)
  To evaluate the performance of the MPT64 antigen detection test for the diagnosis of extrapulmonary tuberculosis in a routine clinical setting in a low-TB-prevalence high-resource setting.
- Study 2 (MPT64 Reproducibility)
  To reproduce an anti-MPT64 antibody for large-scale use of the MPT64 antigen detection test on the biopsy and cytology samples.
- Study 3 (Xpert Ultra Norway)
  To evaluate performance of the fully automated, rapid molecular assay, Xpert Ultra, for the diagnosis of extrapulmonary tuberculosis in a clinical routine setting in a low-TB-prevalence high-resource setting.
- Study 4 (IP-10 treatment response)
  To evaluate the utility of the inflammatory host cytokine IP-10 measured in dried blood spots and dried plasma spots as a biomarker to monitor response to treatment in patients with extrapulmonary tuberculosis.

# 3. Materials and methods

Figure 2 provides an overview of the different studies comprising this thesis.

**Figure 2. Overview of the studies of the thesis.** Abbreviations: EPTB, extrapulmonary tuberculosis; DPS, dried plasma spot; DBS, dried blood spot; HUH, Haukeland University Hospital; SUH, Stavanger University Hospital; OUH, Oslo University Hospital; TB, tuberculosis; IP-10, interferon γ-inducible protein 10; ELISA, enzyme-linked immunosorbent assay.



## 3.1 Setting

Studies 1 and 3 were conducted in Norway from January 2015 through January 2016. In 2015, Norway had a population of 5.2 million people, a low HIV-prevalence [235] and an estimated TB incidence of 6 per 100,000 (3.3 per 100,000 in 2019) [3,236,237]. Thirty percent of all notified TB cases were extrapulmonary in Norway in 2015 [236]. Study 4 was conducted in Zanzibar from August 2014 to September 2015. Zanzibar, which is a semi-autonomous region of the United Republic of Tanzania, has a population of 1.3 million people [238] and a low adult HIV prevalence of 1% [239]. The estimated TB incidence in the United Republic of Tanzania was 306 per 100,000 in 2015 (237 per 100,000 in 2019) [3,240], whereas a national TB survey from 2012 reported an estimated prevalence of bacteriologically confirmed pulmonary TB in Zanzibar of 124 per 100,000 [241]. Twenty-four percent of all notified TB cases in Zanzibar in 2015 were extrapulmonary [242].

## 3.2 Study design

Studies 1, 2 and 3 were diagnostic test accuracy studies. In studies 1 and 3, a single-gated (cohort selection) cross-sectional study design was used to evaluate the accuracy of the MPT64 test (study 1) and the Xpert MTB/RIF Ultra assay (study 3). Samples eligible for enrolment were identified at the laboratory level at several tertiary care hospitals in Norway, and included all samples received for TB diagnostics in a clinical setting at the microbiology laboratory (study 1 and 3) and pathology laboratory (study 3). The samples were prospectively and consecutively included in study 3. In study 1, the samples were partly prospectively and consecutively included, and partly retrospectively included due to logistical issues with the prospective inclusion.

In study 2, a two-gated (case-control selection) cross-sectional study design was used, in which the MPT64 test performance was assessed in a study population comprising TB samples from both clinically diagnosed and confirmed (culture and/or Xpert positive) TB cases and controls with other diseases that were clearly distinct from

TB. The study population was nested in the study population of larger extrapulmonary TB study conducted in Pakistan [243], and the samples were retrospectively included.

In study 4, a single-gated, cohort study design with prospective and longitudinal data collection was used to observe correlation between IP-10 values and TB treatment response in a group of TB patients. Patients eligible for enrolment were consecutive adults (>18 years) diagnosed with extrapulmonary TB at a tertiary care hospital in Zanzibar during the inclusion period.

## 3.3 The reference standard

All the studies in this thesis use a composite reference standard. In study 3, the TB case definition was limited to bacteriologically (culture and/or PCR) confirmed TB. A composite reference standard that included clinical TB diagnosis in addition to culture and PCR status was used in study 1 and 2. In study 1, medical records for all patients with culture and PCR negative samples were checked for a clinical TB diagnosis 8 months after the inclusion of samples had finished. All clinically diagnosed TB patients who responded to treatment, as assessed by the local clinicians, were categorised as clinically diagnosed TB cases in the study. In study 2, patients with presumptive extrapulmonary TB, histology suggestive of TB, and response to treatment, as assessed by the primary investigator, were categorised as clinically diagnosed TB cases. In study 4, a composite reference standard consisting of culture, Xpert, AFB microscopy, histo/cytopathology, diagnostic imaging status and response to treatment was used to categorise patients as confirmed, probable or possible TB cases or non-TB cases. The decision to start treatment was done by local clinicians, whereas evaluation of response to treatment was mostly done by the primary investigator in study 4. For all the studies, the reference standard was interpreted without knowledge of the index test results.

## 3.4 Laboratory methods and assays

### 3.4.1 Antigen production

Detailed protocols and references for culture conditions for mycobacteria, protein expression and purification of MPT64 are provided in the manuscript for study 2. The rationale for choosing to work with *M. bovis* BCG and *M. smegmatis* in study 2 will be briefly discussed in the following section. The extremely slow generation time of approximately 20 hours for *M. tuberculosis*, and the biohazard of handling the pathogen species, challenges laboratory research with pathogenic mycobacteria. This has led to the use of the less virulent *M. bovis* BCG substrains and the non-pathogenic *M. smegamtis* as common surrogates for *M. tuberculosis* in TB research.

The *M. bovis* BCG substrains are laboratory-derived attenuated strains of *M. bovis* that are primarily used in the live attenuated BCG vaccine, which is currently the only licenced vaccine against TB [244,245]. The BCG strains are genetically similar to the other *M. tuberculosis* complex species but only require biosafety level 2 laboratories, which makes them attractive model organisms. However, comparative genome studies between *M. tuberculosis* and BCG strains have revealed numerous regions of gene deletions (regions of difference) in the BCG strains that may limit their use in research [246,247], and the pattern of deletion also varies between substrains. Whereas all the BCG strains lack Region of difference 1, a region that is thought to be important for bacterial virulence [248,249], only the so-called late BCG strains (Prague, Glaxo, Denmark, Tice, Connaught, Frappier, Phipps and Pasteur) lack Region of difference 2, which codes for several antigenic secreted protein, including the MPT64 protein. It has been speculated that this deletion has further attenuated the late strains compared to the early strains (Russia, Moreau, Tokyo, Sweden and Birkhaug) [145,250] thereby contributing to some of the observed heterogeneity in BCG vaccine effectiveness [251,252].

The NTM *M. smegmatis* is a fast-growing, environmental saprophyte. In laboratory research, *M. smegmatis* is a simple and attractive model organism for mycobacterial pathogens because it has a short generation time of 3-4 hours, is non-pathogenic and

shares many genetic, structural, and metabolic properties with *M. tuberculosis* [20]. The mutant, laboratory-derived *M. smegmatis* $mc^2155$ strain is particularly useful for work with gene expression in mycobacteria as it allows efficient plasmid transformation and is readily cultivable in most media [253,254].

### 3.4.2 The MPT64 test

The MPT64 test is an ICC/IHC (immunochemistry)-based test for detection of MPT64 in formalin-fixed tissue sections and ethanol-fixed cell smears. Immunochemistry is a type of immunostaining in which specific antibodies are used to identify antigens (mostly proteins) in cells or tissues. For IHC, the method also provides information about the distribution and quantity of the antigen in the tissue. Immunochemistry is a routine method that is widely used both in diagnostic and research laboratories worldwide. The MPT64 test uses a two-step indirect staining method, which means that an unlabelled primary antibody binds the MPT64 protein in the tissue before labelled secondary antibodies bind the primary antibody. The secondary antibody is labelled with an enzyme, horseradish peroxidase, that reacts with a chromogenic substrate to yield a red brownish colour precipitate that can be visualised by light microscopy (Fig. 3). This method is more sensitive than direct one-step immunochemistry due to the signal amplification provided by binding of several secondary antibodies to one primary antibody.

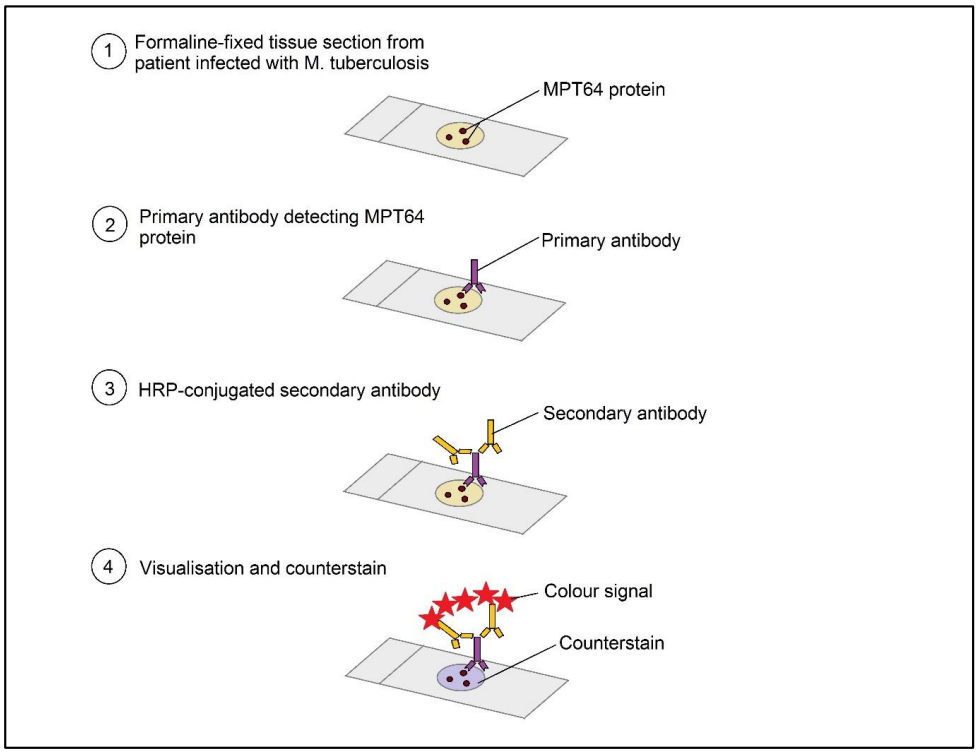



**Figure 3.** The immunohistochemistry-based MPT64 antigen detection test. Abbreviations: HRP, horseradish peroxidase.

The MPT64 test method has been optimised and validated in previous studies [140-142], and previously developed standard of operation was adhered to for all immunostaining runs in studies 1 and 2. In both studies, the same operator performed all runs. Initially, limited validation runs were performed, in which multiple staining runs were performed on serial sections from a small number of positive and negative controls. Based on a subjective evaluation of the consistency in staining patterns in these controls, intra- and inter-run staining variability for the assay was found to be acceptable. For all later runs during the studies, a positive control sample (smear positive confirmed TB tissue section) and a negative control (tissue section from patient with other disease than TB) were included as quality control, in addition to a negative control including no primary antibody.

In study 1, the MPT64 test was interpreted by a designated pathologist with experience in evaluating the test, according to previously developed scoring guidelines [139]. As the study was performed in a clinical setting, clinical information about the patient and results of routine diagnostic tests were sometimes available for the pathologist. In study 2, a pathologist without previous experience with the MPT64 test interpreted the samples after being trained in evaluating the test.

### 3.4.3 The Xpert Ultra assay

The next-generation Xpert MTB/RIF Ultra assay is a semi-quantitative, real-time PCR *in-vitro* diagnostic test for simultaneous detection of *M. tuberculosis* complex species and rifampicin resistance. Compared to the original Xpert MTB/RIF assay, Ultra includes two new multi-copy amplification targets (IS6110 and IS1081) in addition to the original single-copy *rpoB* target, fully nested nucleic acid amplification, and a larger PCR reaction chamber to improve detection and increase the sensitivity of the assay compared with Xpert [255]. The increased sensitivity offered by the multi-copy targets has resulted in the new semi-quantitative category "MTB detected, trace" (trace), which represents the lowest detectable bacillary load. Initial validation of Ultra has shown a limit of detection of 15.6 colony forming units/mL for Ultra versus 112.6 colony forming units/mL for Xpert in *M. tuberculosis* spiked sputum [255]. Additionally, the accuracy of rifampicin resistance detection is increased by incorporating melting temperature-based analysis of the four molecular beacon probes that identify resistance mutations in the *rpoB* gene, instead of the former real-time PCR-based analysis. The test is almost fully automated, requires minimal training of laboratory personnel, and provides results within less than 80 minutes.

Quality control is incorporated in each test cartridge and includes a sample processing control (non-infectious spore), which verifies adequate processing of DNA and detects possible PCR inhibition, and a probe check control, which measures the fluorescence signal from the probes before the start of the PCR reaction. The results of Ultra are generated automatically by the GeneXpert Instrument system from measured fluorescent signals and embedded calculation algorithms.

In study 2, frozen specimens were used for Ultra, and the same operator performed all the runs.

### 3.4.4 The Dried plasma spot/Dried blood spot IP-10 ELISA test

Preparation of plasma, dried plasma spots and dried blood spots were performed according to previously described methods [229,234]. The filter paper cards were transported at room temperature and the plasma samples on dry ice to Bergen, Norway, for analysis.

A previously developed ELISA protocol was used for detection of IP-10 in plasma and eluates from dried plasma spots and dried blood spots [229,234]. The assay is a direct sandwich ELISA, in which two antibodies specific for different epitopes on IP-10 are used. This method can be highly specific and sensitive and is suitable to detect substances in complex samples. A murine anti-IP-10 capture antibody was used to bind IP-10 to the wells of a microtiter plate, and a second horseradish peroxidase - conjugated rat anti-IP-10 detection antibody that binds a different epitope on the IP-10 protein, was used to detect IP-10. Visualisation was performed by adding a substrate to the wells, and a coloured precipitate developed when the substrate reacted with horseradish peroxidase. The presence and quantity of IP-10 was subsequently calculated based on the colour intensity in the wells, as measured by an automated spectrometer.

ELISA characteristics of the test including intra- and inter-run imprecision, IP-10 recovery, linear range, dynamic range, limit of detection and lower limit of quantification, have previously been assessed in an initial validation study [229]. Based on this study, a standard curve that covered the linear range of the assay was included in triplicates in all runs in our study. The blank sample in the standard curve functioned as a negative control and the samples containing recombinant IP-10 functioned as positive controls. The intra-run imprecision of each ELISA run was found to be acceptable if the coefficient of variation of the triplicates of the standard curve was <15%. The ELISA assay was manually performed, and the same operator performed all the runs. The operator was blinded to clinical patient information.

## 3.5 Statistical analyses

In studies 1, 2 and 3, 2x2 cross-tabulation was used to calculate sensitivity, specificity and accuracy against the reference standard. Calculation of 95% confidence intervals for sensitivity and specificity was performed with the exact Clopper-Pearson method.

In study 4, statistical analyses were performed using SPSS Statistics V25 (IBM). Non-parametric statistical methods were applied. Wilcoxon Signed Ranks test was used for repeated measurements to evaluate changes in IP-10 levels at different time points during treatment. For comparison of differences in IP-10 levels in independent groups at baseline, Mann Whitney U test was applied when the groups compared consisted of more than 5 individuals per group. Correlation analyses were performed using Spearman's Rank Correlation Coefficient. A significance level of 0.05 was used.

## 3.6 Ethical considerations

Ethical clearance was obtained from the Regional Committee for Medical and Health Research Ethics, Western Norway (studies 1, 2, 3 and 4), the Institution review board and ethical Committee, Gulab Devi Educational Complex, Lahore, and the National Bioethics Committee Pakistan (study 2), and the Zanzibar Medical Research and Ethics Committee (study 4) before conducting the studies. All methods were carried out in accordance with the relevant guidelines and regulations. For studies 1 and 3, an exemption from informed consent from the participants was granted as the study only included residual material from samples sent for TB diagnostics in a clinical setting. For studies 2 and 4, all patients provided informed written consent. Permission to export plasma, dried plasma spots and dried blood spots out of Tanzania was approved by the Zanzibar Medical Research and Ethics Committee (Ministry of Health, Zanzibar), Mnazi Mmoja Hospital and the study participants.

# 4. Results

In study 1, the diagnostic accuracy of the MPT64 test for diagnosing extrapulmonary TB in low-TB-incidence high-income setting was evaluated against a composite reference standard that included clinically diagnosed TB cases. In formalin-fixed biopsies, the sensitivity of the MPT64 test was 37%, compared to 20%, 37% and 50% for AFB microscopy, PCR-based tests pooled, and culture, respectively. The MPT64 test was positive in 5/7 PCR-positive biopsies, whereas none of the PCR-negative, culture positive, biopsies were positive with the MPT64 test. The specificity of the MPT64 test in formalin-fixed biopsies was excellent (99%) and the test was negative in all NTM cases. The test performance in fine-needle aspirates, pus and fluid samples was generally lower and variable, with sensitivities and specificities between 0-100% and 50-80%, respectively.

Different strategies to reproduce the MPT64 antibody for the MPT64 antigen detection test were investigated in study 2. Several functional polyclonal antibodies were obtained after immunisation of rabbits with a combination of recombinant MPT64 antigen and TiterMax Gold adjuvant through a process of negative screening of pre-immune sera and positive screening of antisera in IHC. By pooling the functional antisera that performed well individually but still displayed slight differences in sensitivity and specificity, we obtained a large volume of a functional antibody with stable performance. The validation of this final antibody in the patient samples showed a high performance with sensitivity in the same range as the reference antibody, but a somewhat lower specificity. We were not able to develop a monoclonal anti-MPT64 antibody applicable in IHC on the formalin-fixed paraffin embedded biopsies with sensitivity and specificity in the same range as the reference antibody.

The diagnostic accuracy of the WHO endorsed rapid molecular test Xpert MTB/RIF Ultra (Ultra) for diagnosing paucibacillary extrapulmonary TB was assessed in a low-prevalence high-income setting in study 3. Using culture and routine-PCR as reference standard, the overall sensitivity and specificity of Ultra were 90% (95% CI

56-100) and 99% (95% CI 93-100), respectively. Ultra was positive in 6/7 smear negative confirmed TB samples. Semi-quantitation of bacillary load by Ultra showed that none of the samples has a high bacillary load and two of the samples were in the new category of "trace" positive samples. The genotypic rifampicin resistance results from Ultra were in concordance with phenotypic drug susceptibility test results.

In study 4, the potential of using IP-10 measured in dried plasma spots and dried blood spots as a biomarker for response to treatment in extrapulmonary TB patients in a TB endemic low-resource setting was investigated. Detectable levels of IP-10 were found in unstimulated plasma, as well as in dried plasma spots and dried blood spots from all of the extrapulmonary TB patients. There was a significant decline in IP-10 levels from baseline to end of treatment in plasma, dried plasma spots and dried blood spots both in extrapulmonary and pulmonary TB patients. For HIV negative patients, the decline was observed already after 2 months of treatment. There was a high correlation between IP-10 measured in plasma and dried plasma spots and dried blood spots, respectively.

# 5. Discussion

In this project we have studied new methods to improve diagnosis and treatment monitoring of extrapulmonary TB. We evaluated the performance of the MPT64 antigen detection test and the Xpert Ultra assay for diagnosing extrapulmonary TB in a high-income low-TB-prevalence setting. The potential of reproducing and improving the MPT64 test was also investigated. Further, the biomarker IP-10 was studied as a marker for response to treatment in extrapulmonary TB.

## 5.1 Methodological considerations

### 5.1.1 Study design

The cross-sectional study design used in studies 1, 2 and 3 is considered a valid study design for clinical assessment of diagnostic accuracy. However, based on several factors such as study setting, study population, sample selection and choice of reference standard, this study design can provide more or less generalisable findings [256,257]. In order to reduce the risk of bias and ensure more complete and transparent reporting of diagnostic test accuracy studies, a quality assessment tool (QUADAS-2) [258] and a guideline for reporting of diagnostic test accuracy studies (STARD) [257] have previously been developed. These guidelines can be of use both when designing and interpreting results of diagnostic test accuracy studies.

In study 2, the case-control selection of samples is likely to have introduced a spectrum bias that can lead to over-estimated test accuracy estimates of the index test. However, in an early exploratory phase of test development, it can be appropriate to initially use a small case-control selection to save time and resources [259]. If the test cannot discriminate between clearly diseased and non-diseased cases, there is no need to perform larger cohort studies in clinical settings.

The cohort selection used in studies 1 and 3 is considered superior to a case-control selection because the index test is evaluated in a population that better reflects the patients that will be tested in clinical practice, and the characteristic of diseased and controls are more similar. In our cohort, samples received for TB diagnostics in a

clinical setting at the laboratories in several tertiary care hospitals in Norway were enrolled, which represents a highly relevant study population reflecting the true test population. The prospective and consecutive inclusion of samples also reduces the risk of sampling bias. However, in study 1, samples were also partly retrospectively collected, which may have introduced a sampling bias. Still, the point of identification of samples was the same as in the prospective and retrospective inclusion (any sample received for TB diagnostics at the microbiology and/or pathology laboratory during the inclusion period), which makes it more likely that the retrospectively included samples were also representative of the true test population.

The single-gated, cohort study design with prospective and consecutive inclusion of participants used in study 4 is a valid study design to provide longitudinal data that can be used to observe correlation between IP-10 values and TB treatment response in TB patients. The study population represented a clinically relevant patient group, as both clinically diagnosed, and microbiologically confirmed TB patients were included.

### 5.1.2 Sample size

A major limitation of the studies in this thesis is the small sample size. Study 2 was an exploratory case-control study, and a calculation of sample size was not performed. The study populations in studies 3 and 4 were nested within the study populations of study 1 and a similar study conducted in Zanzibar (the Zanzibar-study) [139]), respectively. A sample size calculation was performed both for study 1 and the Zanzibar-study but was not performed for the individual subpopulations in study 3 and 4. However, we were not successful in recruiting the targeted number of participants in neither study 1 nor the Zanzibar-study within the inclusion period. This was mainly due to the study design, where inclusion of presumptive extrapulmonary TB patients in routine clinical practice was planned for one year, and it was expected to reach the targeted sample within this period. However, during the implementation, several logistical difficulties were faced, and the number of enrolled patients were less than expected. Scarce sample material and poor sample processing at the laboratories further reduced the sample size. The resulting small sample sizes

led to imprecise estimates of accuracy with wide confidence intervals around them, and also limited the possibility to provide stratified accuracy estimates for key subpopulations (e.g., based on HIV and smear status). However, the samples used in the studies reflect the real-life situation and may be more generalizable in routine clinical practice as compared to the carefully selected samples for a study.

### 5.1.3 The MPT64 test

The reproducibility of ICC/IHC is influenced by several pre-analytic, analytic and post-analytic factors that may introduce variability. Variation in tissue preparation, uneven fixation and antigen retrieval methods are preanalytical factors that can affect the availability and quality of the epitopes of interest. In study 1, we could not control the process of cell smear preparation at the microbiology laboratories, which we think may have been suboptimally performed for some of the included samples (long time before fixation). Analytical factors of variability include incubation time and temperature and concentration of reagents, in addition to operator variation. These factors may be more pronounced when the staining is manually performed, as in our study, in contrast to on environmentally controlled automated platforms. However, the same operator performed all runs in our study, which reduces operator variation. Positive and negative controls were included in all runs for quality control. As the same positive control was used in most of the runs in the study, inter-assay variation was continuously evaluated based on the staining pattern and intensity in the positive control. The same operator performed all runs in our studies, and inter-operator variation was, thus, not investigated.

Post-analytic factors that can affect reproducibility are related to interpretation of test result. The MPT64 test was interpreted according to previously developed scoring guidelines, but still requires some degree of subjective assessment by the reader, which makes the test sensitive to inter- and intra-observer variability. Observer variability has not been systematically evaluated in studies 1 and 2. Clinical information about the patient and results of routine diagnostic tests were sometimes

available for the pathologist who interpreted the MPT64 test in study 1, which may have introduced a bias. However, the pathologist was blinded during interpretation of all the retrospectively included biopsies, and almost all biopsies were retrospectively included. The best performance of the test was observed in biopsies, indicating that confirmation bias during interpretation was less likely to occur.

### 5.1.4 The Xpert Ultra assay

Pre-analytical factors that reduce the number of intact bacilli in the patient samples, such as improper sample collection, sample storage or insufficient concentration of starting material may contribute to test variability. Thirty-two out of 86 specimens in study 3 had a starting volume below the WHO recommended volume requirement for biopsy specimens for Xpert (0.7 mL) [260]. However, cerebrospinal fluid volumes as low as 0.1 mL are recommended for use with Xpert, and specific volume requirements for other extrapulmonary sample materials have not been made, indicating that more knowledge is needed before final sample volume recommendations can be given. Moreover, 30/32 specimens with a low volume in our study had volumes greater than 0.5 mL, which is relatively close to the WHO recommendation. The use of frozen sample material may also have affected the performance of Ultra due to reduced sample quality. However, in previous studies, this effect has been reported to be small [104,105,112,261], and our samples were only subjected to one freeze-thaw cycle before analysis, and we therefore believe this is of less relevance in our study.

Other observed pre-analytic factors that affected the performance of the assay included suboptimal homogenisation of biopsies, which led to clogging and high pressure in the cartridges from particulate matter (ERROR run), and PCR inhibition by blood in the sample (INVALID run). As the test is fully automated, analytic factors that may lead to variability will not be further discussed. The results of Ultra are generated automatically by the GeneXpert Instrument system from measured fluorescent signals and embedded calculation algorithms. Thus, post-analytic factors that can lead to variability are less likely to be an issue with this assay. Still, the

investigator who performed the assay in our study, was blinded to the results of the reference tests.

### 5.1.5 The Dried plasma spot/Dried blood spot IP-10 ELISA test

Sample collection, handling and transportation were mostly performed according to the study protocol, and we believe that pre-analytical factors that can lead to variability is of less importance in study 4. However, long storage time of dried plasma spots and dried blood spots before analysis may lead to degradation of IP-10 in the samples. Albeit a previous validation study has shown that IP-10 is stable in dried plasma spots after four weeks of storage at 37°C [229], our samples were stored for several months before analysis (mostly at -20°C), which may have reduced IP-10 concentration and affected the sensitivity of the assay. The ELISA assay was manually performed, and analytic factors that can cause variability are related to operator (e.g., pipetting accuracy) and environment (incubation temperature and time). The intra-run imprecision of all ELISA runs, as assessed by the coefficient of variation of the standard curve triplicates in each run, was found to be acceptable. Inter-run imprecision was not evaluated in our study but was of less importance because all samples from the same patient were run together on the same plate. The operator was blinded to clinical patient information when performing the assay. Post-analytical factors that can cause variability are less relevant for this assay, as the results are based on automated read-out of light absorbance and require no interpretation.

### 5.1.6 The reference standard

The lack of a true gold standard for the diagnosis of TB is a major challenge for validation of new diagnostic tests. With its high specificity and relatively high sensitivity, automated liquid culture is recommended as the primary reference standard in diagnostic accuracy studies on smear-replacement tests [262]. However, the limited sensitivity of culture can lead to misclassification bias. This can result in overestimated sensitivity of the index test because of false negative culture samples,

and underestimated specificity of the index test if it is more sensitive than culture. As a result, many studies use composite reference standards that include not only culture status, but also PCR and/or smear status and/or clinical TB diagnosis, to better determine true disease status. This is particularly important in paucibacillary TB disease, including paediatric and extrapulmonary TB.

In study 3, the TB case definition was limited to bacteriologically (culture and/or PCR) confirmed TB, which represents an extreme case definition as only clearly diseased cases are classified as TB cases. This may be problematic because the reference standard is not likely to correctly classify all the TB cases in the cohort. The reason for still choosing this reference standard is because information about clinical TB diagnosis was not available for all the study participants. An advantage of using bacteriologically confirmed TB as case definition is that it enables comparison of results between studies and inclusion of data in meta-analyses. There is, however, a risk of overestimating index test sensitivity due to false negative culture samples. Composite reference standards that included clinical TB diagnosis were used in studies 1, 2 and 4. These composite reference standards are likely to correctly classify more true TB cases as compared to the reference standard in study 3 but may also incorrectly classify some non-TB cases as TB cases due to the low specificity of a clinical TB diagnosis. In order to reduce the problem of low specificity, response to treatment was required to be classified as a clinically diagnosed TB case in the studies. A limitation of including clinical TB diagnosis in the reference standard is that the criteria used to define a clinically diagnosed TB case often vary greatly between studies, making it difficult to compare results.

In study 1, routine TB diagnostic tests were performed as part of normal clinical practice at the inclusion sites, and the types of TB diagnostic tests performed varied somewhat based on type of sample material, pre-test probability of TB and specific requests from clinicians. As a consequence, not all patients received the same reference standard. However, the choice to use different diagnostic tests for different samples was not dependent on index test results as the index test was performed after

the reference standard tests, and an introduction of partial or differential verification bias is therefore less likely.

### 5.1.7 Selection bias

Diagnostic accuracy estimates may be biased if not all eligible participants undergo the index test and the reference standard. In studies 1 and 3, any cell-rich non-sputum sample received for TB diagnostics at the microbiology laboratory (and pathology laboratory, study 1) at tertiary care hospitals were eligible for inclusion. These samples are the same samples that will be tested for TB in a clinical setting, and thus reflect the true test population very accurately. However, not all eligible samples underwent the index test or the full reference standard. An evaluation at one of the inclusion sites (Haukeland University Hospital) of the eligible samples that had not been included in the study, showed that at the microbiology department, many lymph node FNAs and biopsies were not included because no sample material was left after routine TB diagnostic tests had been performed. All samples were subjected to culture as a minimum, whereas smear microscopy was only performed if clinical information indicated a high pre-test probability of TB, and routine-PCR was only performed if smear microscopy was positive, or if the clinician specifically requested these tests. Thus, clinically relevant samples (TB lymphadenitis constituted 50% of all notified extrapulmonary TB cases, and 15% of all TB cases in Norway in 2015 [236]) were at higher risk of not being included because of 1) generally low sample volumes of FNAs and biopsies, and 2) an even lower sample volume left after routine diagnostics of the samples with the highest pre-test probability of TB. Firstly, this may have introduced a sampling bias; the proportion of biopsies and FNAs compared to other extrapulmonary sample types was lower in studies 1 and 3 compared to in a routine setting, and, hence, does not fully represent a random sample of the population. Secondly, a disease spectrum bias may also have been introduced. Assuming that samples with higher pre-test probability of TB are more likely to represent true TB and maybe also more severe TB disease, the proportion of TB (and

severe TB disease) in our sample material may have been lower than in a routine setting.

Another group of samples that were eligible for inclusion, but that were not correctly prospectively included in study 1, were formalin-fixed biopsies at the pathology laboratories. A retrospective evaluation showed that many of these samples were missed from inclusion because clinical information stating that TB was a differential diagnosis was often lacking, even when the same sample had been sent for TB culture at the microbiology department in parallel. Based on cross-checking of data on biopsies received for TB diagnostics at the microbiology laboratories with biopsies received at the pathology laboratories, most of the formalin-fixed biopsies that were missed during prospective inclusion were identified and retrospectively included. However, a selection bias was introduced during retrospective inclusion because an extended set of exclusion criteria was applied. Biopsies with a histopathological diagnosis other than TB (e.g., malignancy) or no pathological findings were excluded to narrow down the number of irrelevant non-TB samples included. The use of different eligibility criteria changed the disease spectrum and possibly also increased the TB prevalence in the group of biopsy specimens, but we find it less likely that this selection bias is clinically relevant because these excluded samples with a definite diagnosis other than TB would not have been subjected to TB specific tests at the pathology laboratory in a routine setting.

A selection bias was also introduced in study 3, as pleural fluid samples with a low pre-test probability of TB were excluded. According to local guidelines, many pleural fluid samples are routinely sent for TB diagnostics in our setting, even when the pre-test probability of TB is very low. We therefore only included pleural fluids if TB was mentioned as a diffential diagnosis on the request form or in patient records. This may have changed the disease spectrum in the group of included pleural fluids, but we believe that our selection is in line with the intended use of Ultra, and, thus, represents a clinically relevant cohort.

In study 4, patients eligible for inclusion were prospectively included, thus reducing the risk of selection bias. Subgroup analysis of the patients who refused to participate has not been performed. During the study period, the samples from the study participants who were lost to follow-up due to death ( n = 4), transfer-out (n = 3), refused to give blood (n = 2) and unknown (n = 3) were not available. This may give biased results if the IP-10 levels are different in the group lost-to-follow-up compared to in the group of included participants. Evaluation of the participants lost-to-follow up for other reasons than death did not identify any uniform characteristics with regard to disease severity, age, co-morbidities or outcome, and we think it is less likely that these incomplete datasets have introduced biased results. The patients that were lost-to-follow up due to death presented with more severe disease at baseline (based on patient journals) and had a poorer outcome than the other participants. Hence, a bias may have been introduced by including these incomplete datasets in analysis, but we do not know in which direction such a bias influences the results.

### 5.1.8 Sample flow between reference tests and index test

There can be large variations in bacillary load both between specimens and within specimens from the same patient. For studies 1, 2 and 3 the index test and the reference standard tests were performed on the same specimen, which reduces random variability and provides the best evidence on comparative accuracy [256]. Still, within-specimen variation in bacillary load, due to non-uniform distribution of bacilli, always represents a risk of noise during interpretation, particularly in paucibacillary specimens. A large sample size would have reduced this noise but was not possible to achieve in our studies for reasons explained elsewhere.

### 5.1.9 Modifiers of test accuracy

Clinical characteristics such as gender, age, co-morbidities and spectrum of disease were not available in studies 1 and 3 due to a limited ethical clearance to obtain this information. Some of these characteristics may affect test accuracy in subgroups of the study population. It is possible that not only disease prevalence but also disease spectrum is different in a high-income versus low-income settings. The clinical setting in studies 1 and 3 was tertiary care hospitals, and the patients included in the

study were therefore selected by referral based on symptoms and findings suggestive of TB (or other serious diseases), implying that the test has been assessed in more advanced disease. On the other hand, referral to a hospital may happen at an earlier time point during disease development, i.e., less advanced disease, in high-income settings because of better access to health care system as compared to low-income settings, making it more challenging to compare disease spectrum between our study and previous study settings.

Another potential confounder in study 1, 2 and 3 is HIV TB co-infection, because HIV infection changes immune cell function, bacillary distribution and the histopathology of TB disease, which may potentially also affect the test performance of the MPT64 test and Ultra. Still, in the Norwegian material, the proportion of HIV TB co-infection is believed to be very low. In 2016, HIV status was known for 254/289 TB cases in Norway, of which 10 cases were HIV infected (4%) [263]. HIV TB co-infection is, thus, most likely not a very relevant confounder.

In study 4, clinical characteristics including age, gender and HIV-status were available. HIV TB co-infection was also identified as a potential confounder in this study. Indeed, stratified analysis based on HIV status showed a clearer association between changes in IP-10 levels and response to treatment in the HIV negative group as compared to the HIV infected group. However, the number of HIV infected participants was small, which gives uncertain estimates.

## 5.2 The MPT64 test for diagnosing extrapulmonary TB

Ideally, a new test for diagnosing TB should be simple, low-cost and require little or no instrumentation to make it suitable and sustainable for use at a point-of care level in the low-resource settings with high TB burden. However, low test sensitivity is a major issue in TB diagnostics, indicating that a more complex test for use at reference centre or district/hospital levels may be considered as a valid alternative to a point-of-care test, if it can significantly increase the currently low case detection rate of smear negative TB and reduce empirical treatment which is bound to cause over-treatment

in a substantial proportion of patients. Previous studies performed in low-resource settings suggest that the MPT64 test can play such a role [139-141,143]. In most of these studies, the MPT64 test had a higher sensitivity than conventional smear and culture, and correctly diagnosed a high proportion of clinically diagnosed TB cases.

In study 1, we found that the sensitivity of the MPT64 test for diagnosing extrapulmonary TB was low compared to results obtained in previous studies, both against a composite reference standard that included clinical TB diagnosis, and against culture as reference standard. We believe that the MPT64 accuracy estimates presented in our study are clinically relevant because the study population is representative for the intended test population in a high-income setting, including difficult-to-diagnose paucibacillary TB cases. The discrepant results between study 1 and the previous MPT64 validation studies may be related to differences in study setting, disease spectrum, relative proportion of the different sample types and composition of reference standard among the studies. The disease spectrum is not fully described in any of the MPT64 diagnostic accuracy studies conducted, including study 1, but it is likely that the study populations in the studies conducted in low-resource settings have more severe disease and higher bacillary load at the time of diagnosis as compared to our study population, because patients in a high-resource setting have access to free health services and presumably seek health care at an earlier time during disease progression. This may lead to higher sensitivity of the MPT64 test in low-resource settings as compared to high-resource settings.

The proportion of culture confirmed TB cases also substantially differed between the previous studies conducted in low-resource settings (5-30%) and study 1 (62%). Theoretically, this could reflect more severe disease and a higher bacterial load in our setting, but we find this less likely. As discussed above, the disease spectrum is expected to include fewer cases with severe disease in a high-resource setting, and we have also identified a possible selection bias in our study that led to inclusion of fewer specimens from clearly diseased TB cases. More importantly, the culture procedures used in our setting favour higher bacilli recovery rates and detection rates, which will increase the sensitivity of culture.

Firstly, pre-treatment of samples with N-acetyl-L-cysteine-sodium hydroxide decontamination, which reduces viability, was used more selectively in our setting as compared to previous studies where it was used on all samples. Secondly, most of the previous studies used one solid Lowenstein-Jensen culture per sample, whereas multiple cultures, including both solid and liquid culture of decontaminated and/or non-decontaminated material, was used for a large proportion of the samples in our study. Fully automated liquid culture systems are regarded as superior to solid culture for detection of isolates [186], and the combination of liquid culture and conventional solid culture has been found to provide the highest recovery rates for mycobacteria [264]. Multiple cultures per sample compared to one culture per sample is likely to increase the sensitivity of culture, especially in paucibacillary specimens with non-uniform distribution of bacilli. Thirdly, pre-laboratory factors including long sample transportation time before culture, which reduces viability, and apportioning of scarce sample material between several different tests could also have contributed to reduced sensitivity of culture in previous studies. In our study, many FNAs and biopsies that were eligible for inclusion, were not included because no sample material remained after routine diagnostic tests had been performed. In the previous studies, on the other hand, sample material was distributed equally among all the different TB diagnostic tests that were compared, irrespective of total sample volume. This may lead to insufficient in-put sample volumes for routine diagnostic tests, thereby reducing their sensitivity. Taken together, these methodological differences indicate that culture had a higher sensitivity in our setting, which reduced the sensitivity of the MPT64 test compared to culture. This is further supported by using nested-PCR (targeting IS6110) instead of culture as a comparator between the previous studies and study 1. The sensitivity of the MPT64 test was 71% against nested-PCR positive samples in our study, which is comparable to previous results (72-100%). Nested-PCR was however, only performed on a limited number of samples in our study, and the findings should be interpreted with caution. In contrast to previous studies that only included nested-PCR positive TB samples for validation of the MPT64 test [138,140-144], our study also included several PCR negative, but culture positive TB samples, and all of these samples were negative with the MPT64 test.

Assuming that PCR negative, culture positive samples have lower bacillary load as compared to PCR and culture positive samples, these findings imply that our study has included cases with less severe and paucibacillary disease and that the MPT64 test is not sensitive enough to detect *M. tuberculosis* complex in these samples. The benefit of using the MPT64 test as an add-on test for case detection in a setting with optimal culture methodology is therefore most likely limited, although the test may provide more rapid results than culture. However, study 1 revealed that some tissue samples from patients with symptoms and findings suggestive of TB were not sent for culture in parallel to histopathological examination, presumably due to low TB suspicion or awareness in our setting. In the absence of culture, the MPT64 test may complement other available TB diagnostic tests including AFB microscopy and nested-PCR. Further, *M. tuberculosis* complex specific nested-PCR on formalin-fixed biopsies is not available in all routine pathology laboratories, whereas IHC is.

In low-resource settings with limited culture facilities, the potential of the MPT64 test to improve TB diagnosis is promising [138-144]. Before the potential of the MPT64 test in these settings can be investigated in larger clinical diagnostic test accuracy studies, the issue of test reproducibility must be assessed. This was done in study 2. Test reproducibility and stable antibody supplies are known challenges for tests based on polyclonal antibodies. A common strategy to improve reproducibility and secure infinite supplies of antibodies for antibody-based tests is to replace the polyclonal antibody with a monoclonal antibody. Despite using different combinations of antigen/adjuvants and several screening strategies during hybridoma production, none of the generated monoclonal antibodies in study 2 were fully functional in IHC, albeit they showed good reactivity in ELISA. ELISA was, thus, not an optimal method to select clones that were functional in IHC. Formalin fixation and other tissue processing steps prior to IHC alter epitopes [265], and the antibodies with the strongest binding in ELISA may be non-functional in IHC and vice versa. Important clones for IHC may have been lost in the initial experiments where IHC was not used for screening. However, even after comprehensive screening in IHC in the later experiments, the resulting few monoclonal antibodies that showed some specific

staining in IHC also displayed cross-reactivity, indicating that their target epitopes were not unique to the MPT64 protein. It is possible that our choice of antigen was not optimal to generate antibodies against unique epitopes [266], for instance if the less specific epitopes were more immunogenic than the epitopes of interest [266]. However, as long as the target epitope or epitopes of the MPT64 test are not identified, it is difficult to pin-point the factors that need to be addressed during future antibody production.

When it is not possible to replace a polyclonal antibody with a monoclonal antibody, an alternative strategy to increase both reproducibility and the total volume of the polyclonal antibody is to pool several individual functional antisera, as investigated in study 2. After immunisation of several rabbits with MPT64 antigen to produce MPT64 specific antisera, we observed that the performance of the MPT64 test varied greatly between the different antisera, even when the same batch of MPT64 antigen and immunisation protocol was used. This posed a challenge for reproduction of functional polyclonal antibodies for the MPT64 test, and immunisation and screening of a large number of animals was necessary to identify a sufficiently large number of functional individual antisera. Candidate animals were selected by repeated screening of sera in IHC, both before immunisation to select out sera with non-specific staining, and during immunisation to find the antibodies with the strongest specific staining. Albeit not systematically evaluated, our findings also suggest that the choice of adjuvant and method for production of MPT64 antigen was of importance for obtaining functional antibodies for the MPT64 test. The combination of TiterMax adjuvant and recombinant MPT64 antigen produced in a mammalian cell line vector system resulted in the strongest and most specific immunostaining patterns in IHC among the combinations tested. After pooling the best individual antisera, the antibody test performance in IHC remained similar, which makes this a valid alternative when it is not possible to change to monoclonal antibodies. The diagnostic test accuracy of the final pooled functional polyclonal antibody was assessed in a small number of cases and controls, and the results indicated that the sensitivity was similar, but specificity was somewhat lower compared to the reference antibody. Further modification of the protocol, e.g., optimisation of the blocking of the non-

specific binding sites and increased number of washing steps during IHC has overcome this challenge. However, ongoing studies on a larger cohort will be able to fully evaluate the performance of the new polyclonal antibody in clinically relevant populations.

In addition to reproducibility of the antibody, several other factors related to implementation and quality control of the MPT64 test remain to be more thoroughly investigated. The test requires basic laboratory facilities and trained personnel and is therefore not implementable at a point-of-care level, as is the case with most of the WHO endorsed rapid molecular tests. However, compared to rapid molecular tests, the MPT64 test is less complex, not prone to contamination and can be performed at ambient temperatures using low-cost basic laboratory equipment, which are all advantages in low-resource settings. Still, operational factors including total cost per test, total hands-on time during test performance, test capacity per operator per day, and overall turnaround time for the test (time from the sample is taken to the results are reported back to the clinician) must be evaluated in a real-life setting. Albeit performance of the test itself requires less than one working day, pre-test processing of sample material for biopsies by formalin-fixation, paraffin embedding, and sectioning of slides are all time consuming procedure. Lack of qualified personnel to perform the test or interpret the immunostained sections may also be a limiting factor in some settings. Time needed to train personnel must be investigated, and inter- and intra-operator and -observer variability must also be assessed. Finally, the clinical impact of the MPT64 test remains to be investigated in a so-called phase IV diagnostic study [259]. Will introduction of the test result in earlier diagnosis and earlier initiation of treatment, and if so, will this affect TB mortality and morbidity rates. Also, the use of the test in patients previously treated for TB must be established. It is unknown if MPT64 remains detectable after treatment, and if so, for how long. If MPT64 would prove to be a marker that is only present in active disease, the test could provide very important information.

## 5.3 The Xpert Ultra assay for diagnosing extrapulmonary tuberculosis in high-resource settings

In study 3, we found that the test performance of Ultra was very good in extrapulmonary samples. With a sensitivity that was almost as high as culture, and a laboratory turnaround time of less than two hours for results, the test may contribute to a more rapid TB diagnosis in a high-resource setting compared to other routine TB diagnostics. Moreover, the high specificity of the test (99%) in our study, indicates that it may be suiteable for use in low TB prevalence countries, where the majority of samples tested would be non-TB. There are, however, issues related to the specificity of Ultra that remain to be explored.

Despite a generally high performance of Ultra for the diagnosis of extrapulmonary TB in previous studies [108-114,117,118,267], several studies report that the specificity of Ultra is slightly reduced compared to Xpert against culture as a reference standard [109,113,114,116,267]. In a low TB prevalence setting, even a small reduction in specificity may have clinical implications, and it is not given that the advantage of earlier diagnosis and treatment of a few extrapulmonary TB cases outweights the disadvantages of inappropriate TB treatment of several false positive cases. Moreover, as culture is known to be an imperfect reference standard, it is not clear if, or under which conditions, Ultra positive, but culture negative, samples should be interpreted as false positive or true positive. In patients recently treated for TB, Ultra positive, culture negative samples may represent detection of non-viable bacilli left after treatment, and not relapse of active TB disease. This is a well-known issue for all tests based on detection of DNA, and they are concidered less suiteable for diagnosis of TB relapse. However, Ultra may also have limited usefullness for detection of TB reinfection, even many years after TB treatment, as shown in a study by Dorman and collegues [106]. They found that the specificity of Ultra compared to Xpert was reduced up to seven years after treatment in patients previously treated for pulmonary TB, possibly due to the presence of non-viable or non-replicating bacilli several years after treatment. It has also been speculated that Ultra positive culture negative samples could represent non-replicating or non-viable bacilli from patients

with self-contained/cured minor TB disease or latent TB infection in the lower end of the TB disease spectrum, which raises issues about how, or if, these cases should be treated [268]. In the study by Dorman and colleagues, clinical follow-up of a subgroup of patients with Ultra positive, culture negative samples who had not started TB treatment, showed improvement or resolution of symptoms in 17/18 patients after 2 months, further questioning the relevance of an isolated positive Ultra test result. As many of these presumed false positive Ultra samples were categorised as trace, the authors suggested repeated testing, or the use of various algorithms that categorise all or some trace positive samples as non-TB cases to improve the specificity of the assay (with some loss of sensitivity), a strategy which has later been used in other Ultra studies.

Updated WHO guidelines now state that trace positive samples should be considered true positive among people with presumptive extrapulmonary TB, children and people living with HIV, whereas repeated testing is recommended in other patient groups [107]. This strategy is considered a trade-off between increased numbers of false positives and maximisation of case detection in patient groups that will potentially benefit most from Ultra. These recommendations are, however, based on a low level of evidence. Modelling studies performed by WHO show that replacement of Xpert with Ultra will lead to 49 incremental unnecessary treatments per incremental death averted in an Indian TB centre setting, 10 unnecessary treatments per TB death averted in a South-African HIV clinic setting, and as much as 501 unnecessary treatments per death averted in a Chinese primary care setting [107]. These data suggest that the consequences of implementing Ultra will vary greatly depending on the clinical setting and TB prevalence, and that more setting specific approaches to implementation may be necessary for Ultra. We followed the WHO recommendation to categorise trace positive samples from patients with presumptive extrapulmonary TB as Ultra positive in study 3, and we still obtained a high specificity. However, as clinical information about previous history of TB was not available, the high specificity may reflect that our study population included few patients previously treated for TB. Previous history of TB is an important confounder for Ultra

specificity in pulmonary TB that should be systematically assessed, alongside other potential confounders, in further studies of extrapulmonary TB.

In addition to a high test performance, the simple and automated design of Ultra is one of its main advantages, whereas the high price is a major limitation. The price is currently reduced to US$9.98 per cartridge in eligible high burden low- and middle-income countries as a result of the 2012 donor-negotiated concessional pricing program [269], but it is substantially higher in high-income countries including Norway. Compared to the cost of reagents and equipment required for the MPT64 test, Ultra is much more expensive. On the other hand, the total cost of a test also includes labour cost, and the MPT64 test requires specially trained personnel to perform the test and interpret the results, while the fully automated Xpert/Ultra offers low personell cost (very short laboratory hands-on time for sample preparation, followed by automated sample analysis and reporting of results, and hence low use of "scarce" labour resources). In settings where the availability of trained personnel is limited and/or salary costs are high, labour cost may be of great importance and possibly outweight the high cost of the Ultra cartridge itself. Still, despite the user-friendly design of Ultra, there are operational challenges related to the roll-out of Xpert/Ultra worldwide [118]. According to a 2017 Médecins Sans Frontières report, nationwide implementation of Xpert as the initial TB diagnostic test is lagging behind. Only 15/28 high TB burden countries evaluated in the report had guidelines that recommended use of Xpert as the initial test, out of which only 7 countries had implemented the policy widely [93]. Some of the main challenges for implementation and sustaineability were unstable power supplies and temperature control, and lack of resources for cartridges, calibration and repair of the machines, which are all factors that are of less concern with the MPT64 test. Further, several studies that evaluate outcome of Xpert implementation have shown that Xpert testing does not increase the number started on treatment and does not greatly impact TB mortality and morbidity rates [270-272]. This may be partly explained by non-functional health care systems and pre-treatment delays. Similarly to the MPT64 test, Xpert/Ultra is performed at the district/hospital level far away from the patient, which leads to delays before the patient receives the test results. The low clinical impact of introducing Xpert/Ultra may also partly be

explained by the fact that many patients are started on empirical TB treatment before test results are available. The recently released GeneXpert Edge machine, and the soon expected GeneXpert Omni machine, which are portable and battery-operated smaller systems that can be used at a rural point-of care level, are very promising and may overcome some of these implementational challenges of Xpert/Ultra in low-resource settings [273-275]. In high-income countries with functional health care systems, low risk of pre-treatment delays and sufficient funding for cartridges and machine maintenance, these implementational issues of Ultra are of less concern, and the potential clinical impact of introducing a sensitive and more rapid test can, hence, be larger. While the clinical usefullness of the MPT64 test in a high-resource setting is limited due to its low sensitivity compared to culture, our results suggest that implementation of Ultra as an add-on test will most likely be beneficial and lead to earlier diagnosis of extrapulmonary TB for the majority of culture positive patients in our setting. However, the clinical impact on diagnostic delay, TB morbidity and mortality should be investigated to ensure that implementation of a new and expensive test is actually cost-effective. As discussed above, the issue with reduced specificity must also be handled, and only selected samples from patients with a high pre-test probability of extrapulmonary TB should be subjected to Ultra.

Finally, other potential serious consequences of Ultra implementation that need to be addressed, are the down-scaling or replacement of microscopy and culture facilities and networks due to increased use of Ultra. Ultra is not suitable for monitoring treatment response, and before better methods are developed, culture and or smear microscopy is still needed for this purpose. Reduced access to microscopy and/or culture may threaten the quality of other important parts of TB care, including treatment monitoring and drug susceptibility testing. Ideally, these effects of Ultra implementation should be studied alongside clinical impact on TB mortality and morbidity in further clinical phase IV diagnostic accuracy studies of Ultra.

## 5.4 Biomarkers for response to treatment in extrapulmonary tuberculosis

Development of alternative methods that can replace culture and smear for evaluation of treatment response in extrapulmonary TB patients is needed for timely and proper management, as many of these patients are started empirically on treatment due to the low sensitivity of the conventional tests. A new marker for treatment response should detect treatment failure, e.g., caused by drug-resistant TB, low adherence to treatment or substandard/counterfeit tuberculosis drugs, and ideally should also be able to predict relapse-free cure. In study 4, we found that IP-10 levels significantly decline after two months of treatment and upon completion of successful treatment in HIV negative extrapulmonary TB patients, which is in accordance with several other studies that evaluate IP-10 response during treatment of TB [219,221,224-227]. However, the small sample size in our study gives reduced power to the estimates. Detectable IP-10 levels were present in both dried plasma spots and dried blood spots prepared from unstimulated blood samples, indicating that the concentration of the marker is sufficiently high in whole blood to allow for direct detection (as opposed to sample processing with antigen stimulation to increase cytokine responses). Moreover, taking a whole blood sample from a finger prick is an easily available sample material that can be stored and transported as dried blood spots at ambient temperatures to regional/hospital level laboratories for analysis, making it suitable for low-resource settings. Dried blood spots are already being used worldwide for newborn screening of congenital diseases[276], and has also been tested for HIV screening and viral load monitoring[277,278].

A weakness of study 4 is that it did not include a control group of non-responders. As a consequence, our study only shows that there is a correlation between declining IP-10 levels and response to treatment, and we cannot assess if IP-10 levels remain unchanged or increase during treatment failure or progression of disease, albeit limited data from previous studies suggest that this does occur [227,279]. In one of these studies, a higher level of IP-10 at 2 months of treatment was found in pulmonary TB patients with high risk of treatment failure as defined by a positive sputum smear at 2

months and cavitations on initial chest x-ray[227]. However, the utility of IP-10 monitoring to detect treatment failure is potentially much larger in smear negative pulmonary and extrapulmonary TB, but this remains to be investigated. As our cohort was only followed until end of treatment, it is not possible to evaluate the role of IP-10 kinetics as a predictor for relapse-free cure.

The low specificity of IP-10 is an issue that can affect interpretation, as observed in our study. Unstimulated IP-10 is a non-specific and dynamic marker of inflammation vis-à-vis C-reactive protein. High levels of IP-10 are not only associated with several infections [280-283], but also observed in a variety of inflammatory diseases [284-286] and other conditions that lead to changes in cytokine profiles [287]. IP-10 levels may also vary between subgroups of TB patients. Studies in cell-models[288,289] and humans[290] have shown that antibiotics can affect cytokine production, and TB patients on various drug regimens may show different cytokine patterns during treatment depending on the antibiotics used, albeit this has not been specifically studied for IP-10. For TB HIV co-infected patients, changes in IP-10 levels may be influenced not only by response to TB treatment, but also by changes in CD4 cell count and immunomodulatory effects of antiretroviral drugs. It has also been reported that drug resistant strains can cause other immune responses than drug sensitive strains [291,292], which could be of importance if cytokines, including IP-10, are used to monitor treatment response in MDR-TB patients. IP-10 must, thus, be interpreted in a clinical context. Still, a non-specific marker may be useful during treatment monitoring, which is best exemplified by C-reactive protein. C-reactive protein is conventionally used to monitor treatment response in numerous infectious diseases, and albeit non-specific, a decline in the marker during treatment indicates improvement. Of note, C-reactive protein has also been shown to significantly decrease at two months of treatment in TB patients with clinical response [293,294]. However, the marker may not be sensitive enough to monitor treatment response as detectable levels of C-reactive protein are not found in all patients at baseline [295]. In contrast, detectable levels of IP-10 were found for all the participants in our study, indicating that IP-10 may be a more sensitive marker. However, apparent stable or weakly fluctuating low levels of

plasma IP-10 (< 300 pg/mL) throughout treatment were observed in a few extrapulmonary TB patients despite good clinical response. TB patients represent a heterogenous group of patients, both in terms of infection sites and variation in systemic inflammation. Thus, low systemic plasma IP-10 levels in some patients could reflect low disease activity already at baseline, or compartmentalized production and degradation of cytokines at the site of infection, both limiting the use of the assay in the subgroups of extrapulmonary TB patients. This remains to be further examined.

In the study, we found a high positive correlation between IP-10 measured in plasma and IP-10 extracted from dried plasma spots and dried blood spots. However, discordant results between the three methods were observed for some patients, mostly HIV-infected. This may be related to assay variability, even though the coefficient of variation for the standard curve triplicates in all runs was low, indicating a high intra-assay precision. Still, coefficient of variation could not be calculated for individual patient samples, because the patient samples were assayed as singlets due to limited sample material. The use of triplicates in further studies would provide important information about whether assay varability or some other factor is causing discordant results between the methods.

Empirical treatment of extrapulmonary TB and smear negative pulmonary TB is extensively used globally, particularly in low-resource settings [86,90]. A biomarker that can predict treatment response at an early time point would be very useful for timely management of patients with presumptive TB in order to reduce overtreatment. A limitation of our study was that it did not include assessment of IP-10 treatment response at earlier time points than 2 months. Changes in immune responses, including IP-10, have been reported to occur as early as one week after start of treatment[228,296], albeit the correlation between the early decline in IP-10 levels and clinical outcomes including treatment failure and successful treatment were not assessed in the studies. These data suggest that it may be possible to evaluate response to treatment at very early time points, but this remains to be investigated. The need for new biomarkers for assessment of treatment response is lower in high-

income settings compared to low-resource settings because of reduced risk of failure to treatment caused by drug-resistant TB and overtreatment (more patients are culture confirmed before initiation of treatment), lower risk of non-adherance (stricter regimens of direct observed treatment), and extensive use of diagnostic imaging modalities to aid in clinical evaluation of treatment response. Still, as much as 29% of patients diagnosed with extrapulmonary TB in Norway in 2018 were not culture confirmed [17], indicating an uncertain diagnosis in a substantial proportion of patients with extrapulmonary disease even in high-income settings. Evaluation of IP-10 response during treatment would be particularly usefull in these patients and should be further studied.

# 6. Conclusions

- The usefulness of introducing the MPT64 test as an add-on test for diagnosing extrapulmonary TB in a high-resource setting with optimal culture performance is limited due to low sensitivity compared to culture. However, the test may provide an earlier TB diagnosis as compared to culture and may be a useful tool in the absence of culture.
- Acceptable reproducibility of a polyclonal antibody for the MPT64 test can be achieved through comprehensive screening of individual pre-immune sera and antisera from immunised rabbits, followed by pooling of the best functional antibodies to provide large volume of antibodies with stable performance. The choice of adjuvant and method for antigen production is also likely to affect the performance of the antibodies.
- The use of Xpert Ultra as an add-on test may lead to earlier diagnosis of various forms of extrapulmonary TB in a high-resource setting, and may also provide earlier information about drug resistance. Caution must be made when interpreting Ultra trace positive results, particularly in patients with a previous history of TB which can give false positive results.
- IP-10 levels measured in plasma, dried plasma spots and dried blood spots during TB treatment have the potential to serve as a biomarker for treatment response in extrapulmonary TB patients. However, further studies are needed to evaluate IP-10 responses during treatment failure and relapse and the low specificity of the marker may be an issue.
- Dried blood spots represent an easy method for collection, storage and transportation of patient samples that can contribute to better sample flow in TB care in rural low-resource settings.

# 7. Future perspectives

Even though the sensitivity of the MPT64 test in our study was low compared to culture, the results are uncertain, and a more controlled study with a larger sample size and complete reference standard data for all samples is needed to fully evaluate the test performance in our setting. Several other test issues including operator and observer variability, total test cost (including labour cost), overall turnaround time for test results, test capacity per day, time needed to train personnel and pathologist, and, finally, the clinical impact on TB morbidity and mortality, must also be determined before the cost-effectiveness of the MPT64 test can be compared to other tests. Further validation studies in different settings with head-to-head comparison with the reference antibody are also needed to confirm the non-inferiority of the new polyclonal antibody for the MPT64 test. An ongoing validation study of the new antibody in a clincally relevant study population is currently being undertaken in a high TB burden low-resource setting.

Our findings indicate that Xpert Ultra has the potential to provide earlier diagnosis for extrapulmonary TB patients in a low-prevalent high-resource setting, but larger studies in similar settings are needed to confirm this. Further, the test is expensive, and cost-effectiveness analyses that include evaluation of the clinical impact of Ultra on diagnostic delay, morbidity, and mortality for extrapulmonary TB patients must be performed. The issue of reduced specificity of Ultra in patients with a previous history of TB warrants additional TB diagnostic testing in this group of patients to avoid overdiagnosis.

In order to fully evaluate IP-10 as marker for response to treatment in extrapulmonary TB patients, further studies must include control groups of treatment failure and relapse. Patient samples should also be collected at earlier time points during treatment to explore the marker's potential for early prediction of relapse-free cure. Further, IP-10 kinetics including normal variation in IP-10 levels in healthy controls, clinically significant cut-off levels and clinically significant changes in IP-10 levels during TB treatment needs to be determined.

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# 9. Papers

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RESEARCH ARTICLE Open Access

# Diagnosis of extrapulmonary tuberculosis using the MPT64 antigen detection test in a high-income low tuberculosis prevalence setting

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## Abstract

**Background:** Extrapulmonary tuberculosis (EPTB) poses diagnostic challenges due to the paucibacillary nature of the disease. The immunochemistry-based MPT64 antigen detection test (MPT64 test) has shown promising results for diagnosing EPTB in previous studies performed in low-resource settings, with higher sensitivity than microscopy and culture. The aim of this study was to investigate the performance of the MPT64 test in a routine clinical setting in a high-income low TB prevalence country.

**Methods:** Extrapulmonary samples sent for TB diagnostics to microbiology and pathology laboratories at three regional tertiary care hospitals in Norway in a one-year period were included and subjected to the MPT64 test in parallel to the routine TB diagnostic tests.

**Results:** Samples from 288 patients were included and categorised as confirmed TB cases ($n = 26$), clinically diagnosed TB cases ($n = 5$), non-TB cases ($n = 243$) and uncategorised ($n = 14$), using a composite reference standard (CRS). In formalin-fixed biopsies, the sensitivity (95% CI) of the MPT64 test, microscopy, PCR-based tests pooled, and culture was 37% (16–62), 20% (4–48), 37% (16–62) and 50% (23–77), respectively, against the CRS. The MPT64 test showed a good positive predictive value (88%) and an excellent specificity (99, 95% CI 92–100) in formalin-fixed biopsies. In fine-needle aspirates, pus and fluid samples, the test performance was lower.

**Conclusions:** The MPT64 test was implementable in pathology laboratories as part of routine diagnostics, and although the sensitivity of the MPT64 test was not better than culture in this setting, the test supplements other rapid diagnostic methods, including microscopy and PCR-based tests, and can contribute to strengthen the diagnosis of EPTB in formalin-fixed biopsies in the absence of culture confirmation.

**Keywords:** Extrapulmonary tuberculosis, Diagnostic test, Immunohistochemistry, MPT64, Antigen detection test

## Background

While tuberculosis (TB) remains a global health problem, the incidence in Norway and many other high-income countries is low [1]. Still, diagnosis and control of TB disease poses significant challenges in high-income settings. Although TB rates have been continuously declining in the Norwegian-born population since the middle of the past century, the overall TB incidence in Norway and other high-income countries has remained relatively stable over the last years because of immigration from TB prevalent countries [2–5]. Several studies also report that the increase in foreign-born TB cases is associated with a rise in the proportion of extrapulmonary TB (EPTB) [3, 5–7]. In the European region, EPTB has increased from 16,4% of all TB cases in 2002 to 22,8% in 2016 [6, 8]. In the Netherlands, England, Australia and Norway, EPTB currently accounts for as much as 40% of all TB cases [1, 2].

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The diagnosis of EPTB is challenging. Clinical and radiological findings are often non-specific and the sensitivity of routine TB diagnostic tests, including microscopy for acid fast bacilli (AFB) and culture, is low in paucibacillary disease [9]. Culture also requires advanced laboratory facilities, and results could be delayed up to 8 weeks. Globally, the use of rapid molecular tests for detection of TB is increasing, albeit most commercially available PCR-based tests are only approved for pulmonary TB. The only World Health Organization (WHO) endorsed PCR-based test for diagnosing EPTB, Xpert MTB/RIF (Cepheid, Sunnyvale, CA), has shown variable sensitivity in extrapulmonary samples [10] and is only recommended for subgroups of EPTB [11]. A recently launched new version, Xpert MTB/RIF Ultra (Xpert Ultra), performs better in smear negative, culture positive sputum samples [12], but so far, few studies have investigated its use in EPTB [13–18]. Histopathological findings suggestive of TB may support the EPTB diagnosis, but these are also present in other diseases including sarcoidosis and non-tuberculosis mycobacteria (NTM) infections. The incidence of NTM infections is also increasing in western countries [19–22]. Due to these diagnostic challenges, a definite diagnosis of EPTB is often difficult to obtain. Many EPTB patients are diagnosed clinically and EPTB is associated with diagnostic delay [23–25]. Thus, better diagnostic tests are needed to improve early case detection and management of EPTB patients.

An immunochemistry-based test for detection of the mycobacterial secreted protein MPT64 (MPT64 test) from biopsies, fine-needle aspirates (FNAs) and fluid samples has shown high sensitivity for diagnosing EPTB in previous studies compared to culture and a TB specific nested-PCR [26–31]. The MPT64 test is robust and fast, and can differentiate between NTM and TB disease, as the MPT64 protein is specific for *Mycobacterium tuberculosis* complex (MTBC) species, and not found in NTM [32–34]. A recent study conducted in Zanzibar, Tanzania, has also shown that the MPT64 test is implementable in a routine TB diagnostic setting in a TB high-endemic low-resource country [35]. However, the performance of the MPT64 test has not yet been evaluated in a routine clinical setting in a low TB burden high-income country. The objective of the study was to evaluate performance of the MPT64 test and whether the test would provide an added value to EPTB diagnostics when implemented in routine TB diagnostics in the high-resource health care system in Norway.

## Methods

### Sample inclusion

Formalin-fixed biopsies, FNAs and fluid samples sent for TB diagnostics to microbiology and pathology laboratories at three regional tertiary care hospitals (Haukeland University Hospital (HUH), Oslo University Hospital (OUH) and Stavanger University Hospital (SUH)) from January 2015 until January 2016 were prospectively included in the study, provided there was enough material left after routine diagnostics to prepare a minimum of one cell smear or tissue section for the study (Fig. 1). Acellular fluid samples and all samples from patients that had received TB treatment during one year prior to the study, were excluded.

Because very few formalin-fixed biopsies from patients with presumptive TB had been prospectively included, a retrospective inclusion of biopsy specimens was also performed. At HUH and SUH, all samples included in the study from the microbiology laboratories were cross-checked with the pathology laboratory registers to see if the same sample, or a different sample material collected from the same location at the same time, had been sent to the pathology laboratories. At OUH, a list of all biopsies sent for TB diagnostics to the microbiology laboratory during 2015 was cross-checked with the pathology register to find the samples that had been sent for both departments. Based on these searches, formalin-fixed biopsies from the pathology departments were included if they showed any type of inflammation or necrosis. Biopsies with a histopathological diagnosis other than TB (e.g. malignancy) or no pathological findings were not included as these samples will not be subjected to TB specific tests at the pathology laboratory in a routine clinical setting.. Additionally, all formalin-fixed biopsies that had been subjected to a TB specific nested-PCR as part of routine diagnostics at Department of Pathology at HUH, were retrospectively included.

### Sample processing and routine TB diagnostic procedures

All samples were subjected to routine TB diagnostics at the inclusion hospitals according to local diagnostic algorithms. At the microbiology laboratories, FNAs in saline and fluid samples were used unconcentrated if volume < 10 mL and concentrated by centrifugation before resuspension of sediment in saline if the sample volume was > 10 mL. Biopsy specimens were mechanically homogenized and resuspended in saline. Cell smears were stained using the Ziehl-Neelsen or Auramine method for detection of AFB. For the study, a minimum of one cell smear from fluid samples was prepared on a Superfrost glass, air-dried for 20 min, fixed in absolute ethanol for 20 min and stored at room temperature. A standard NALC-NaOH decontamination procedure was performed on the remaining sample material if the sample was non-sterile, before appropriate sample volumes were seeded in liquid medium (BACTEC MGIT), and for most of the samples, also onto solid medium (Lowenstein-Jensen). At HUH, all lymph node specimens, sterile fluids and aspirates and most biopsies were cultured both before and after

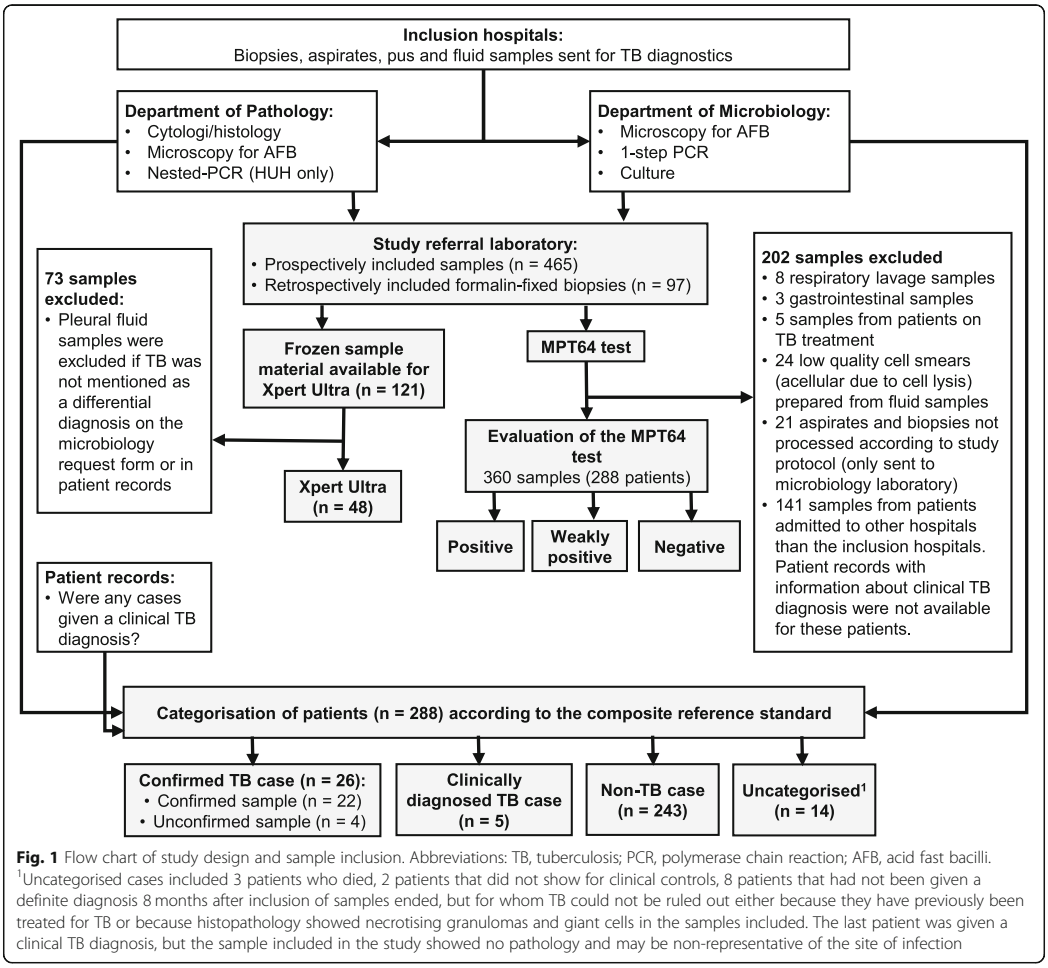


**Fig. 1** Flow chart of study design and sample inclusion. Abbreviations: TB, tuberculosis; PCR, polymerase chain reaction; AFB, acid fast bacilli. [1]Uncategorised cases included 3 patients who died, 2 patients that did not show for clinical controls, 8 patients that had not been given a definite diagnosis 8 months after inclusion of samples ended, but for whom TB could not be ruled out either because they have previously been treated for TB or because histopathology showed necrotising granulomas and giant cells in the samples included. The last patient was given a clinical TB diagnosis, but the sample included in the study showed no pathology and may be non-representative of the site of infection

NALC-NaOH decontamination, and lymph node specimens were also cultured at 28 °C. If PCR was requested by the clinician, a 1-step PCR-based tests (1-step PCR) including Cobas Taqman MTB (Roche, Switzerland) at OUH, Abbott Real Time MTB (Abbott, United States) at SUH and Genotype MTBDR plus (Hain Lifescience, Germany) at HUH, was performed. All samples with a remaining volume of > 0.5 mL, were stored at − 80 °C for later analysis with Xpert Ultra.

At the pathology laboratories, biopsy specimens were routinely fixed in PBS buffered formalin and embedded in paraffin before tissue sections were prepared for histology. Fine-needle aspiration from lymph nodes was performed by local clinicians or pathologists and cell smears for cytology were directly prepared and fixed after sample collection. If microscopy for AFB was requested by the pathologist, the Ziehl-Neelsen (HUH, SUH), Auramine (OUS) or Fite Faraco [36] (OUS) method was used. Additionally, a previously developed in-house nested-PCR (n-PCR) for detection of the MTBC-specific *IS6110* sequence in DNA extracted from archived material [37] was also performed on the samples as part of routine diagnostics at HUH only, if requested by the pathologist.

### Xpert ultra

Xpert Ultra was performed on all the frozen sample material during the autumn 2018, except for pleural fluid samples, which were only subjected to Xpert Ultra if TB was mentioned as a differential diagnosis on the request form or in patient records. This was done to exclude clinically irrelevant samples, as many pleural fluid samples are routinely sent for TB diagnostics, even when the

pre-test probability of TB is very low. Samples were thawed at room temperature and processed according to the manufacturer's protocol. All but two samples (both volume 0.25 mL) had a sample volume of minimum 0.5 mL. Samples with volume < 0.7 mL ($n$ = 20) were added sample reagent to sample in a 3:1 ratio, whereas a ratio of 2:1 was used for samples with a volume of 0.7 mL or more ($n$ = 28).

### Immunostaining with MPT64

The MPT64 test was performed by a laboratory technician in parallel to routine TB diagnostics at Department of Pathology at HUH. The request form with clinical information, sometimes including results of TB diagnostic tests, was available to the technician. The test was performed using an in-house polyclonal rabbit anti-MPT64 antibody at 1:250 dilution together with the Dako Envision + System-HRP kit (Agilent, Santa Clara, CA), according to the manufacturer's protocol with some modifications as earlier described [26, 28]. Briefly, tissue sections were deparaffinized with xylene, before tissue sections and cell smears were rehydrated through decreasing grades of alcohol. Microwave antigen retrieval in citrate buffer, pH 6.2, was then performed on tissue sections only. Further, tissue sections and cell smears were washed in distilled water for 10 min and incubated with hydrogen peroxide for 20 min. The primary anti-MPT64 antibody was applied and the slides were incubated for 60 min, before horseradish conjugated secondary anti-rabbit antibody was applied for 45 min. Thereafter, the substrate (3-amino-9-ethylcarbazol) was added to the slides for 10 min for smears and 15 min for biopsies, followed by counterstaining with Mayer's haematoxylin and mounting with Immu-Mount (Thermo Fisher Scientific, United States). Slides were washed with wash buffer (0.05 mol/L Tris/HCl buffered saline with 0.05% Tween 20, pH 7.6) between incubation steps.

### Evaluation of immunostaining

A laboratory technologist was trained to screen the MPT64 test stained cell smears prepared from fluid samples. Screening was performed at a total magnification of 200x and more detailed evaluation at 400x. Fluid samples screened possibly positive were examined by a designated pathologist, who also evaluated all biopsies and FNAs, according to a previously developed guideline for interpretation [35]. Briefly, a sample was positive if a minimum of two granular red-brown coloured spots, either observed intracytoplasmic in inflammatory cells or extracellularly in necrotic material, were present in the sample. If only one typical spot was present, or if the staining was not strongly granular, the test was evaluated as weakly positive. No staining, nuclear staining or extracellular granular staining in non-necrotic areas were interpreted as negative. Clinical information on the request form, which sometimes included information about results of routine TB diagnostics, was available to the pathologist.

### Categorisation of samples and patients according to a composite reference standard

A composite reference standard (CRS), including both microbiologically confirmed TB and clinically diagnosed TB, was used to define a TB case. Results of routine TB diagnostic tests and cyto/histopathological examination were obtained from the laboratory information systems. Medical records for all patients with culture and PCR negative samples were checked for a clinical TB diagnosis 8 months after the inclusion of samples had finished. According to the CRS, a patient was defined as a *confirmed TB case* if a culture and/or PCR (1-step PCR and/or n-PCR) positive sample was registered during the inclusion period or on a repeat sample until 8 months afterwards. Culture and/or PCR positive samples were classified as *confirmed samples from confirmed TB cases*, whereas culture and PCR negative samples from patients that were diagnosed with microbiologically confirmed TB within 8 months after end of inclusion, were classified as *unconfirmed samples from confirmed TB cases*. A patient that had been given a clinical TB diagnosis and successfully completed a full course of TB treatment, was defined as a *clinically diagnosed TB case*. Patients with culture and PCR negative samples that improved without treatment, or were given a diagnosis other than TB, or had a negative interferon-gamma-release assay, or had stable symptoms and negative results of TB diagnostics at repeated controls until 8 months after the inclusion had finished, were defined as *non-TB cases*. Patients that did not fit into any of these categories were classified as *uncategorised cases*.

### Statistical analysis

Test performance was evaluated using one sample per case. When multiple samples were included from one case, the first sample collected from non-TB cases and the first confirmed TB sample collected from TB cases was chosen for analysis. For TB cases with multiple unconfirmed samples included, the sample with cyto/histopathological findings most strongly suggestive of TB was chosen. Sensitivity, specificity and accuracy were calculated using the CRS as reference method.

## Results

### Clinical samples

A total of 465 samples received for TB diagnostics at the inclusion hospitals were consecutively sent to the HUH during the study period (Fig. 1). Additionally, 97 samples were retrospectively included from the same hospitals.

After exclusion of 202 samples for various reasons, the remaining 360 samples from 288 patients were classified using the CRS. Twenty-six patients were confirmed TB cases, 5 clinically diagnosed TB cases, 243 non-TB cases and 14 uncategorised cases. Uncategorised cases were excluded, leaving samples from 274 patients for analysis. Clinical characteristics for the included samples are shown in Table 1. Pleural fluid was the most common sample type. The MPT64 test was performed on all samples, whereas the type and number of routine TB diagnostic tests performed on the samples varied. HIV status was unknown for the study participants.

Among the 97 retrospectively included formalin-fixed biopsies, 13 biopsies were included because they had been subjected to a TB specific n-PCR at the pathology laboratory at HUH, due to histopathological findings suggestive of TB. These samples had not been sent for TB diagnostics at the microbiology laboratory and TB was not mentioned as a differential diagnosis on the request form. Four of 13 samples were n-PCR positive, and TB was thus, an unexpected finding in these cases.

**Table 1** Characteristics of samples included (one sample per patient)

| | | TB cases[1] | non-TB cases |
|---|---|---|---|
| | | *n* = 31 | *n* = 243 |
| Sample material | | | |
| Lymph node aspirates | | 7 | 4 |
| Lymph node biopsies | | 7 | 17 |
| Other biopsies | | 12 | 52 |
| Pus | | 2 | 13 |
| Pleural fluid | | 3 | 133 |
| Ascites | | 0 | 8 |
| Pericardial fluid | | 0 | 3 |
| Synovial fluid | | 0 | 10 |
| Other fluids | | 0 | 3 |
| Number of samples per patient[2] | | | |
| 1 sample | | 24 | 200 |
| 2 samples | | 6 | 33 |
| 3 samples | | 0 | 6 |
| 4 samples | | 1 | 3 |
| 5 samples | | 0 | 1 |
| | Biopsies | Fine-needle aspirate | Puss and fluid samples |
| | *n* = 88 | *n* = 11 | *n* = 175 |
| Sample sent to both microbiology and pathology laboratories | **68** | **9** | **83** |
| Microscopy[3] | 59 | 9 | 43 |
| 1-step PCR | 32 | 6 | 8 |
| Nested-PCR | 12 | 4 | 1 |
| Culture | 68 | 9 | 83 |
| Sample only sent to the microbiology laboratory | **N/A** | **N/A** | **92** |
| Microscopy | N/A | N/A | 51 |
| 1-step PCR | N/A | N/A | 12 |
| Culture | N/A | N/A | 92 |
| Sample only sent to the pathology laboratory | **20** | **2** | **N/A** |
| Microscopy | 4 | 0 | N/A |
| Nested-PCR | 17 | 1 | N/A |

Abbreviations: TB, tuberculosis; PCR, polymerase chain reaction, N/A, not applicable
[1]Includes 5 clinically diagnosed patients
[2]Number of samples per patient included at different time points. Patients with the same material from the same site (*n* = 27), different material from the same site (*n* = 13), material from different locations (*n* = 6), material from different locations, multiple samples collected from some of these locations (*n* = 4)
[3]Microscopy performed at pathology and/or microbiology laboratory. A sample with discordant microscopy results between the laboratories (*n* = 2) is registered as positive

## MPT64 test performance compared to routine TB diagnostics and Xpert ultra

### *Biopsy specimens*

Using the CRS, the sensitivity (95% CI) of the MPT64 test in formalin-fixed biopsies was 37% (16–62), compared to 20% (4–48), 37% (16–62) and 50% (23–77) for microscopy, PCR-based tests pooled and culture respectively (Tables 2 and 3). Against PCR (1-step PCR and n-PCR pooled) as a reference standard, the sensitivity of the MPT64 test was 71% (5/7, 95% CI 29–96). However, in PCR negative, culture positive biopsies (*n* = 6), the MPT64 test was negative in all samples. One of the 69 non-TB biopsies was MPT64 test positive, yielding a positive predicitive value of 88% (7/8 MPT64 test positive biopsies were from TB cases) and an excellent specificity of 99% (95% CI 92–100). Granulomatous inflammation with necrosis, the most specific histopathological finding suggestive of TB, was present in 13/19 biopsies from TB cases and 13/69 non-TB biopsies (Table 4). This gives histopathology a sensitivity, specificity and positive predicitive value of 68% (43–87), 81% (70–90) and 50% (36–64) respectively against the CRS. Among biopsies from non-TB cases, 5 samples were bacteriologically confirmed NTM infections and another 3 samples came from patients with a probable, though not confirmed, NTM infection. The MPT64 test was negative inn all these samples.

### *Fine needle-aspirates and fluid samples*

Abundant non-specific staining was observed in the cell smears prepared from FNAs, pus and fluid samples, and the MPT64 test performance was lower in these materials compared to the biopsies. Using the CRS, the sensitivity and specificity of the MPT64 test in lymph node FNAs was 29% (95% CI 4–71) and 50% (95% CI 7–93) respectively. Cytopathological findings suggestive of TB had low

**Table 2** Results of routine TB diagnostic tests, Xpert Ultra and the MPT64 test performed on samples

| | | Positive samples/total samples (%) | | | | | |
|---|---|---|---|---|---|---|---|
| | Number of cases | Micro-scopy[a] | Culture | 1-step PCR | Nested-PCR | Xpert Ultra | MPT64 test |
| TB cases | | | | | | | |
| All samples | 31 | 6/27 (22) | 16/26 (62) | 6/18 (33) | 7/11 (64) | 4/7 (57) | 10/31 (32) |
| Lymph node biopsies | 7 | 1/6 (17) | 2/6 (33) | 0/5 (0) | 2/2 (100) | N/A | 3/7 (43) |
| Other biopsies | 12 | 2/9 (22) | 5/8 (63) | 1/7 (14) | 4/6 (67) | N/A | 4/12 (33) |
| Pus samples | 2 | 0/2 (0) | 1/2 (50) | 1/1 (100) | N/A | 1/2 (50) | 1/2 (50) |
| Lymph node aspirates | 7 | 3/7 (43) | 5/7 (71) | 4/4 (100) | 1/3 (33) | 3/4 (75) | 2/7 (29) |
| Fluid samples | 3 | 0/3 (0) | 3/3 (100) | 0/1 (0) | N/A | 0/1 (0) | 0/3 (0) |
| Confirmed TB case -confirmed sample | 22[b] | 5/19 (26) | 16/17 (94) | 6/13 (46) | 7/9 (78) | 4/5 (80) | 8/22 (36) |
| Culture positive sample | 16 | 4/16 (25) | 16/16 (100) | 5/12 (42) | 2/4 (50) | 4/5 (80) | 3/16 (19) |
| Culture positive, PCR positive sample | 6 | 3/6 (50) | 6/6 (100) | 5/5 (100) | 2/2 (100) | 3/3 (100) | 3/6 (50) |
| Culture positive, PCR negative sample | 8 | 1/8 (13) | 8/8 (100) | 0/7 (0) | 0/2 (0) | 1/2 (50) | 0/8 (0) |
| 1-step PCR positive sample | 6 | 4/6 (67) | 5/6 (83) | 6/6 (100) | 1/1 (100) | 3/3 (100) | 4/6 (67) |
| Nested-PCR positive sample | 7 | 1/4 (25) | 2/2 (100) | 1/1 (100) | 7/7 (100) | 1/1 (100) | 5/7 (71) |
| Confirmed TB case -unconfirmed sample | 4 | 0/3 (0) | 0/4 (0) | 0/3 (0) | N/A | N/A | 2/4 (50) |
| Clinically diagnosed TB case | 5 | 1/5 (20) | 0/5 (0) | 0/2 (0) | 0/2 (0) | 0/2 (0) | 0/5 (0) |
| Non-TB cases | | | | | | | |
| All samples | 243 | 7/139 (5) | 0/226 (0) | 0/40 (0) | 0/24 (0) | 1/41 (2) | 39/243 (16) |
| Lymph node biopsies | 17[c] | 1/13 (8) | 0/14 (0) | 0/4 (0) | 0/4 (0) | 0/2 (0) | 0/17 (0) |
| Other biopsies | 52[d] | 2/35 (6) | 0/40 (0) | 0/16 (0) | 0/17 (0) | 1/8 (13) | 1/52 (2) |
| Pus samples | 13[e] | 3/13 (23) | 0/13 (0) | 0/4 (0) | 0/1 (0) | 0/8 (0) | 4/13 (31) |
| Lymph node aspirates | 4[f] | 1/2 (50) | 0/2 (0) | 0/2 (0) | 0/2 (0) | 0/1 (0) | 2/4 (50) |
| Fluid samples | 157 | 0/76 (0) | 0/157 (0) | 0/14 (0) | N/A | 0/22 (0) | 32/157 (20) |

Abbreviations: TB, tuberculosis; PCR, polymerase chain reaction

[a]Microscopy performed at microbiology laboratory and/or pathology laboratory. A sample with discordant microscopy results between the laboratories (n = 2) is registered as microscopy positive

[b]One of the nested-PCR positive patients was not started on TB treatment, and only followed by controls

[c]6 cases of NTM infection (2 NTM culture positive, 1 NTM specific PCR positive, 3 given a clinical diagnosis)

[d]2 cases of NTM infection (2 NTM specific PCR positive)

[e]3 cases of NTM infection (3 microscopy and NTM culture positive)

[f]1 case of NTM infection (microscopy, NTM culture and NTM specific PCR positive. This case was also MPT64 positive)

sensitivity and specificity for diagnosing TB. In pus and fluid samples, the sensitivity of all tests methods was difficult to evaluate due to few TB cases. All three pleural fluids from TB cases were culture positive and negative with all other tests. Two pus samples from TB cases were included. One was microscopy negative and positive with culture, 1-step PCR, Xpert Ultra and the MPT64 test, whereas the other sample was negative with all tests. Many non-TB pus and fluid samples were interpreted as weakly positive ($n$ = 33) or positive (n = 3), and the specificity of the MPT64 test was 80% (95% CI 72–86) and 69% (95% CI 39–91) in fluid and pus samples, respectively.

### Head-to-head comparison of various diagnostic methods

As the number of TB diagnostic tests performed on the samples varied greatly, the diagnostic performance of the different tests was also evaluated based on head-to-head comparisons (Table 5). There was no difference in the overall test performance between microscopy and the MPT64 test, which both detected the same number of TB cases as 1-step PCR, and fewer TB cases than n-PCR, Xpert Ultra and culture. Further, the subgroup comparisons of culture, 1-step PCR, microscopy and the MPT64 test showed that some samples were positive in one test and negative in the other and vice versa. The MPT64 test was positive in 4 microscopy negative samples, 2 1-step PCR negative samples and 3 culture negative samples, indicating added value of combining various TB diagnostic tests.

## Discussion

This is the first study in which the MPT64 test, an immunochemistry-based test for diagnosing EPTB, has been implemented in parallel to routine TB diagnostics in a low TB prevalence country with a high-resource health care system. Using a CRS that included clinically

**Table 3** Test accuracy for various routine diagnostic tests, Xpert Ultra and the MPT64 test using a composite reference standard

| | Test performed on number of samples | Sensitivity % (95% CI) | Specificity % (95% CI) | Overall accuracy % |
|---|---|---|---|---|
| All samples ($n = 274$) | | | | |
| Microscopy | 166 | 22 (9–42) | 95 (90–98) | 83 |
| Culture | 252 | 62 (41–80) | 100 (98–100) | 96 |
| PCR (1-step PCR and n-PCR pooled) | 87 | 44 (26–65) | 100 (94–100) | 83 |
| Xpert Ultra | 48 | 57 (18–90) | 98 (87–100) | 92 |
| MPT64 test | 274 | 32 (17–51) | 84 (79–88) | 78 |
| Lymph node biopsies (n = 24) | | | | |
| Microscopy | 19 | 17 (0–64) | 92 (64–100) | 68 |
| Culture | 20 | 33 (4–78) | 100 (77–100) | 80 |
| PCR (1-step PCR and n-PCR pooled) | 15 | 29 (4–71) | 100 (63–100) | 67 |
| Xpert Ultra | 2 | N/A | 100 (16–100) | – |
| MPT64 test | 24 | 43 (10–82) | 100 (80–100) | 83 |
| All biopsies (n = 88) | | | | |
| Microscopy | 63 | 20 (4–48) | 94 (83–99) | 76 |
| Culture | 68 | 50 (23–77) | 100 (93–100) | 90 |
| PCR (1-step PCR and n-PCR pooled) | 57 | 37 (16–62) | 100 (91–100) | 79 |
| Xpert Ultra | 10 | N/A | 90 (56–100) | – |
| MPT64 test | 88 | 37 (16–62) | 99 (92–100) | 85 |
| Lymph node aspirates (n = 11) | | | | |
| Microscopy | 9 | 43 (10–82) | 50 (1–99) | 44 |
| Culture | 9 | 71 (29–96) | 100 (16–100) | 78 |
| PCR (1-step PCR and n-PCR pooled) | 9 | 67 (22–96) | 100 (29–100) | 78 |
| Xpert Ultra | 5 | 75 (19–99) | 100 (3–100) | 80 |
| MPT64 test | 11 | 29 (4–71) | 50 (7–93) | 36 |
| Pus samples ($n = 15$) | | | | |
| Microscopy | 15 | 0 (0–84) | 77 (46–95) | 67 |
| Culture | 15 | 50 (1–99) | 100 (75–100) | 93 |
| PCR (1-step PCR and n-PCR pooled) | 6 | 100 (3–100) | 100 (48–100) | 100 |
| Xpert Ultra | 10 | 50 (1–99) | 100 (63–100) | 90 |
| MPT64 test | 15 | 50 (1–99) | 69 (39–91) | 67 |
| Fluid samples ($n = 160$) | | | | |
| Microscopy | 79 | 0 (0–71) | 100 (95–100) | 96 |
| Culture | 160 | 100 (29–100) | 100 (98–100) | 100 |
| PCR (1-step PCR and n-PCR pooled) | 15 | 0 (0–98) | 100 (77–100) | 93 |
| Xpert Ultra | 23 | 0 (0–98) | 100 (85–100) | 96 |
| MPT64 test | 160 | 0 (0–71) | 80 (72–86) | 78 |

Abbreviations: PCR, polymerase chain reaction; CI, confidence interval

diagnosed TB cases, the test had a sensitvity (95% CI) of 37% (16–62) in formalin-fixed biopsies, compared to 37% (16–62) and 50% (23–77) for PCR-based tests pooled and culture, respectively. The specificity of the test was excellent (99, 95% CI 92–100) in formalin-fixed biopsies. In cell smears prepared from FNAs, pus and fluid samples, the test performance was low. Culture was found to be the most sensitive method for diagnosing TB in the study, with a disadvantage of long turnaround time. The study revealed that in this low TB incidence setting, many EPTB cases are incidentally detected based on histopathological findings in biopsy specimens that

**Table 4** Cyto/histopathological findings in biopsy and fine-needle aspirate samples

| | TB cases | | | | Non-TB cases |
|---|---|---|---|---|---|
| | Confirmed sample | Unconfirmed sample | Clinically diagnosed sample | Total TB cases | |
| Histomorphology, biopsies (n = 88) | n = 13 | n = 3 | n = 3 | *n* = 19 | *n* = 69 |
| Granulomatous inflammation with necrosis | 9 | 3 | 1 | 13 | 13 |
| Granulomatous inflammation without necrosis | 1 | | 2 | 3 | 13 |
| Subacute or chronic inflammation and necrosis | 3 | | | 3 | 4 |
| Abundant necrosis | | | | | 3 |
| Subacute or chronic inflammation | | | | | 22 |
| Acute inflammation | | | | | 3 |
| Malignant tumor | | | | | 4 |
| Benign tumor | | | | | 2 |
| Fibrosis | | | | | 1 |
| Foreign body granuloma | | | | | 1 |
| Lymph node hyperplasia | | | | | 1 |
| Mesothelial proliferation | | | | | 1 |
| No pathology | | | | | 1 |
| Cytomorphology, FNAs (n = 11) | n = 5 | n = 1 | n = 1 | *n* = 7 | n = 4 |
| Granulomatous inflammation with necrosis | | | 1 | 1 | 1 |
| Abundant necrosis | 3 | | | 3 | 1 |
| Fluid background, mostly RBCs | 1 | | | 1 | 1 |
| Subacute or chronic inflammation | 1 | | | 1 | 1 |
| Acute inflammation | | 1 | | 1 | |

Abbreviations: TB, tuberculosis; FNA, fine-needle aspirate

have not been sent for culture in parallel. Histopathological findings alone cannot confirm a TB diagnosis, and in these cases, the MPT64 test can supplement other rapid tests, including microscopy and n-PCR. This test is less prone to contamination than PCR and, in contrast to microscopy, can differentiate between MTBC and NTM infections. Thus, the MPT64 test may strengthen the TB diagnosis in a pathology laboratory in the absence of culture confirmation.

The MPT64 test performance was lower in the present study compared to previous studies [28–30, 35]. Against a CRS, the overall sensitivity was 32% (95% CI 17–51) for the MPT64 test, compared to 67–100% in previous studies [28–30, 35]. The use of different composite reference standards and variable TB prevalence across the studies may contribute to this variation. All previous studies were conducted in high TB burden settings, in which a higher pre-test probability of TB combined with potentially more advanced stage of TB disease at the time of diagnosis, may lead to higher test sensitivity. Still, also when using culture as a reference standard, the overall MPT64 test sensitivity was lower (19, 95% CI 4–46) compared to previous studies (75–100%) [26, 28, 30, 35, 37]. This could partly be explained by different procedures for culture used across the studies. Apportioning of smaller sample volumes for culture and long transportation time to the TB laboratory, potentially reducing the viability of the bacilli, may have reduced the sensitivity of culture in previous studies [35]. In most of the previous studies, all samples were decontaminated and seeded onto only 1 tube of solid medium, whereas 2–8 culture tubes per sample were used for most samples in the present study, including culturing of material not treated with NALC-NaOH for many samples. These factors can lead to increased sensitivity of culture in our study, especially in paucibacillary specimens with non-uniform distribution of bacilli. Further, the use of different reference standards makes it challenging to compare the studies. For validation of the MPT64 test, n-PCR has been used as a reference standard in most previous studies, yielding a sensitivity of 72–100% [26–29, 31, 35]. In the present study, n-PCR was only performed on a subgroup of samples and could not be used for validation alone. However, when using n-PCR as a reference standard in this subgroup, the sensitivity of the MPT64 test was 71%

**Table 5** Head-to-head comparison of different TB diagnostic tests, including Xpert Ultra and the MPT64 test among TB cases[1]

| | All TB cases n = 31 | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | MPT64 test performed | | Microscopy performed | | 1-step PCR performed | | Nested-PCR performed | | Culture performed | |
| | n = 31 | | n = 27 | | *n* = 18 | | n = 11 | | n = 26 | |
| | MPT64 test + | MPT64 test - | Micro-scopy + | Micro-scopy - | 1-step PCR+ | 1-step PCR- | Nested-PCR+ | Nested-PCR- | Culture + | Culture - |
| | *n* = 10 | *n* = 21 | n = 6 | n = 21 | n = 6 | *n* = 12 | n = 7 | n = 4 | n = 16 | n = 10 |
| Microscopy + | 2 | 4 | | | | | | | | |
| Microscopy - | 4 | 17 | | | | | | | | |
| 1-step PCR + | 4 | 2 | 4 | 2 | | | | | | |
| 1-step PCR - | 2 | 10 | 2 | 9 | | | | | | |
| Nested-PCR + | 5 | 2 | 1 | 3 | 1 | 0 | | | | |
| Nested-PCR - | 0 | 4 | 0 | 4 | 0 | 1 | | | | |
| Culture + | 3 | 13 | 4 | 12 | 5 | 7 | 2 | 2 | | |
| Culture - | 3 | 7 | 2 | 7 | 1 | 5 | 0 | 2 | | |
| Xpert Ultra + | 2 | 2 | 2 | 2 | 3 | 0 | 1 | 1 | 4 | 0 |
| Xpert Ultra - | 0 | 3 | 0 | 3 | 0 | 1 | 0 | 1 | 1 | 2 |

Abbreviations: TB, tuberculosis; PCR, polymerase chain reaction
[1]TB cases include both microbiologically confirmed TB cases (n = 26) and clinically diagnosed TB cases (n = 5)

(95% CI 29–96). This is close to previous findings. Moreover, all culture positive samples were n-PCR positive in previous studies, whereas the present study included several culture positive, but PCR negative samples ($n = 8$). The MPT64 test was negative in all these culture positive, PCR negative samples. Assuming that culture positive, PCR negative samples have a lower bacterial load than culture positive, PCR positive samples, these results indicate that the MPT64 test is not sensitive enough to detect samples with very low bacterial load. However, the long turnout time of culture does not help clinicians to make a timely diagnosis. Further, although culture performed under optimal conditions is the most sensitive method for diagnosing EPTB in the present study, TB culture facilities are not available in most TB endemic areas, in which TB diagnostics are most needed.

The specificity of the MPT64 test in biopsy specimens was very high and comparable to results observed in previous studies, whereas the specificity in cell smears prepared from FNAs, pus and fluid samples was lower. In lymph node FNAs, the specificity was only 50%, However, this was based on only two MPT64 test positive non-TB cases of a total of four non-TB cases, which gives low power to the estimate. In pus and fluid samples, non-specific false positive staining was observed in a large proprotion of the smears and made interpretation challenging. The non-specific staining may have been caused by suboptimal sample handling at the microbiology laboratories where samples could be stored cold for more than one day before preparation of smears, as indicated by cell lysis in many samples. Long storage time may have affected the antigen integrity and increased non-specific binding. In contrast, smears in previous studies were prepared immediately after sample collection.

The low specificity of the test in cell smears has a greater impact in this low prevalence setting compared to a high prevalence setting because more false positive cases and unecessary treatment must be accepted for every true positive case detected. Thus, the results of the present study indicate that the MPT64 test is not useful for diagnosis of EPTB in cell smears. In biopsy specimens, on the other hand, the test was highly specific. It was negative in clinically relevant non-TB samples with various types of inflammation and in all samples from patients with NTM infections. NTM infection is an important differential diagnosis to EPTB, as 31% of the microbiologically confirmed mycobacterial infections were NTM in the present study.

There are limitations to the study. The low number of TB cases gives low power to sensitivity estimates.. Further, the exlusion of culture and PCR negative samples because information about clinical TB diagnosis was not available (Fig. 1), in addition to the exclusion of biopsies with a histopathological diagnosis other than TB or no pathological findings, leads to a selection bias in favour of samples with a higher pre-test probability of TB, which could affect specificity estimates. As the study was designed to evaluate the MPT64 test performance in a routine setting, we did not intervene in sample handling or TB diagnostic algorithms at the inclusion sites, leading to many suboptimally prepared samples for the MPT64 test. Samples from patients with presumptive

EPTB were often not sent for TB diagnostics both to microbiology and pathology laboratories, as would have been expected according to good clinical practice. Not only may this lead to diagnostic delay since the available diagnostic tools are not fully utilized in difficult-to-diagnose cases, but it also makes it difficult to compare test performance in the present study because the types and number of tests performed per sample varied greatly. Hence, more controlled validation studies with larger cohorts are needed to fully asses MPT64 test performance in a low TB incidence high-resource setting.

## Conclusions

The diagnosis of EPTB is challenging in a high-resource, low-TB incidence country. The awareness of TB is often low and routine TB diagnostic tests are not able to identify all EPTB cases. The MPT64 antigen detection test has a good positive predictive value and an excellent specificity in formalin-fixed biopsies and is implementable in pathology laboratories. In the absence of culture, the MPT64 test may contribute to strengthen the TB diagnosis in formalin-fixed biopsies when used in combination with microscopy and PCR-based tests, and thus, has an added value in TB diagnostics in this setting.

### Abbreviations

AFB: Acid fast bacilli; CRS: Composite reference standard; EPTB: Extrapulmonary tuberculosis; FNA: Fine needle aspirate; HUH: Haukeland University Hospital; MTBC: *Mycobacterium tuberculosis* complex; n-PCR: Nested-PCR; NTM: Non-tuberculosis mycobacteria; OUH: Oslo University Hospital; SUH: Stavanger University Hospital; TB: Tuberculosis; WHO: World Health Organisation

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### Authors' contributions

TM conceptualised the study. Study design and methodology was developed by TM, LS, HW, MDJ and IMH. AMDR, IS, HS, PJ and IMH collected data. LS and TM interpreted and validated the results of the study. IMH performed formal analysis of data. IMH and TM wrote the original draft. IMH, LS, HS, AMD, IS, PJ, MDJ, HW and TM reviewed and edited the manuscript. All authors have read and approved the final manuscript.

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### Availability of data and materials

The datasets generated and analysed during the current study are available from the corresponding author on reasonable request.

### Ethics approval and consent to participate

The study was approved by the Regional Committee for Medical and Health Research Ethics of Western Norway (REK Vest) (2014/46/REK vest). An exemption from informed consent from the patients was granted as the study only included residual material from samples sent for TB diagnostics in the clinical setting.

### Consent for publication

Not applicable

### Competing interests

The authors declare that they have no competing interests.

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## Publisher's Note

# Immunochemistry-based diagnosis of extrapulmonary tuberculosis: A strategy for large-scale production of MPT64-antibodies for use in the MPT64 antigen detection test

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**Abstract:** Tuberculosis (TB) is a global health problem. The immunohistochemistry (IHC)-based MPT64 antigen detection test has shown promising results for diagnosing extrapulmonary TB in previous studies. However, the anti-MPT64 antibody currently used in the test is in limited supply, and reproduction of a functional antibody is a prerequisite for further large-scale use. Various antigen-adjuvant combinations and immunisation protocols were tested in mice and rabbits to generate monoclonal and polyclonal antibodies. Antibodies were screened in IHC, and the final new antibody was validated on clinical human specimens. We were not able to generate monoclonal antibodies that were functional in IHC, but we obtained multiple functional polyclonal antibodies through careful selection of antigen-adjuvant and comprehensive screening in IHC of both pre-immune sera and antisera. To overcome the limitation of batch-to-batch variability with polyclonal antibodies, the best performing individual polyclonal antibodies were pooled to one final large-volume new anti-MPT64 antibody. The sensitivity of the new antibody was in the same range as the reference antibody, while the specificity was somewhat reduced. Our results suggest that it possible to reproduce a large-volume functional polyclonal antibody with stable performance, thereby securing stable supplies and reproducibility of the MPT64 test, albeit further validation remains to be done.

**Keywords:** extrapulmonary tuberculosis; diagnostics; antigen detection; MPT64; immunohistochemistry; polyclonal antibody; monoclonal antibody.

## 1. Introduction

Tuberculosis (TB) is a global health problem with an estimated 10 million new cases and 1.4 million deaths in 2019 (1). Approximately one third of the estimated new TB cases each year are not diagnosed or reported.

Extrapulmonary TB (EPTB), which is more common in children and people with HIV (2-6), pose a special diagnostic challenge due to the paucibacillary nature of the disease. This leads to variable and generally low sensitivity of routine microscopy, PCR and culture (1, 7), and new improved diagnostic tests are needed. Tests based on detection of mycobacterial antigens are of special interest as they have the potential to provide rapid and direct evidence of active TB disease (8). Two antigen-detection tests for TB diagnosis are currently commercially available (Alere Determine TB LAM and Fujifilm SILVAMP TB LAM, both detecting lipoarabinomannan in urine) but their clinical use is restricted due to suboptimal sensitivity (9, 10). The novel MPT64 antigen-detection test (the MPT64 test) for the diagnosis of EPTB, which is based on detection of the mycobacterial antigen MPT64 in tissue samples from the site of infection, has shown promising results in previous validation studies (11-18), with higher sensitivity than routine smear and culture in high TB incidence settings (12-17). The test is feasible to implement in the low-resource setting and can contribute towards timely and accurate diagnosis of EPTB (16). These results warrant further research to evaluate the diagnostic test accuracy in larger cohorts and to investigate the potential for large-scale use and clinical roll-out of the test. However, the in-house polyclonal rabbit anti-MPT64 antibody used in the test so far is in limited amount, and reproduction of an antibody with stable performance, which is a pre-requisite for large-scale use, can be challenging due to batch-to-batch to variations in polyclonal antibodies (19). The aim of the study was to develop an anti-MPT64 antibody to secure stable supplies and performance of the MPT64 test. Here, we describe different aspects to be considered when developing antibodies for use in immunohistochemistry (IHC), the challenges faced with the production of a monoclonal antibody and strategies to make large volumes of a functional polyclonal antibody.

## 2. Materials and Methods

*Production of MPT64 antigen*

Several strategies were used to produce MPT64 antigen for immunisation. Native MPT64 was produced because the conformational epitopes, which may be important targets in IHC, are conserved in native proteins. Native MPB64 protein was obtained from cultures of *Mycobacterium bovis* bacillus Calmette-Guérin Moreau (BCG Moreau), according to previously developed protocols for culturing and purification (20-22), with some modifications (Supplementary text). MPB64 is homologous to the *M. tuberculosis* derived MPT64 protein used as an antigen when the reference anti-MPT64 antibody was generated (23), and the two proteins are hereafter collectively referred to as MPT64. Because production of native MPT64 is time-consuming due to the slow growth of the bacilli and the resulting low yield of MPT64 protein, we also produced recombinant MPT64 antigen in several expression systems. In our laboratory, untagged recombinant MPT64 protein was expressed in the non-pathogenic, fast-growing *M. smegmatis* $mc^2$ 155 (24) transformed with the mycobacterial plasmid (pUV15tetORm (25)) modified to contain the mpb64 gene with its predicted secretion signal sequence (GenBank Accession No. AM412059.2; BCGM locus 1981c), according to previously developed protocols (25-27), (Supplementary text, figure S1). His-tagged recombinant MPT64 was produced in *E. coli* (by

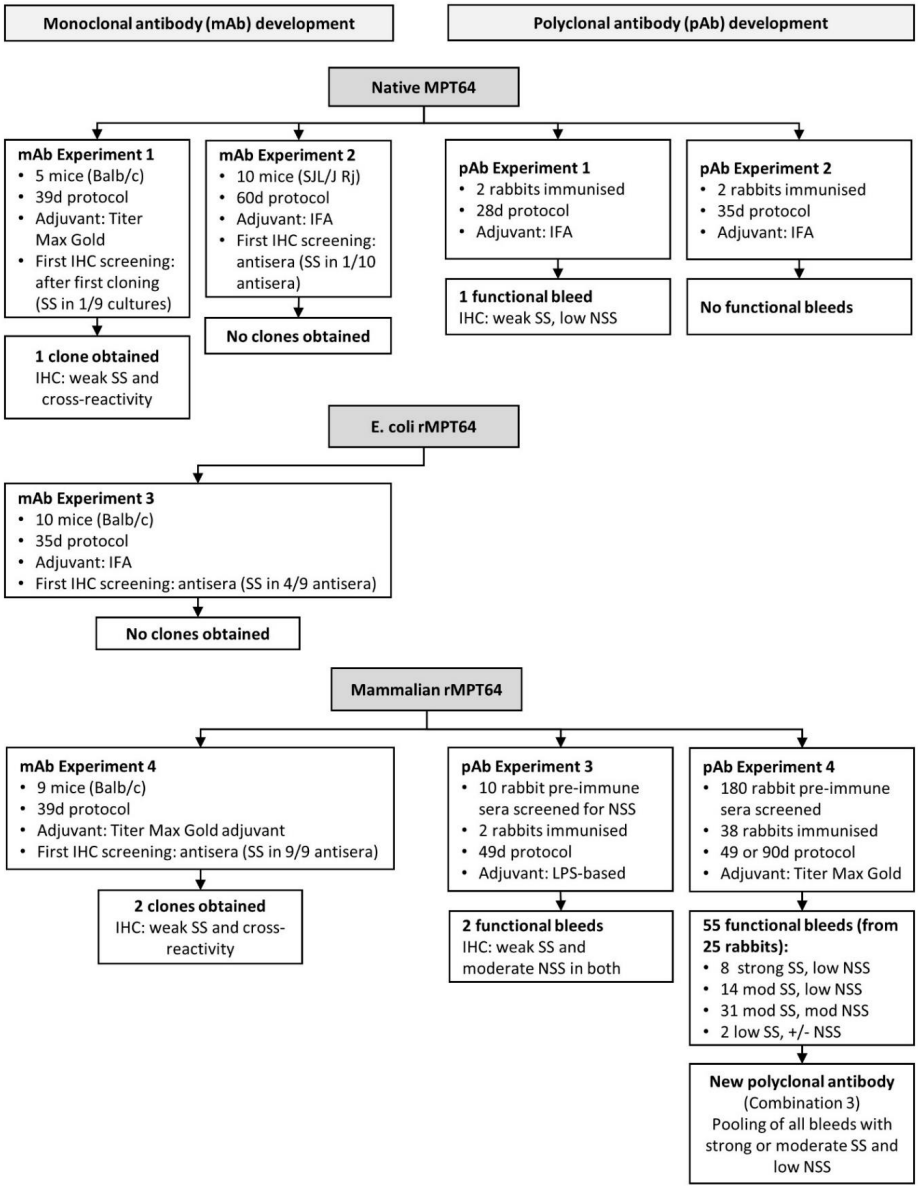
Trenzyme Life Science Services, Konstanz, Germany) and in a mammalian cell line (by InVivo Biotech Services, Berlin, Germany), based on the MPT64 amino acid sequence from *M. bovis* BCG Moreau without the signal sequence (Supplementary text). Table 1 shows the predicted amino acid sequence of the different MPT64 proteins that were used as antigens in the study.

**Table 1. Amino acid sequence of the various forms of MPT64 protein used as antigen in the study.** The native MPT64 sequence is derived from *M. bovis* BCG Moreau and includes a N-terminal cleavable protein secretion signal sequence (bold), which is not present in the final, secreted form of the protein. The sequence of the recombinant protein produced in *Escherichia coli* (*E. coli* rMPT64) includes a C-terminal Thrombin-cleavable 8XHis-tag (underlined) to simplify purification. The sequence of the recombinant protein produced in human HEK cells (mammalian rMPT64) includes a N-terminal HSA signal peptide (bold) to secrete the protein, and a C-terminal 6XHis-tag (underlined) to simplify purification.

| Protein | Amino acid sequence |
|---|---|
| Native MPT64 | **MRIKIFMLVTAVVLLCCSGVATA**APKTYCEELKGTDTGQACQIQMSDPAYNINISL PSYYP DQKSLENYIAQTRDKFLSAATSSTPREAPYELNITSATYQSAIPPRGTQAVVLKV YQNAGGTHPTTTYKAFDWDQAYRKPITYDTLWQADTDPLPVVFPIVQGELSKQTG QQVSIAPNAGLDPVNYQNFAVTNDGVIFFFNPGELLPEAAGPTQVLVPRSAIDSMLA |
| *E. coli* rMPT64 | MAPKTYCEELKGTDTGQACQIQMSDPAYNINISLPSYYPDQKSLENYIAQTRDKFLSAA TSSTPREAPYELNITSATYQSAIPPRGTQAVVLKVYQNAGGTHPTTTYKAFDWDQAYRK PITYDTLWQADTDPLPVVFPIVQGELSKQTGQQVSIAPNAGLDPVNYQNFAVTNDG VIFFFNPGELLPEAAGPTQVLVPRSAIDSMLAVLVPRGSAAALEHHHHHHHH |
| Mammalian rMPT64 | **MKWVTFISLLFLFSSAYS**APKTYCEELKGTDTGQACQIQMSDPAYNINISLPSYYPDQKSL ENYIAQTRDKFLSAATSSTPREAPYELNITSATYQSAIPPRGTQAVVLKVYQNAGGTHPTT TYKAFDWDQAYRKPITYDTLWQADTDPLPVVFPIVQGELSKQTGQQVSIAPNAGLDPV NYQNFAVTNDGVIFFFNPGELLPEAAGPTQVLVPRSAIDSMLAHHHHHH |

*Development of a monoclonal anti-MPT64 antibody*

Monoclonal antibodies from mice were generated by hybridoma technology according to standard methods (28) by commercial companies (Biogenes, PharmAbs and InVivo). Four different strategies to develop a functional monoclonal antibody for the MPT64 test were investigated (mAb experiment 1-4, figure 1). Different combinations of MPT64 antigen and adjuvants were tested, and an increasing number of screening steps in IHC were added in subsequent experiments. Antibodies were screened in parallel in indirect enzyme-linked immunosorbent assay (ELISA) by the commercial companies, and in IHC at our laboratory, to identify the hybridomas that produced MPT64-specific antibodies. Antibody performance in IHC was assessed in positive and negative control tissue sections using serial dilution to find the optimal working dilution, by several readers (T.M. and I.M.H.). Several antigen retrieval methods were tested to optimise the MPT64 test protocol for murine monoclonal antibodies (Supplementary text). Figure 2 provides an overview of the different stages of hybridoma production and target points for screening of clones for monoclonal antibodies.

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127 **Figure 1. Overview of the different strategies used to develop monoclonal and polyclonal anti-MPT64 antibodies.**
128 Abbreviations: d, day; IFA, incomplete Freund adjuvant, IHC, immunohistochemistry; mod, moderate; NSS, non-
129 specific staining; SS, specific staining; rMPT64, recombinant MPT64.

130

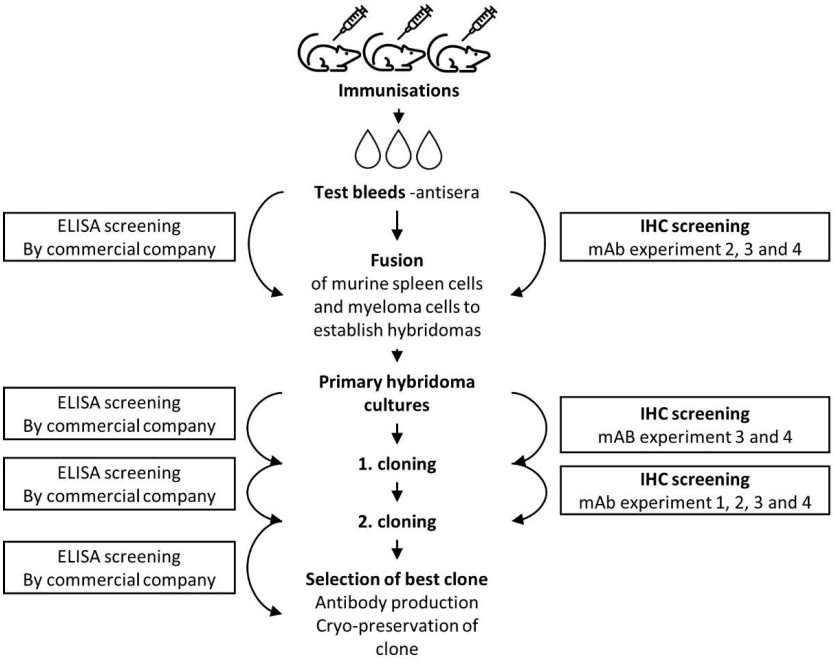


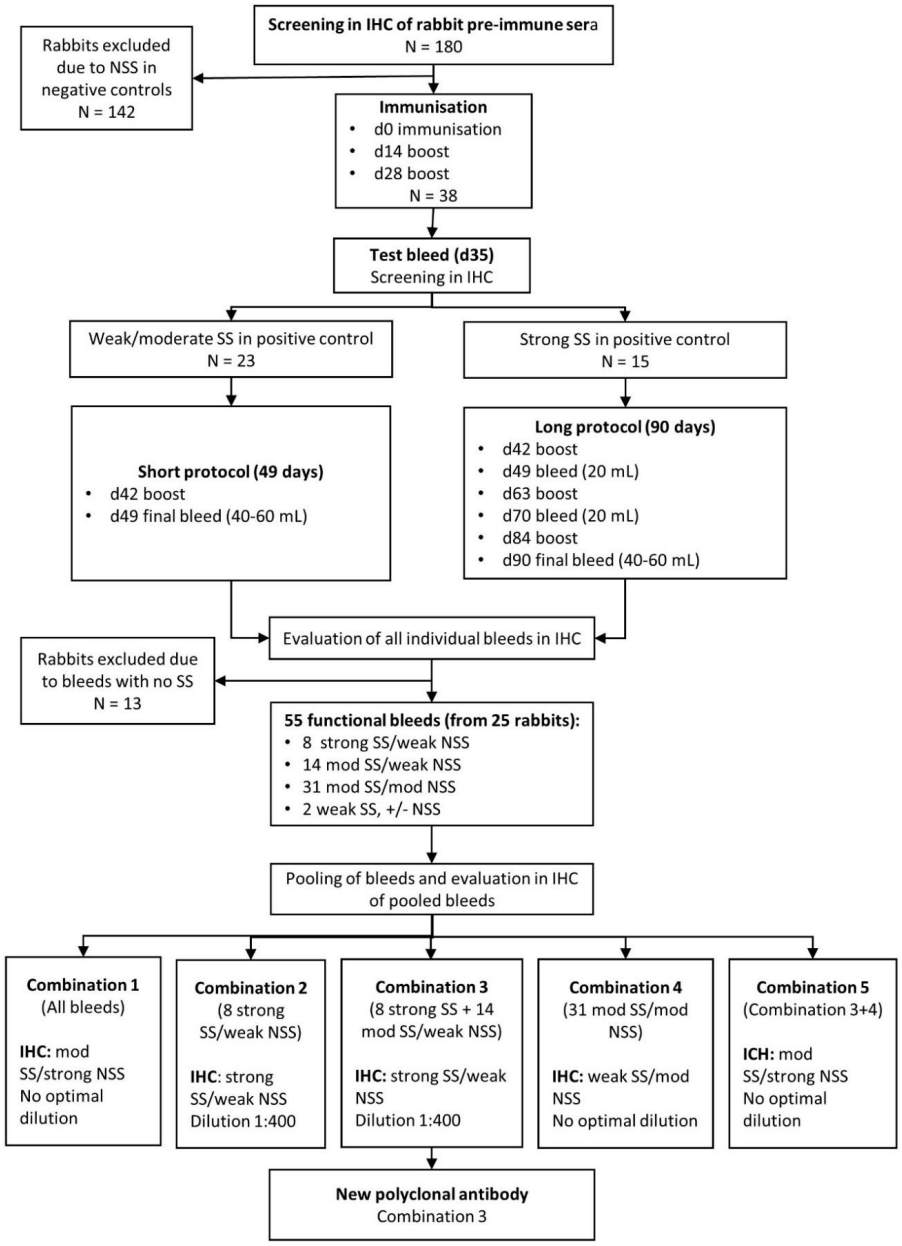

**Figure 2. The steps of hybridoma production and different target points for screening of clones during development of monoclonal antibodies.** Abbreviations: ELISA, enzyme-linked immunosorbent assay, IHC, immunohistochemistry; mAb, monoclonal antibody.

*Development of polyclonal rabbit anti-MPT64 antibodies*

Immunisations

Immunisation of rabbits to produce polyclonal anti-MPT64 antibodies was performed by commercial companies (Biogenes, PharmAbs and InVivo). Figure 1 shows the four different strategies for development of polyclonal MPT64-antibodies that were investigated (pAb experiment 1-4). In experiments 1-2, the original protocol for development of the reference anti-MPT64 antibody was followed (29), with the exception that the antigen was not immunoprecipitated with polyclonal rabbit anti-MPT64 antibodies before immunisation. In brief, outbred female rabbits were immunised intradermally with native MPT64 emulsified in incomplete Freund adjuvant (IFA) using a standard immunisation protocol. In later experiments, recombinant MPT64 and other adjuvants were also tested, rabbits whose pre-immune sera gave non-specific staining were excluded from immunisation, and the immunisation protocols were longer for rabbits whose antisera tests bleeds gave particularly strong specific staining (figure 3). In all experiments, pre-immune sera were collected at baseline, antisera test bleeds were collected

seven days after the second or third immunisation, and the final bleed was taken seven days after the last immunisation. Pre-immune sera and all individual bleeds were tested by indirect ELISA by the companies, and in IHC in our laboratory on positive and negative control tissue sections. The staining in IHC was evaluated by several readers (T.M., I.A.M.A. and I.M.H.). The optimal working dilution was determined using serial dilution.

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160 **Figure 3. Flow chart showing immunisation protocols, screening and pooling strategies used to develop the new**
161 **polyclonal antibody (polyclonal antibody experiment 4).** Abbreviations: d, day; IHC, immunohistochemistry; mod,
162 moderate; NSS, non-specific staining; SS, specific staining.

Selection and pooling of MPT64 specific antibodies

The performance of the different bleeds in IHC were evaluated according to previously developed guidelines for interpretation of the MPT64 test (16). The antibodies were categorised as 1) strong specific staining (SS) and low non-specific staining (NSS), 2) moderate SS and low NSS, 3) moderate to strong SS and moderate to strong NSS, and 4) not functional, defined as no or weak SS and various degrees of NSS. In order to reduce batch-to-batch variation and to enable large-scale use of the polyclonal antibodies, the bleeds were pooled. Different combinations of pooled bleeds were tested in IHC (figure 3), and the combination of bleeds with the best sensitivity and specificity in IHC, hereafter referred to as the new polyclonal antibody, was chosen for further experiments.

*Background blocking and antibody absorption experiments*

Blocking experiments were performed to reduce the non-specific binding of the new polyclonal antibody. Before application of the MPT64-antibody, the tissue sections were incubated with blocking solutions containing either 1) bovine serum albumin and serum free protein block with casein (Dako, Agilent), or 2) normal goat serum, or 3) recombinant Fc domain protein (Hu Fc block pure, BD, Becton Dickinson). Table 2 provides an overview of the different dilutions, incubation times and combinations of blocking solutions that were tested. Experiments with negative absorption were carried out at our laboratory by mixing the new polyclonal antibody with different proteinaceous solutions to allow non-specific antibodies or antibodies with cross-reactivity to bind proteins in the solutions and precipitate (Supplementary text).

**Table 2. Strategies employed to reduce non-specific staining in immunohistochemistry with the new polyclonal antibody.**

| | Level of non-specific staining | |
|---|---|---|
| **Strategy** | **Positive TB control** | **Negative non-TB control** |
| **Blocking experiments** | | |
| Serum free block (12 min, 30 min, 60 min, or overnight) | - | - |
| BSA 3% or 10% and NGS 10% (60 min), followed by serum free block (60 min) | -/↓ | -/↓ |
| BSA 3% or 10% and NGS 10% (overnight), followed by serum free block (12 min) | ↓↓ | ↓↓ |
| Fc block | - | - |
| **Absorption experiments** | | |
| *M. bovis* BCG Copenhagen, culture filtrates | -/↓ | -/↓ |
| *M. bovis* BCG Copenhagen, cell sonicate | - | - |
| Homogenised non-TB lung and lymph node tissue sections (deparaffinised and hydrated) | - | ↑ |

Abbreviations: TB, tuberculosis; BSA, bovine serum albumin; NGS, normal goat serum; NSS, non-specific staining; SS, specific staining; *M. bovis*, *mycobacterium bovis*; (-), no change; (↑), increased; (↓), decreased.

*Immunohistochemistry (the MPT64 test)*

The MPT64 test was performed using the Dako Envision + System-HRP kit (Agilent, Santa Clara, CA), according to the manufacturer's protocol with some modifications. Briefly, 4 μm thick tissue sections on Superfrost Plus slides (Thermo Fisher Scientific, MA) were deparaffinized with xylene and rehydrated through decreasing grades of alcohol. When rabbit antibodies were used as primary antibody, heat induced antigen retrieval (HIER) was performed by microwave boiling the sections in citrate buffer at pH 9, for 20 minutes. For murine antibodies, HIER was performed by pressure cooker boiling at 125°C in TE-buffer at pH 9, for 1 min. The sections were left to cool for 20 minutes at RT, washed in distilled water for 10 minutes and incubated with peroxidase block for 20 minutes. For IHC with the new polyclonal antibody, an additional protein blocking step was then added to the protocol, in which a combination of 10% normal goat serum and 3% bovine serum albumin was applied to the sections overnight at 4°C, followed by serum-free protein block (Dako) for 12 minutes at RT the next day. The primary antibody was applied, and the slides were incubated for 60 minutes, before horseradish peroxidase conjugated secondary anti-rabbit antibody was applied for 45 minutes. Thereafter, the substrate (3-amino-9-ethylcarbazol) was added to the slides for 15 minutes, followed by counterstaining with Mayer's haematoxylin and mounting with Immu-Mount (Thermo Fisher Scientific, United States).

Slides were washed with wash buffer (0.05 mol/L Tris/HCl buffered saline with 0.05% Tween 20, pH 7.6) between all incubation steps. In each IHC run, tissue sections from known TB and non-TB cases were added as controls. In addition, the primary antibody was substituted with antibody diluent on one non-TB tissue section, to assess any non-specific binding of the secondary antibody or other reagents during IHC.

*Validation of the new polyclonal antibodies*

Human clinical samples were used for the validation of the new anti-MPT64 polyclonal antibody. Twenty extrapulmonary biopsies from TB cases with a confirmed (culture and/or Xpert MTB/RIF positive) or clinical TB diagnosis (defined as patients with presumptive extrapulmonary TB, and histology suggestive of TB, and response to treatment, as assessed by the primary investigator) were used. These materials were collected as part of another project where the clinical samples were obtained from a cohort of EPTB patients (30). Twenty-four non-TB samples with histopathological diagnoses other than TB were used as controls. The immunostaining was screened at a total magnification of 200x and evaluated in detail at 400x by one designated reader (S.I.) according to previously developed guidelines for interpretation of the MPT64 test (16). Briefly, a sample was positive if a minimum of two granular red-brown coloured spots, either observed intracytoplasmic in inflammatory cells or extracellularly in necrotic material, were present in the sample. No staining, nuclear staining or extracellular granular staining in non-necrotic areas were interpreted as negative.

*Statistical methods*

The sensitivity and specificity of the new polyclonal antibody were calculated using 2x2 cross-tabulation against a reference standard that included culture, Xpert MTB/RIF and clinical TB diagnosis.

## 3. Results

### *3.1. Production of MPT64 antigen*

During a period of one year, 156 litres of BCG Moreau culture filtrates were produced and subsequently purified by chromatography (figure S2), resulting in a yield of approximately 15 mg MPT64 protein with a purity of > 90%, based on visual evaluation of Coomassie stained SDS-PAGE gels (figure S3). Untagged recombinant MPT64 was expressed in *M. smegmatis* at our laboratory, and the presence of MPT64 in the cultures was confirmed by positive MGIT TBc identification test. No band of the expected size of MPT64 was found on Coomassie stained SDS-PAGE gel, neither from the concentrated culture filtrate nor cell lysate solution, but both solutions gave a band of the right size in Western blot. This indicated that soluble MPT64 had been expressed, but in low quantities. Purified HIS-tagged recombinant MPT64 protein from *E. coli* (Trenzyme) and mammalian HEK-cells (InVivo) were provided from commercial companies.

### *3.2. Development of monoclonal MPT64 antibody*

Figure 1 provides the results from the four strategies that were tested to develop a monoclonal anti-MPT64 antibody. Monoclonal antibodies that were functional in ELISA were obtained with all the strategies, but most of these antibodies gave no specific staining in IHC. As ELISA was not suitable to select clones that were functional in IHC, we included earlier and more frequent screening in IHC in the latter experiments (figures 1 and 2). In experiment 2-4, only mice whose antisera showed strong SS in IHC were used for fusion, and we observed that the combination of mammalian recombinant MPT64 and Titer Max Gold adjuvant resulted in particularly good polyclonal antisera in the mice (SS in 9/9 mice), as compared to native MPT64 and IFA (SS in 1/10 mice), or *E. coli* recombinant MPT64 and IFA (SS in 4/9 mice). However, hybridoma cultures from mice with particularly good antisera did not result in more SS on IHC despite very good reactivity on ELISA. The few clones that gave possible SS with IHC, also displayed cross-reactivity, and development of a monoclonal antibody was not further pursued after the fourth experiment.

### *3.3. Development of a polyclonal antibody*

The results from the four strategies used to develop a polyclonal antibody are summarised in figure 1. Based on the results from experiment 1-2 where non-specific staining and weak specific staining were observed in the majority of antibodies, the strategy of screening was modified in experiment 3-4 (figures 1 and 4). To minimize non-specific binding, we adopted a strategy of only selecting the rabbits whose pre-immune sera showed minimal or no staining in IHC for further immunisations. Additionally, the adjuvant was changed to Titer Max Gold together with mammalian recombinant MPT64 as antigen in experiment 4, as particularly good polyclonal antisera had been obtained with this combination in mice during development of monoclonal antibodies. Further, the rabbits whose antisera gave particularly strong specific staining in IHC in experiment 4, were selected for a longer immunisation protocol (90 days) to generate larger volumes of antisera (figure 3). Using this strategy, a total of 38 rabbits were immunised in experiment 4 (whereas 142 rabbits were excluded after screening of pre-immune sera and released for other projects within the company), resulting in 55 bleeds from 25 animals that were functional in IHC (figure 3). These bleeds were further pooled in different combinations (combination 1-5), to increase the total antibody volume and reduce batch-to-batch variation. Combination 2 and 3 gave the best results in IHC, both showing strong SS that was comparable to the staining from the reference antibody in the positive control, and weak NSS. Combination 3 was chosen as the new polyclonal antibody because of the larger volume as compared to combination 2, making it suitable for future large-scale use. Among the various strategies employed to reduce NSS in the new polyclonal antibody, blocking with bovine serum albumin 3% and normal goat serum 10% overnight, followed by serum-free block for 12 min gave the best results with clearly reduced NSS (table 2). This blocking step was incorporated into the MPT64 test protocol for the new polyclonal antibody. Negative absorption of the new antibody with different proteinaceous solutions, including *M. bovis* BCG Copenhagen components, did not reduce NSS.

### *3.4. Validation of the new polyclonal antibody*

Validation of the new polyclonal antibody was performed on human clinical extrapulmonary specimens, including 20 biopsies from confirmed or clinically diagnosed TB cases and 24 biopsies from non-TB cases. In the TB samples, the sensitivity of ZN, Xpert, Culture and the new polyclonal MPT64 antibody was 11% (1/9), 67% (6/9), 40% (6/15) and 95% (19/20), respectively (table 3). The new polyclonal antibody was positive in all the culture and/or Xpert positive TB samples (n = 12), and positive in 7/8 samples from clinically diagnosed TB cases. Non-specific staining was observed in 4/24 non-TB samples with the new polyclonal antibody, yielding an overall specificity of 83%.

**Table 3. Results of routine diagnostic tests and the MPT64 test performed on clinical TB and non-TB samples.**

| | | **Routine diagnostic tests** Positive/total (%) | | | **The MPT64 test** Positive/total (%) |
|---|---|---|---|---|---|
| | n= | **Ziehl-Neelsen** | **Xpert MTB/RIF** | **Culture LJ** | **New polyclonal MPT64 antibody** |
| **TB cases total** | **20** | 1/9 (11) | 6/9 (67) | 6/15 (40) | 19/20 (95) |
| Lymph node biopsies | 14 | 1/6 (17) | 3/4 (75) | 6/12 (50) | 14/14 (100) |
| Other biopsies | 6 | 0/3 (0) | 3/5 (60) | 0/3 (0) | 5/6 (83) |
| Confirmed TB cases | 12 | 0/5 (0) | 6/6 (100) | 6/11 (55) | 12/12 (100) |
| Clinically diagnosed TB cases | 8 | 1/4 (25) | 0/4 (0) | 0/3 (0) | 7/8 (88) |
| **Non-TB cases total** | **24** | N/A | N/A | N/A | 4/24 (17) |
| Lymph node biopsies | 7 | N/A | N/A | N/A | 2/7 (29) |
| Other biopsies | 17 | N/A | N/A | N/A | 2/17 (12) |

Abbreviations: LJ, Lowenstein Jensen; TB, tuberculosis.

## 4. Discussion

In this study we have investigated different strategies to develop a new functional antibody for use in the TB diagnostic MPT64 test, which has shown promising results for diagnosing EPTB in low-resource settings (11-17). The test uses an in-house polyclonal antibody for detection of the mycobacterial antigen MPT64, but the antibody is in limited supply and further large-scale use of the test requires reproduction of the antibody. Despite generation of several monoclonal anti-MPT64 antibodies with good reactivity in ELISA, none of the antibodies were fully functional in IHC. We therefore opted for development of polyclonal antibody. By careful selection of animals for

immunisation, optimal antigen-adjuvant combination, screening of antibodies in IHC and pooling of the best performing individual antibodies, generation of a sensitive new polyclonal antibody in a large volume was achieved. The new antibody was more sensitive than routine microscopy, Xpert and culture when validated on a small number of clinical samples, albeit a reduced specificity warrants more work with background reduction.

The choice of antigen and adjuvant can greatly affect the performance of the resulting antibody (31-34). We used native MPT64 as antigen in the initial experiments because the conformational epitopes, which may be important targets in IHC (35), are conserved in native proteins. However, as the resulting antibodies were neither sensitive nor specific in IHC, and the production of native MPT64 was time-consuming, we changed to recombinant MPT64 in the latter experiments. Recombinant protein expression allows for rapid production but may alter conformational epitopes depending on the choice of host system. Recombinant MPT64 from *E. coli* has been reported to elicit weaker immune responses than native MPT64 and recombinant MPT64 from *M. smegmatis* (36), possibly due to different post-translational modification systems (37-43), suggesting that important MPT64 epitopes can be affected by the host system. Despite this possible drawback, immunisation of mice with recombinant MPT64 from both *E. coli* and mammalian cells resulted in polyclonal antisera with as strong, or stronger, specific staining in IHC as compared to immunisation with native MPT64 (strong specific staining was observed in 9/9, 4/9 and 1/10 murine antisera with mammalian MPT64, *E. coli* MPT64 and native MPT64 as antigen, respectively). Several adjuvants, which can enhance and prolong the immune response (33, 34), were also tested in the study (fig 2). Based on the particularly strong specific staining obtained after immunisation of mice with mammalian recombinant MPT64 and Titer Max Gold adjuvant, we chose to change to this antigen-adjuvant combination in rabbits as well. This resulted in some of the best individual antisera in our study, with almost as strong specific staining as the reference antibody, and low non-specific staining.

The main reason for choosing monoclonal antibodies in a diagnostic test is to avoid batch-to-batch variability, thereby securing reproducible test performance. Still, polyclonal antibodies offer some important advantages (19). Development of polyclonal antibodies is relatively simple, fast and inexpensive and can results in highly sensitive antibodies because several epitopes are recognised simultaneously, leading to efficient signal amplification and improved detection. High test sensitivity is of great importance in TB diagnostics, as the sensitivity of routine TB diagnostic tests is low in paucibacillary EPTB disease (44). Further, the biological diversity of polyclonal antibodies allows for use under a wide range of chemical conditions and temperatures, which is an advantage in low-resource settings. Thus, as long as batch-to-batch consistency is managed through standardised validation of all new batches, or through creation of a large batch as in this study, polyclonal antibodies may be used, and are being used, for diagnostic purposes (45-47). Still, cross-reactivity is a common issue with polyclonal antibodies, and antibody purification or background blocking is often required. Surprisingly, negative absorption with BCG Copenhagen culture filtrate proteins, which successfully reduced non-specific staining in the reference antibody, had no effect on the new polyclonal antibody. We

experienced that a combined strategy of careful selection of animals for immunisation and optimised background blocking prior to IHC were the most effective measures to reduce non-specific staining. The specificity of the new antibody is still not optimal, but ongoing work indicates that increased duration of the washing steps in IHC removes the non-specific staining without reducing the specific staining of the antibody. This will be further explored in an up-coming validation study.

The development of monoclonal antibodies for use in IHC can be challenging, as demonstrated in our study. The screening of hybridoma cultures in ELISA was not an optimal method to select clones that are functional in IHC. Clones with high performance in ELISA may not detect relevant epitopes in IHC, because formalin fixation prior to IHC can mask or alter the three-dimensional conformation of the epitopes that were exposed during ELISA (48). Antigen retrieval partly reverses these alterations, but the effect varies between epitopes, and antibody screening should therefore preferably be performed directly in IHC. This was done in the latter experiments but is more time-consuming than ELISA and requires large numbers of positive control tissue sections. In the latter experiments, antisera from several immunised mice gave relatively strong specific staining in IHC, but the resulting few monoclonal antibodies that showed some specific staining also displayed cross-reactivity, indicating that their target epitopes were not unique to the MPT64 protein. We may have lost clones that recognised unique MPT64 epitopes during fusion or hybridoma selection, especially in the initial experiments where IHC was not used for screening. Another possible explanation is that our choice of antigen was not optimal to generate antibodies against unique epitopes (49). If the less unique epitopes are immunodominant, most of the antibodies in the mice will be generated against these, whereas less immunogenic, but unique epitopes could be missed by the immune system. To avoid this, so-called subtractive immunisation techniques can be applied, in which the undesirable antibodies are used to mask their epitopes on the antigen before immunisation, so that antibodies are only generated against other epitopes (49). This remains to be explored during further development of monoclonal MPT64-antibodies for IHC.

## 5. Conclusions

Reproduction of a functional polyclonal MPT64 antibody for large-scale use of the TB diagnostic MPT64 test was achieved through a combination of careful selection of antigen-adjuvant for the immunisation protocol, comprehensive screening in IHC of both pre-immune sera and antisera to find the best performing antibodies, followed by pooling the best individual antisera to obtain a large volume of polyclonal antibodies with stable performance, thereby securing stable supplies and reproducibility of the MPT64 test. Further validation of the new polyclonal antibody in clinically relevant larger populations remains to be done.

**Supplementary Materials:** Supplementary text: Detailed protocols for production and purification of MPT64 antigen, development of antibodies, and immunohistochemistry background reduction, Figure S1: The completed vector construct, pUV15tetORmMpt64, expressing MPT64 protein, Figure S2: Chromatograms from the three-step chromatography purification strategy applied to

purify native MPT64 protein, Figure S3: Overview of purity of the MPT64 containing fractions after each step of chromatography.

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**Institutional Review Board Statement:** The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the National Bioethics Committee of Pakistan (Islamabad, Pakistan), and the Regional Committee for Medical and Health Research Ethics of Western Norway (REK vest) (2014/46/REK vest). All animal experiments were performed by commercial companies that are certified according to ISO 9001. The animal keeping and corresponding works were performed according to German/Belgian country specific, European and US NIH/OLAW guidelines.

**Informed Consent Statement:** A written informed consent was obtained from all subjects involved in the study.

**Data Availability Statement:** The data presented in this study are available on request from the corresponding author.

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# Supplementary materials to manuscript "Immunochemistry-based diagnosis of extrapulmonary tuberculosis: A strategy for large-scale production of MPT64-antibodies for use in the MPT64 antigen detection test"

## Supplementary text

### Detailed protocols for production and purification of MPT64 antigen, development of antibodies, and immunohistochemistry background reduction

#### Production of native MPT64 protein

The Brazilian BCG vaccine strain *M. bovis* bacillus Calmette-Guérin Moreau (BCG Moreau) was used for production of endogenous, native MPB64 protein. Briefly, lyophilised BCG cells were suspended in isotone saline and inoculated onto Lowenstein-Jensen slants and subsequently onto wholly synthetic Sauton's liquid medium to be grown as surface pellicles at 37°C without shaking. The cultures were harvested after three weeks, as the peak concentration of secreted MPT64 protein from *in vitro* grown *M. tuberculosis* is obtained at this time point (20). The bacilli were removed with paper filter from the culture filtrates which were sterile filtered (0.2 µm) before further use.

#### Purification of native MPT64 protein

The methods for protein purification were based on previous protocols (21, 22), with some modifications. To concentrate the proteins in the culture filtrates, solid ammonium sulphate was added to 80% saturation (533 g/L at 4°C). Proteins were left to precipitate overnight, followed by centrifugation at 10,000 x *g* at 4°C for 45 minutes. The protein pellet was resuspended in phosphate-buffered saline (PBS) and dialysed against PBS. Protein concentration was measured on Direct Detect Spectrometer (Merck Millipore, MA, USA).

In order to purify the untagged, native MPT64 protein (nMPT64) to the required level of purity for antibody production (80-90%), a three-step chromatography strategy, carried out under native conditions, including ion-exchange chromatography (IEX), hydrophobic interaction chromatography (HIC) and size exclusion chromatography (SEC), was used. All purification steps were performed on fast liquid protein chromatography (FPLC) machines (Äkta Explorer or

Purifier) at room temperature (RT), using filtered (0.45 µm) and degassed buffers and protein samples. The pH of all buffers was adjusted at RT.

Before the first step IEX, the protein concentrate was dialysed against IEX binding buffer (20 mM Tris-HCl, pH 8.3), and loaded onto a Q Sepharose Fast Flow XK 50/20 column that was subsequently washed with binding buffer until unbound proteins were removed. For elution, a linear gradient of 0 to 0.3 M NaCl in 20 mM Tris-HCl, pH 8.3, was applied, and 10 mL fractions of the eluate were consecutively collected. Between each chromatography step the collected fractions were subjected to SDS-PAGE under non-reducing conditions and Western blot with polyclonal rabbit anti-MPT64 antibody to assess purity and identify MPT64. MPT64 positive fractions were pooled and concentrated by ultrafiltration (Amicon Ultra spin-filter, molecular weight cut-off 3000, Merck, Germany) at 3200 x *g* for 25 minutes at RT. To prepare the sample for the intermediate HIC step, the sample buffer was exchanged to HIC binding buffer (20 mM Tris-HCl, 1.7 M ammonium sulphate, pH 7.5) by adding HIC binding buffer to the concentrated protein sample in the spin-filter, followed by ultrafiltration. This procedure was repeated twice. The protein sample was then loaded onto a Phenyl HP HiTrap column (1 mL bed volume) in HIC binding buffer. The column was washed with binding buffer until unbound proteins were removed, followed by elution of bound proteins by applying a linear gradient of 0-100% elution buffer (20 mM Tris-HCl, pH 7.5). 2 mL fractions of the eluate were collected during the run, and the fractions containing MPT64 were further pooled. Concentration and buffer exchange to SEC buffer (20 mM Tris-HCl, 0.3 M NaCl, pH 7.5) of the pooled protein sample were carried out by ultrafiltration as described above. For the final SEC polishing step, the protein sample was loaded onto a Superdex 75pg HiLoad 26/600 column in SEC buffer, separated according to molecular size as the sample passed through the medium over a volume of 400 mL SEC buffer at a flow rate of 2,5 mL/min, and collected as 5 mL fractions. The level of purity in the fractions containing MPT64 was visually evaluated in Coomassie stained SDS-PAGE gels. Fractions with a purity of approximately >90% were selected for later use as antigen. Endotoxin levels were measured (Pierce LAL chromogenic endotoxin quantitation kit, ThermoFisher Scientific) before and after endotoxin affinity purification (Pierce high capacity endotoxin removal spin columns, ThermoFisher Scientific). Protein concentration was measured by Direct Detect. The samples were stored at -80°C.

**Production of recombinant MPT64 protein**

**Mycobacterial vector system**

All laboratory kits were used according to the manufacturer's recommended protocols, unless otherwise stated. Genomic DNA from BCG Moreau was prepared using FastDNA SPIN Kit (MP Biomedicals, OH, USA). An 888 bp segment of genomic DNA containing the mpb64 gene with its

predicted secretion signal sequence (GenBank Accession No. AM412059.2; BCGM locus 1981c), was amplified using KAPA HiFi HotStart Readymix PCR kit (Roche) and the primers mpt64UP1 5'-ATCGCGGCAATCCAATCTCC-3' and mpt64LP1 3'-TCTCTAGCGACGATTCTTGAGC-5' (Initial denaturation 3 min 95°C. 30 amplification cycles: 20 sec 98°C, 15 sec 63°C, 20 sec 72°C. Final extension 1 min 72°C). Following purification with UltraClean 15 DNA Purification kit (MO BIO Laboratories), the PCR product was used as template to amplify an 853 bp segment containing the mpt64 coding region with PacI and PstI restriction sites, using the primers mpt64adaptUP1 5'-GCTTAATTAACTACTCCCGGAGGAA-3' and mpt64LP1 3'-TCTCTAGCGACGATTCTTGAGC-5' with the same PCR and purification protocols. The purified PCR product and the mycobacterial inducible plasmid pUV15tetORm were separately digested with PacI and PstI (New England BioLabs, MA, USA) and purified. Cloning was performed by incubating the plasmid backbone and insert (insert:backbone ratio 5:1), T4 DNA ligase and T4 DNA Ligase Buffer (Invitrogen) at 4°C overnight. Correct insertion was controlled by sequencing. Figure S1 shows the final plasmid construct and table 1 shows the predicted amino acid sequence of the expressed protein. Chemically competent E. coli cells (One Shot TOP10, ThermoFisher Scientific, MA, USA) were transformed with the plasmid by heat shock, plated on selective Luria Bertani (LB) agar, expanded in selective liquid LB medium (both containing 200 mg/L hygromycin (ThermoFisher Scientific)) and purified using QIAprep Spin Miniprep Kit (QIAGEN, Netherlands).

Electrocompetent M. smegmatis $mc^2$ 155 was cultured in Middlebrook 7H9 supplemented with OADC, 0.05% Tween-80 and 0.2% glycerol, and prepared for transformation in glycerol as described by Goude and colleagues (26). The pUV15tetORmMpt64 plasmid was transformed into M. smegmatis $mc^2$ 155 by electroporation (Bio-Rad Gene Pulse II) in a 0.2 cm gap electroporation cuvette subjected to one single pulse of 2,5kV, 25µF, with the pulse-controller resistance set at 1000Ω. After 3 hours cell recovery in 7H9 without antibiotics, the cells were harvested by centrifugation at 3000 x g for 10 minutes and plated out in suitable dilutions on

Middlebrook 7H10 agar plus 50 mg/L hygromycin. At day 3, transformant colonies were used to inoculate 5 mL 7H9 plus 50 µg/mL hygromycin and incubated on a shaker (100 rpm) at 37°C to reach logarithmic phase ($OD_{600}$ 0.8-1). The culture was then inoculated into 200 mL 7H9 plus 50µg/mL hygromycin and 200ng/mL anhydrotetracycline, to induce expression of rMPT64 (27), and was incubated on a shaker (100 rpm) at 37°C for 3 days. Expression of rMPT64 was confirmed by subjecting 10µL of the culture diluted in 90 µL 7H9, to the MGIT TBc identification test (Becton Dickinson, NJ, USA), a rapid lateral-flow immunochromatographic assay normally used for detection of MPT64 antigen in MTBC cultures.

To assess if soluble rMPT64 had been expressed, the protein content in culture filtrates and cell sonicates were analysed. Culture filtrate was prepared from half of the culture as previously described. The other half was centrifuged at 3000 x *g* for 10 min at 4°C to pellet out bacterial cells. The bacteria were dissolved in PBS and probe sonicated in bursts of 30 sec over 10

minutes in a rosette cooling cell in an ice bath. The resulting cell lysate was centrifugated at 3000 x *g* for 10 min at 4°C and the supernatant was sterile filtered. Solid ammonium sulphate was added to 80% saturation (533 g/L at 4°C) to the culture filtrate and cell lysate supernatant and gently mixed by stirring. Proteins were left to precipitate overnight, followed by centrifugation at 10,000 x *g* at 4°C for 45 minutes. Each protein pellet was resuspended in 2 mL PBS, and 10 µL of the solution was analysed by SDS-PAGE and Western blot with polyclonal rabbit anti-MPT64 antibody to identify any rMPT64 protein bands.

To avoid protein contamination of the antigen from the culture medium, transformant colonies were also cultured in protein-free, wholly synthetic Sauton's medium instead of 7H9, otherwise using the same protocol as described above.

**E. coli vector system**

A recombinant MPT64-His protein was produced in an *E. coli* expression system (E. coli rMPT64) by Trenzyme Life Science Services (Konstanz, Germany). The E. coli rMPT64 was designed based on the MPT64 amino acid sequence from *M. bovis* BCG Moreau without the signal sequence and with a C-terminal 8xHis-tag (table 1). Cloning, transformation of *E. coli* and expression of the protein was performed according to standard protocols. The rMPT64 was purified under non-denaturing conditions using affinity chromatography, followed by dialysis against tris-buffered saline (TBS), pH 7.5. Purity (> 98%) was measured by densitometry of Coomassie stained SDS-PAGE gel. Endotoxin content was measured by LAL before and after endotoxin removal by affinity purification. The protein was stored at -80°C.

**Mammalian cell line vector system**

A recombinant MPT64 protein expressed in a human cell line (mammalian rMPT64) was produced by InVivo Biotech Services (Berlin, Germany). The mammalian rMPT64 was made based on the MPT64 amino acid sequence from *M. bovis* BCG Moreau without the signal sequence, with an HSA signal peptide at the N-terminal and a 6xHis-tag at the C-terminal (table 1) using an expression vector that was expanded in E. coli and then transfected into HEK cells, a cell line derived from human embryonic kidney cells, to express recombinant protein. Affinity chromatography was used to purify the rMPT64, followed by dialysis against TBS, pH 7.4. A purity of >90% was obtained, as determined by analysis of Coomassie-stained SDS-PAGE. Endotoxin levels were measured by LAL. The protein was stored at 2-8° C.

**Development of a monoclonal and polyclonal anti-MPT64 antibody**

**Antigen retrieval experiments**

Tissue sections were subjected to several antigen retrieval protocols prior to IHC to optimise the MPT64 test protocol for murine monoclonal antibodies. After deparaffinisation with xylene and

rehydration through decreasing grades of alcohol, the positive control tissue section were treated with 1) proteinase K digestion for 5 min, 2) heat induced epitope retrieval (HIER) in microwave oven in citrate buffer, pH 9, 20 min, 3) HIER in pressure cooker at 125°C in Tris-EDTA buffer, pH 9, for 1 min, and 4) no retrieval. The method that resulted in the strongest specific staining was used in further IHC experiments with monoclonal antibodies.

**Background blocking experiments**

In addition to the blocking of endogenous peroxidase, which is part of the original MPT64 test protocol, further blocking experiments were performed to reduce non-specific binding to tissue components when the new polyclonal antibody was used. Before application of the primary antibody, the tissue sections were incubated with different blocking solutions containing 1) proteins that bind readily to non-specific sites, including bovine serum albumin (BSA) and serum free protein block with casein (Dako, Agilent), 2) normal goat serum (NGS), in which antibodies in non-immune serum bind non-specific sites in the tissue, and 3) recombinant Fc domain protein (Hu Fc block pure, BD, Becton Dickinson) to block Fc receptors on immune cells in the tissue sections which could otherwise bind the Fc domain of the primary antibodies. Table 2 provides an overview of the different dilutions, incubation times and combinations of blocking solutions that were tested.

**Absorption experiments**

Experiments with negative absorption were carried out at our laboratory by mixing the new polyclonal antibody with different proteinaceous solutions to allow non-specific antibodies or antibodies with cross-reactivity to bind proteins in the solutions and precipitate. The new polyclonal antibody was mixed with 1) culture filtrates (3 mg/mL) from *M. bovis* BCG Copenhagen, a BCG sub-strain that lacks Region of Difference 2 and therefor does not express MPT64 protein, in ratio 1:1, or 2) BCG Copenhagen cell sonicate and antibody in ratio 1:5, or 3) homogenised non-TB lung and lymph node tissue sections (deparaffinised and hydrated) in PBS in ratio 1:1. The mixture was gently vortexed and left overnight at 4°C. The absorbed antibodies were then carefully pipetted off the precipitated antibody-antigen complexes and tested in IHC.

**Protocol for preparation of homogenised tissue solutions**

Formalin-fixed and paraffin-embedded non-TB lung and lymph node biopsies were selected for absorption experiments because the new polyclonal antibody showed a high degree of non-specific staining in these biopsies. Six 10 µm thick sections from each biopsy were placed in a tube, deparaffinized with xylene and rehydrated through decreasing grades of alcohol. After three minutes in distilled water, the tubes were centrifuged, the water pipetted off and 1 mL PBS was added to each tube. The tissue/PBS mixture was transferred to a lysing matrix tube A (MPbio.com) containing garnet matrix and ¼ ceramic sphere and homogenized for 40 seconds

by a Fastprep-24-5G homogenizer. The lysed tissue mixture was subsequently used directly for absorption experiments as described above.

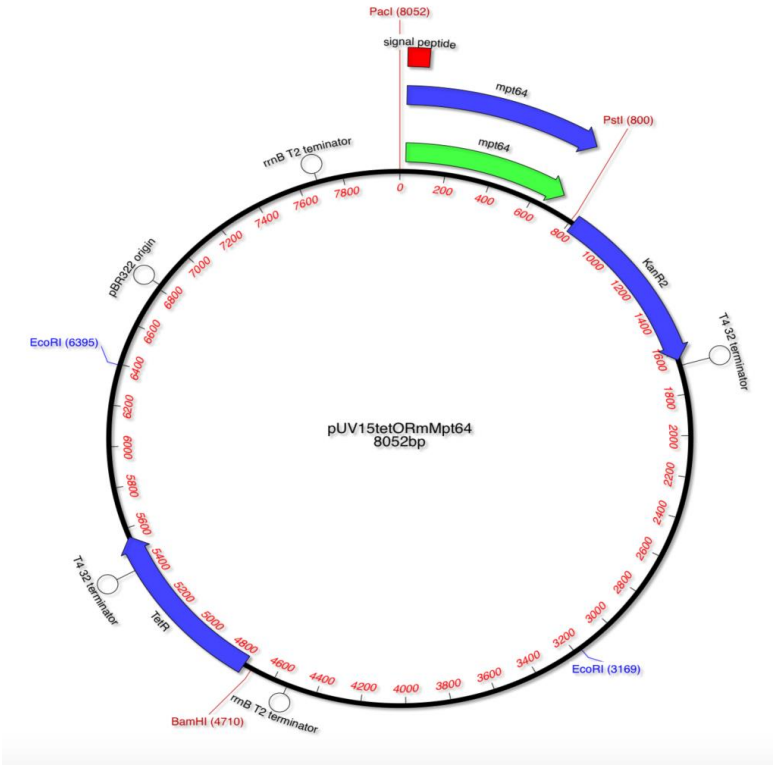


**Figure S1. The completed vector construct, pUV15tetORmMpt64, expressing MPT64 protein.** An 800 bp fragment containing the gene encoding the MPT64 protein, including its secretion signal sequence, was inserted between the PacI and PstI sites of the pUV15tetORm plasmid, a vector stable in *M. smegmatis* and *E. coli*.

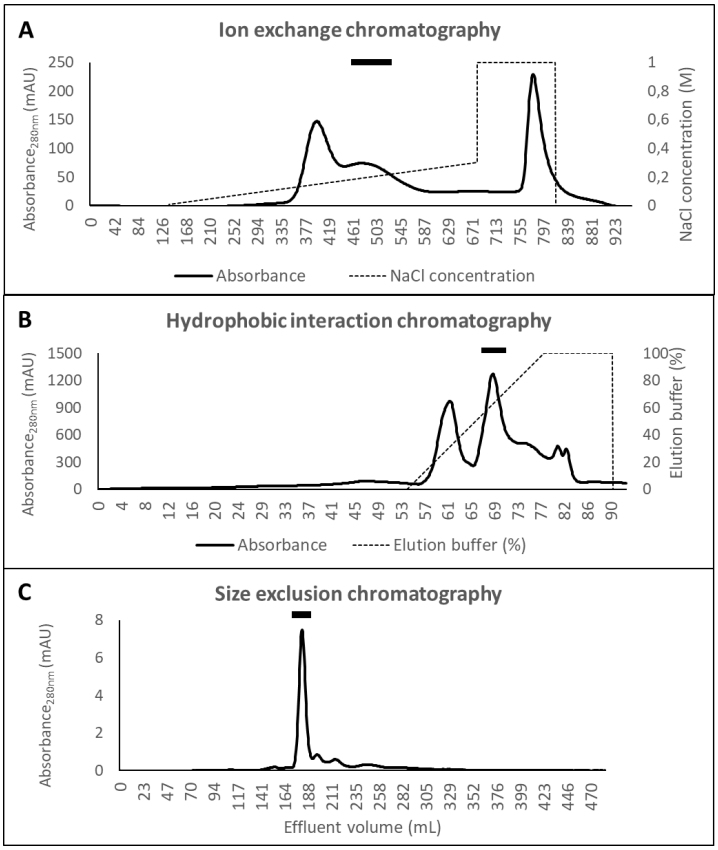


**Figure S2. Chromatograms from the three-step chromatography purification strategy applied to purify native MPT64 protein.** The elution pattern from the first step separation of culture filtrate proteins from M. bovis BCG Moreau by anion exchange chromatography on a Q Sepharose Fast Flow column is shown in (A). Fractions containing MPT64 were pooled and further purified in the intermediate hydrophobic interaction chromatography step on a Phenyl HP HiTrap column (B), before the final polishing step size exclusion chromatography (C) on a Superdex 75pg HiLoad column. Fractions of the eluate were consecutively collected during all runs and subjected to SDS-PAGE and Western blot to identify the MPT64 containing fractions. The bars in the chromatograms indicate the part of the eluate that contained MPT64.

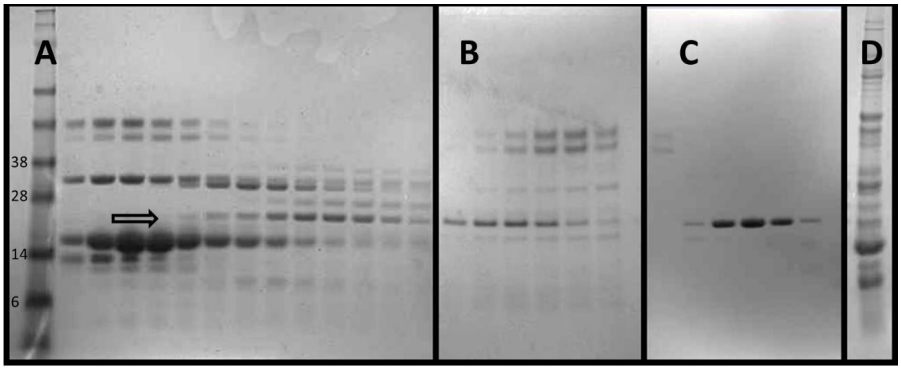


**Figure S3. Overview of purity of the MPT64 containing fractions after each step of chromatography.** The figure shows the purity of the fractions containing MPT64 protein in Coomassie-stained SDS-PAGE after (A) ion exchange chromatography, (B) hydrophobic interaction chromatography and (C) size exclusion chromatography. (D) shows concentrated culture filtrates from M. bovis BCG. Molecular mass is given for the bands in the standard (SeeBlue Plus2, Invitrogen, CA, USA) at the left in (A). The MPT64 band is located at approximately 24 kDa (arrow).

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# Xpert MTB/RIF ultra for rapid diagnosis of extrapulmonary tuberculosis in a high-income low-tuberculosis prevalence setting

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**The diagnosis of extrapulmonary tuberculosis (EPTB) is often challenging due to paucibacillary nature of the disease. Xpert MTB/RIF Ultra (Ultra) has been developed to improve detection of *Mycobacterium tuberculosis* complex (MTC) in paucibacillary specimens. The objective of the study was to assess the performance of Ultra for the diagnosis of EPTB in a high-income low TB prevalence country. Extrapulmonary samples received for TB diagnostics at two hospitals in Norway between January 2015 and January 2016 were prospectively and consecutively included. Defrosted samples were subjected to Ultra. Culture and routine PCR tests were used as reference standard. A total of 82 samples, 10 culture and/or routine PCR positive (confirmed TB) samples and 72 culture and routine PCR negative samples were included in analysis. The overall sensitivity and specificity of Ultra were 90% (9/10, 95% CI 56–100) and 99% (71/72, 95% CI 93–100), respectively. Ultra was positive in 6/7 smear negative confirmed TB samples. To conclude, Ultra showed a high sensitivity and specificity in extrapulmonary specimens and may contribute to a rapid diagnosis of EPTB in a low TB prevalence setting.**

Tuberculosis (TB) is a global health problem[1]. Extrapulmonary TB (EPTB) accounts for approximately 15% of notified TB cases globally[1], whereas as much as 40% of TB cases are extrapulmonary in several high-income countries, including Norway[1,2]. Due to paucibacillary nature of the disease, the diagnosis of EPTB is often challenging. The worldwide roll-out in 2010 of the new PCR-based assay, Xpert MTB/RIF (Xpert; Cepheid, Sunnyvale, CA), represented a breakthrough in TB diagnostics[3]. The rapid and fully automated assay simultaneously detects *Mycobacterium tuberculosis* complex (MTC) species, the causative agents of TB, and rifampicin resistance (RIF-R), and has a high sensitivity for diagnosing pulmonary TB (PTB) in smear positive sputum samples[4]. However, the sensitivity of Xpert in paucibacillary specimens, including smear negative PTB and many forms of EPTB, is limited[4–6]. To improve the performance of Xpert in smear negative samples, an upgraded version of the assay, Xpert MTB/RIF Ultra (Ultra), has been developed[7]. Ultra was launched in 2017 and is recommended by the World Health Organization as a replacement for Xpert in all settings[8]. The increased sensitivity of Ultra is achieved by incorporation of two new PCR assays targeting the multicopy genes *IS6110* and *IS1081* for the diagnosis of TB, a larger DNA reaction chamber and transformation from hemi-nested to fully nested PCR reactions[7]. A number of studies report increased sensitivity of Ultra compared to Xpert in smear negative PTB[9–16], and several studies also show promising results for diagnosing EPTB[11,14,15,17–25]. However, most of the studies that investigate Ultra for diagnosing EPTB have been conducted in low-resource settings with high TB incidence. The aims of the present study were to (1) evaluate the diagnostic accuracy of Ultra for diagnosing EPTB compared to routinely used culture and PCR tests in a clinical setting in the high-income low TB prevalence country Norway[26], and (2) investigate the potential of Ultra as an add-on test to the existing routine tests to improve the rapid diagnosis of EPTB.

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## Methods

**Sample inclusion.** The present study was performed on frozen specimens that had been collected as part of a larger prospective study conducted at Haukeland University Hospital, Bergen, Norway, between January 2015 and June 2016[27]. The study includes 47 specimens that were also included in the larger study, and 39 additional specimens that did not meet the inclusion criteria in the larger study. The eligibility criteria and reference standard were designed prior to Ultra testing. Samples eligible for inclusion were identified at the microbiology laboratories at two regional tertiary care hospitals, Haukeland University hospital and Stavanger University hospital. All consecutive extrapulmonary samples received for TB diagnostics from patients of all ages were included during the study period, provided there was enough material left after routine diagnostics. As the diagnosis of EPTB almost always requires invasive sample collection, which is only performed on symptomatic patients with abnormal tissue masses or fluids, we assumed that the pre-test probability of TB was generally quite high in these samples. One exception was pleural fluid samples, which accounted for a large proportion of the samples, but often had a very low pre-test probability for TB because most of the samples were malignant pleural effusions routinely sent for TB diagnostics before initiation of cancer chemotherapy. Hence, pleural fluid samples were only included and subjected to Ultra if TB was mentioned as a probable differential diagnosis on the request form, whereas all other sample types were included without selection. Multiple samples were taken from some patients, and individual patients were allowed to contribute to the dataset multiple times. Results of routine TB diagnostic tests were obtained from the microbiology laboratory information systems. A microbiological reference standard was used in this study. Culture and/or routine PCR test positive samples were categorised as confirmed TB samples, and culture and routine PCR test negative samples were categorised as non-TB samples. Because information about clinical TB diagnosis was not available for all the samples in the cohort, we could not include a clinical TB diagnosis as part of the reference standard, and any sample included from a clinically diagnosed TB case was therefore categorised as a non-TB sample in this study.

**Sample processing and routine TB diagnostic procedures.** Laboratory personnel at the inclusion hospitals performed routine TB diagnostics according to local diagnostic algorithms. They were blinded to Ultra results. Fine needle aspirates (FNAs) and fluid samples with a volume of < 10 mL were used unconcentrated, whereas samples with a volume of > 10 mL were concentrated by centrifugation (3,800×*g* for 15 min) before resuspension of the sediment in saline. Biopsy specimens were mechanically homogenized and resuspended in saline. The Ziehl–Neelsen (ZN) method was used for detection of acid fast bacilli by smear microscopy. At Haukeland University hospital, a standard NALC-NaOH decontamination procedure was performed if the sample was assumed to be non-sterile, before seeding of appropriate sample volumes in liquid medium (BACTEC MGIT) and solid medium (Lowenstein-Jensen containing glycerol and sodium pyruvate). All lymph node specimens, sterile fluids and aspirates and most biopsies were cultured both before and after NALC-NaOH decontamination, and lymph node specimens were also cultured at 28 °C. At Stavanger University hospital, most extrapulmonary samples were NALC-NaOH decontaminated and only cultured on liquid medium (Bactec MGIT 960; Becton Dickinson, Towson, MD). If the clinician requested PCR, appropriate sample volumes were further used for Cobas Taqman MTB (Roche, Switzerland), Abbott Real Time MTB (Abbot, Des Plaines, IL) or Genotype MTBDR plus (Hain Lifescience, Nehren, Germany), hereafter collectively referred to as routine PCR. Any remaining sample material was stored at − 80 °C for later analysis with Ultra.

**Xpert ultra.** We performed Xpert ultra on the frozen sample material during the autumn 2018, blinded for results of routine TB diagnostics and clinical information. Samples were thawed at room temperature and processed according to the manufacturer's protocol. All but two samples (both volume 0.25 mL) had a sample volume of minimum 0.5 mL. In samples with volume < 0.7 mL (n = 32), sample reagent was added in a 3:1 reagent to sample ratio, whereas a ratio of 2:1 was used for samples with a volume of 0.7 mL or more (n = 54).

**Statistical analysis.** Sensitivity, specificity and accuracy were calculated using culture and routine PCR as the reference standard. A minimum of one valid (positive or negative) culture result and a valid Ultra result were required to include a sample in this analysis. Calculation of 95% confidence intervals for sensitivity and specificity was performed with the exact Clopper-Pearson method.

**Ethical considerations.** The study was approved by the Regional Committee for Medical and Health Research Ethics of Western Norway (REK Vest), (2014/46/REK vest), and carried out in accordance with relevant guidelines and regulations. REK Vest granted an exemption from informed consent from the patients, as the study only included residual material from samples sent for TB diagnostics in a clinical setting.

## Results

Figure 1 provides the study overview. Frozen material was available from a total of 177 samples received for TB diagnostics during the study period, and comprised 21 biopsies, 16 lymph node FNAs, 16 pus samples and 124 fluid samples. Pleural fluid samples accounted for more than half of all the specimens (n = 109), but TB was only mentioned as a probable differential diagnosis on the request form for 18/109 samples. We excluded the 91 pleural fluids with assumed low pre-test probability of TB and analysed the remaining 86 extrapulmonary samples (from 80 cases) with Ultra. All these samples had a minimum of one valid culture result (positive or negative) available. Four samples were excluded from further analysis due to invalid Ultra result (ERROR).

The type and number of routine TB diagnostic tests performed on the analysed samples varied (Table 1). All samples were subjected to culture, 85% to ZN microscopy (n = 70) and 24% to a routine PCR (n = 20). MTC was

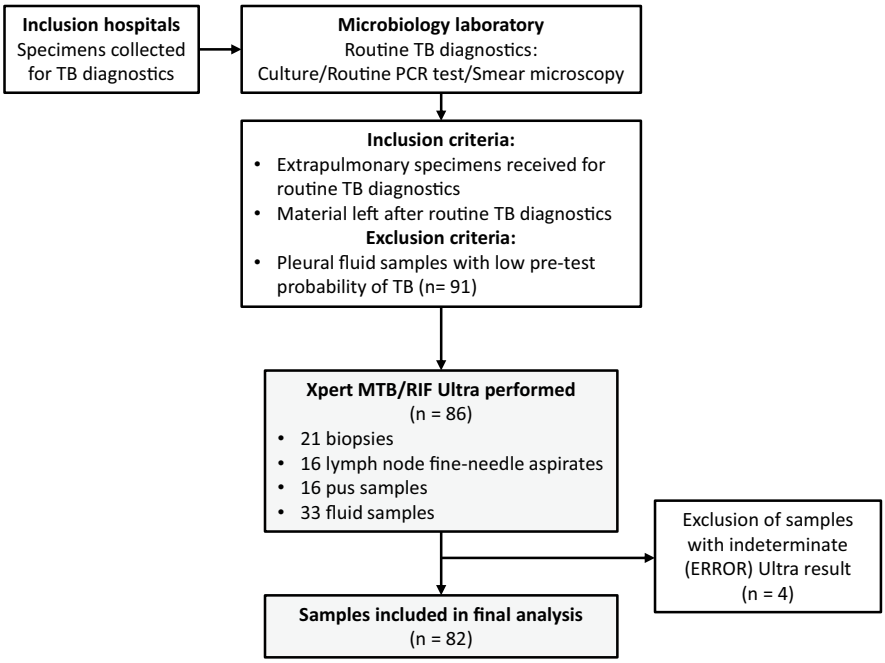


**Figure 1.** Overview of study design. The type of routine PCR test performed varied between the inclusion hospitals, and included Cobas Taqman MTB (Roche, Switzerland), Abbott Real Time MTB (Abbot, Des Plaines, IL) and Genotype MTBDR plus (Hain Lifescience, Nehren, Germany). *TB* tuberculosis, *PCR* polymerase chain reaction.

detected in ten samples (positive culture and/or routine PCR), hereafter called confirmed TB samples, while 72 samples were culture and routine PCR negative (Table 1). HIV status was unknown for all cases.

**Test performance of ultra compared to routine TB diagnostic tests.** Using culture and/or routine PCR as reference standard, Ultra was positive in 9 of 10 confirmed TB samples, giving an overall sensitivity of 90% (95% CI 56–100) (Table 2). Among the nine culture positive TB samples, Ultra was positive in eight (Fig. 2). All the smear positive (3/3) and 6/7 (86%) smear negative confirmed TB samples were Ultra positive. Semi-quantitation of bacillary load by Ultra categorised the TB samples as medium (n = 3), low (n = 3), very low (n = 1) and trace (n = 2). None of the TB samples had a high bacillary load. Among the 8 culture and Ultra positive samples, genotypic RIF-R was detected by Ultra in two samples, not detected in four samples and indeterminate in the two samples semi-quantitated as "trace". The genotypic RIF-R results were in concordance with the phenotypic drug susceptibility test (DST) results. The two samples with indeterminate RIF-R by Ultra were both sensitive to first line TB drugs in phenotypic DST.

One of the 72 culture and routine PCR negative samples was Ultra positive, yielding an overall specificity of 99% (95% CI 93–100) for Ultra. This sample was a lymph node biopsy from a patient with lymphadenitis following BCG vaccination. The lymphadenitis healed spontaneously and was eventually interpreted as an immune reaction to the vaccine. Among the culture and routine PCR negative samples were also three non-tuberculous mycobacteria (NTM) culture positive samples, identified as *Mycobacterium avium*. All three were Ultra negative.

## Discussion

In the present study we have investigated the performance of Ultra compared to routine TB diagnostic tests for diagnosing EPTB in a small cohort of prospectively collected extrapulmonary specimens in a high resource setting with a low TB prevalence. Using culture and/or routine PCR as reference standard, we found that Ultra had an overall sensitivity and specificity of 90% (95% CI 56–100) and 99% (95% CI 93–100), respectively. In smear negative confirmed TB samples, the sensitivity of Ultra was 86% (95% CI 42–100).

Several studies have been published on the diagnostic accuracy of Ultra in extrapulmonary specimens[11,14,15,17–25,28,29]. Most of the studies were performed in TB endemic settings[14,17,19–25,28,29], and only three studies have investigated Ultra test performance in low TB prevalence settings[11,15,18]. However, two of these studies were retrospective and performed on selected sample material. A strength of our study is that it is a prospective cohort study with consecutive inclusion of samples in a clinical routine setting, and the study population is thus more likely to be representative of the true test population in a low TB prevalence setting. The majority

| Culture and/or PCR positive samples[a] | Number of samples | Routine TB diagnostic tests (positive/total number) | | | Xpert Ultra |
|---|---|---|---|---|---|
| | | Microscopy | Routine PCR | Culture | |
| Lymph node biopsy | 1 | 0/1 | 1/1 | 1/1 | 1/1 |
| Lymph node aspirate | 4 | 3/4 | 3/3 | 4/4 | 4/4 |
| Pus samples | 2 | 0/2 | 2/2 | 1/2 | 2/2 |
| Other biopsies (pleura) | 1 | 0/1 | 0/1 | 1/1 | 1/1 |
| Fluid samples | 1 | 0/1 | 0/1 | 1/1 | 0/1 |
| Gastrointestinal lavage | 1 | 0/1 | 1/1 | 1/1 | 1/1 |
| Total | 10 | 3/10 | 7/9 | 9/10 | 9/10 |
| **Samples per inclusion site** | | | | | |
| Samples from HUH | 8 | 3/8 | 5/7 | 8/8 | 7/8 |
| Samples from SUH | 2 | 0/2 | 2/2 | 1/2 | 2/2 |
| **Culture and PCR negative samples[b]** | | | | | |
| Lymph node biopsy | 4 | 1/4 | 0/1 | 0/4 | 1/4 |
| Lymph node aspirate | 11 | 2/11[c] | 0/3 | 0/11 | 0/11 |
| Pus samples | 14 | 1/14[d] | 0/1 | 0/14 | 0/14 |
| Other biopsies | 13 | 0/13 | 0/4[e] | 0/13 | 0/13 |
| Fluid samples | 28 | 0/17 | 0/3 | 0/28 | 0/28 |
| Gastrointestinal lavage | 2 | 0/1 | N/A | 0/2 | 0/2 |
| Total | 72 | 4/60 | 0/12 | 0/72 | 1/72 |
| **Samples per inclusion site** | | | | | |
| Samples from HUH | 58 | 3/53 | 0/9 | 0/58 | 0/58 |
| Samples from SUH | 14 | 1/7 | 0/3 | 0/14 | 1/14 |

**Table 1.** Distribution of routine TB diagnostic tests performed and results of routine TB diagnostic tests and Xpert MTB/RIF Ultra. *HUH* Haukeland University Hospital, *SUH* Stavanger University Hospital. [a]Two of the samples (culture positive biopsy and culture positive fluid sample) are from the same TB case. [b]Includes three samples from clinically diagnosed TB cases (one pus sample, one biopsy and one lymph node aspirate). [c]The two microscopy positive samples were both culture positive for *Mycobacterium avium.* [d]Microscopy positive sample was culture positive for *Mycobacterium avium.* [e]Five culture negative biopsy samples were subjected to routine PCR, of which one PCR test result was indeterminate (technical failure). The indeterminate PCR result has been excluded from the results and analysis.

| Sample material | Sensitivity<br>% (95% CI)<br>TP/(TP + FN) | Specificity<br>% (95% CI)<br>TN/(TN + FP) | PPV<br>%<br>TP/(TP + FP) | NPV<br>%<br>TN/(TN + FN) | Accuracy<br>% (95% CI)<br>(TP + TN)/(TP + FP + FN + FP) |
|---|---|---|---|---|---|
| All samples | 90 (56–100)<br>9/10 | 99 (93–100)<br>71/72 | 90<br>9/10 | 99<br>71/72 | 98 (91–100)<br>80/82 |
| Lymph node biopsy | 100 (3–100)<br>1/1 | 75 (19–99)<br>3/4 | 50<br>1/2 | 100<br>3/3 | 83 (6–100)<br>4/5 |
| Lymph node aspirate | 100 (40–100)<br>4/4 | 100 (72–100)<br>11/11 | 100<br>4/4 | 100<br>11/11 | 100 (78–100)<br>15/15 |
| Pus samples | 100 (16–100)<br>2/2 | 100 (77–100)<br>14/14 | 100<br>2/2 | 100<br>14/14 | 100 (79–100)<br>16/16 |
| Other biopsies | 100 (3–100)<br>1/1 | 100 (75–100)<br>13/13 | 100<br>1/1 | 100<br>13/13 | 100 (77–100)<br>14/14 |
| Fluid samples | 0 (0–98)<br>0/1 | 100 (88–100)<br>28/28 | 0<br>N/A | 97<br>28/29 | 97 (82–100)<br>28/29 |
| Gastrointestinal lavage | 100 (3–100)<br>1/1 | 100 (16–100)<br>2/2 | 100<br>1/1 | 100<br>2/2 | 100 (30–100)<br>3/3 |

**Table 2.** Validation of Xpert MTB/RIF Ultra using culture and/or routine PCR tests as a reference standard. 95% confidence intervals for sensitivity, specificity and accuracy were calculated using the exact Clopper–Pearson method. *CI* confidence interval, *TP* true positive, *FN* false negative, *TN* true negative, *FP* false positive.

of extrapulmonary samples tested for TB in Norway are from patients with other diseases than TB. Hence, it may be just as important to avoid false positive test results in the large group of non-TB cases, which can lead to overtreatment and potentially severe side effects, as to obtain a more rapid TB diagnosis in the small group of TB cases. Our study can provide useful information about test performance in these clinically relevant non-TB cases, which we think is of particular importance in our setting. Indeed, the specificity of Ultra in our study was

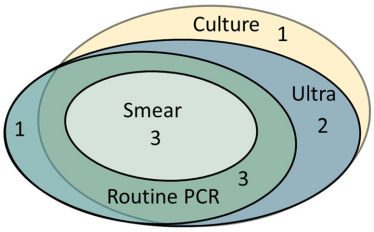


**Figure 2.** Euler diagram illustrating overlap of positive results of routine TB diagnostic tests and Xpert MTB/RIF Ultra in confirmed TB samples (n = 10).

high (99%). This is in concordance with the specificities (97–100%) found in other low TB prevalent settings where culture was used as reference standard[15,18]. However, in most of the studies that directly compare the performance of Xpert and Ultra, the specificity of Ultra is reduced compared to Xpert, both in extrapulmonary specimens[14,19,20,22,29] and sputum[9,10,12,13]. In low TB prevalent settings, even a small reduction in specificity could lead to unacceptable high rates of false positive cases, emphasizing that Ultra should be performed on selected samples with a high pre-test probability of TB disease.

Only one culture and routine PCR negative sample in our cohort was Ultra positive ("trace"). The sample came from a patient with regional lymphadenitis following BCG vaccination. As the BCG strains are also members of the MTC[30,31], the Ultra result may have been true positive for this case. However, Ultra is not able to separate non-viable from viable bacilli and cannot provide information as to whether the DNA present in the sample represented active mycobacterial disease or remnants of old DNA from the vaccine. This illustrates the challenges in interpreting positive Ultra results in certain clinical contexts. The inability of Ultra to separate viable from non-viable bacilli also limits its use for detection of relapse and reinfection. Dorman and colleagues reported a reduced specificity of Ultra in previously treated TB patients up to seven years after completion of treatment compared to patients with no previous history of TB[9]. This underlines the importance of culture for TB diagnosis.

Most, but not all[28,29], of the studies that investigate Ultra for the diagnosis of EPTB suggest increased sensitivity of Ultra compared to Xpert in different extrapulmonary specimens[11,14,15,17,19–23,25]. When culture is used as reference standard, the reported sensitivity of Ultra varies, but is generally high in lymph node specimens (90–94%)[18,24], FNAs and tissue samples (87–95%)[18,22], cerebrospinal fluid (80–100%)[17,18,21,25,29] and pus specimens (65–95%)[18,19], and lower in pleural fluid samples (48–84%)[18,20,22,23], which is in line with our findings. Several of the studies also show that Ultra can detect MTC in culture negative specimens from clinically diagnosed TB cases[17,19–25,28,29], thus, contributing to improved diagnosis of TB in samples with a very low bacillary load.

The ability of Xpert and Ultra to simultaneously detect MTC and RIF-R is considered one of the strengths of the assays. In addition to detection of the single-copy *rpoB* gene for the simultaneous diagnosis of TB and rifampicin resistance, Ultra includes two new and more sensitive PCR assays that target the multicopy *IS6110* and *IS1081* genes to improve MTC detection in paucibacillary samples[7]. However, the increased sensitivity of the test comes with an expense, because information about RIF-R is not available in *IS6110 or IS1081* positive and *rpoB* negative samples, which are categorised as "trace" by Ultra[7,18]. Two of the culture confirmed TB samples in our study were Ultra "trace" positive. For these samples, information about drug resistance was only provided by culture, and both were drug sensitive. The incidence of multidrug resistant (MDR)-TB is low in our setting[26], but in high MDR-TB incidence settings, MDR-TB cases can be missed among the "trace" positive samples. Therefore, Ultra should complement, but not replace culture.

This study has some limitations. The small sample number leads to uncertain sensitivity estimates. The study was performed in a clinical routine setting, and the combination of routine TB diagnostics used and sample processing varied slightly between the inclusion hospitals. The number of cultures performed per sample and the use of decontaminated versus not-decontaminated material may affect the sensitivity of culture. At both inclusion sites, fluid samples and FNAs with a volume of > 10 mL were concentrated before use. Hence, we cannot evaluate the overall effect of sample concentration on Ultra test performance, as some samples were concentrated and others were not. The use of frozen sample material for Ultra may also affect the performance of the assay due to reduced sample quality. However, studies that have specifically investigated the Xpert/Ultra test performance on frozen samples report discordant results, but the effect is small in the studies that find a difference[6,12,18,32]. Furthermore, DNA remains quite stable under frozen conditions, and our samples were only subjected to one freeze–thaw cycle. These findings imply that the impact of frozen samples on Ultra performance is small. Larger and more controlled studies with uniform sample processing should be conducted to adjust for these limitations. As culture is known to be an imperfect reference standard in paucibacillary cases of EPTB, future studies should focus on diagnostic accuracy of TB diagnostic tests both in microbiologically confirmed and clinically diagnosed TB subjects to better reflect the true test performance in all clinically relevant groups of the test population.

To conclude, the sensitivity and specificity of Ultra was comparable to culture in most sample materials in this study with the advantage of results within hours. These promising findings suggest that Ultra is a useful add-on test that can contribute to a rapid diagnosis of EPTB in our setting. However, the inability of Ultra to separate viable from non-viable bacilli and the lack of information about drug resistance for "trace" positive samples, limits its use in some clinical cases, especially in patients previously treated for TB.

## Data availability

The datasets generated and analysed during the current study are available from the corresponding author on reasonable request.

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## Author contributions

T.M. designed the study. I.S. and H.S. collected data. I.M.H. performed the study and analysis of data. I.M.H. wrote the original draft. I.M.H., H.S., I.S. and T.M. reviewed and edited the manuscript. All authors have read and approved the final manuscript.

## Competing interests

The authors declare no competing interests.

## Additional information

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# IP-10 dried blood spots assay monitoring treatment efficacy in extrapulmonary tuberculosis in a low-resource setting

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**Treatment efficacy is difficult to evaluate in extrapulmonary tuberculosis (EPTB) patients. Interferon-γ inducible protein (IP-)10 has been suggested as a biomarker for response to treatment. We have investigated if IP-10 from dried plasma spots (DPS) or dried blood spots (DBS) can be used in treatment monitoring of EPTB patients in a low-resource setting of Zanzibar. IP-10 levels in plasma, DPS and DBS samples collected before, during (2 months) and after TB treatment of 36 EPTB patients (6 culture and/or Xpert MTB/RIF positive and 30 clinically diagnosed) and 8 pulmonary tuberculosis (PTB) patients, were quantified by an enzyme-linked immunosorbent assay. There was a high positive correlation between IP-10 measured in plasma and DPS and DBS, respectively. We found a significant decline in IP-10 levels from baseline to end of treatment in plasma, DPS and DBS, both in EPTB and PTB patients. The declines were observed already after 2 months in HIV negative patients. In conclusion, the DPS/DBS IP-10 assay allows for easy and manageable monitoring in low-resource settings and our findings suggest that IP-10 may serve as a biomarker for treatment efficacy in EPTB patients, albeit further studies in cohorts of patients with treatment failure and relapse are needed.**

Tuberculosis (TB) is a global health problem with an estimated 10.0 million new cases of active TB and 1.6 million deaths because of TB in 2017[1]. Rapid and robust tests for TB diagnosis and treatments efficacy are needed to achieve global TB control. Although smear microscopy and culture are frequently used to assess treatment responses, the sensitivity of these methods is low in paucibacillary disease[2], and they also have limited ability to predict treatment outcomes[3–5]. Extrapulmonary TB (EPTB), which accounts for approximately 15–40% of all TB cases[6–9] with higher numbers in young children and people living with HIV[10–14], is typically paucibacillary. As smear or culture conversion cannot be used to monitor treatment efficacy in EPTB patients, clinicians must rely on clinical evaluation to detect treatment failure, which is often challenging due to the nonspecific symptoms in EPTB disease. The emergence of multidrug resistant (MDR-)TB[15] further emphasises the importance of early detection of ineffective treatment or low treatment adherence. Thus, there is a need for new assays to better predict treatment outcomes such as failure, cure and relapse. Biomarkers for early treatment efficacy may also provide surrogate end points in clinical trials for development of more effective and shorter personalized treatment regimens[16].

Numerous host biomarkers are being investigated as markers for treatment response in TB[17–19], including the pro-inflammatory chemokine interferon-γ inducible protein (IP-)10, also known as CXCL-10. Several studies show that plasma and serum IP-10 levels decline upon efficient treatment of TB[20–26]. Further, IP-10 is a robust marker expressed at higher levels than many other candidate biomarkers[20,27], which allows for IP-10 detection using simple test platforms.

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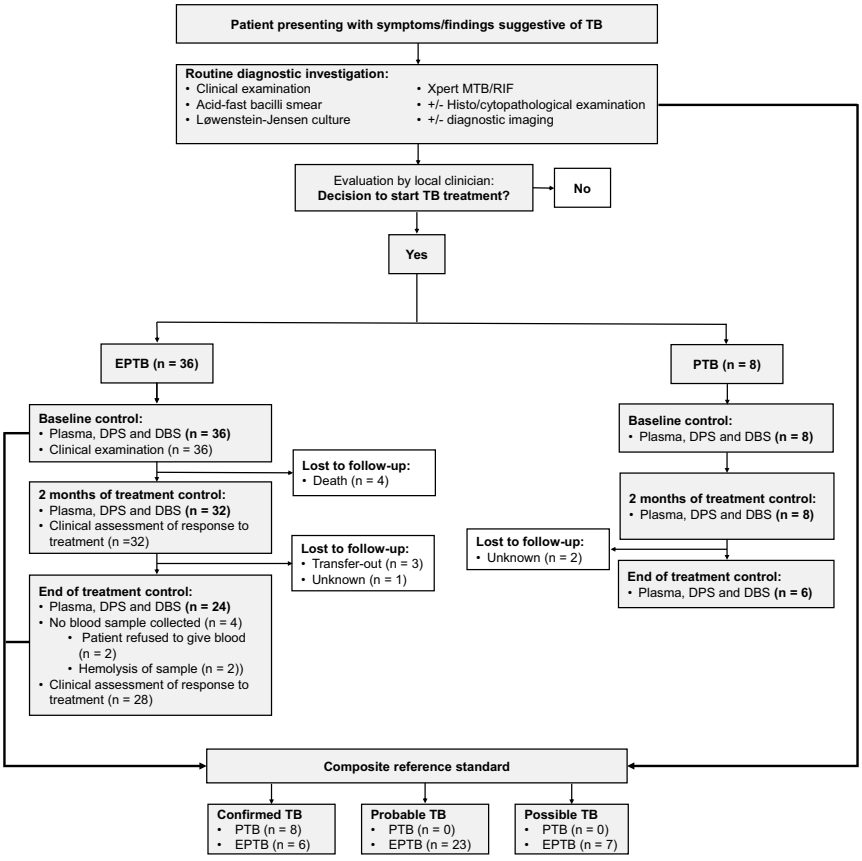


**Figure 1.** Flow chart of patient inclusion and data collection during the study. Abbreviations: TB, tuberculosis; EPTB, extrapulmonary TB; PTB, pulmonary TB; DPS, dried plasma spot; DBS, dried blood spot.

Dried plasma spots (DPS) and dried blood spots (DBS) applied on filter paper is a simple and robust method for storage and transportation of blood specimens. Several substances, including IP-10, are stable in DPS and DBS, even when kept at ambient temperatures[27,28], and IP-10 extracted from DPS and DBS can be quantified using ELISA-based methods[27,29,30]. Moreover, a user-friendly quantitative lateral flow assay for detection of IP-10 from antigen stimulated blood in TB patients has shown promising results[31,32], indicating that IP-10 based assays, eventually combined with other biomarkers, have the potential to be developed into simple point-of-care (POC) tests.

We have previously shown that IP-10 levels in DPS decline already after two weeks of therapy both in pulmonary TB (PTB) and EPTB[24]. Still, most of the studies on IP-10 as a marker for response to treatment are on PTB and data on EPTB in resource-constrained settings is scarce. The objective of this study was to compare IP-10 obtained from DPS and DBS to IP-10 measured directly in plasma in order to evaluate the performance of the IP-10 DBS assay as a biomarker for response to treatment in EPTB patients in a clinical low-resource setting.

## Results

**Study participants and clinical outcome.** Eight patients with PTB and 36 patients with EPTB were longitudinally followed during TB treatment (Fig. 1). Clinical characteristics are presented in Table 1. All PTB patients and six EPTB patients had bacteriologically confirmed TB (positive culture and/or Xpert MTB/RIF) and 30 EPTB patients had a clinical TB diagnosis, categorised as a probable TB case (n = 23) and possible TB case (n = 7), based on a composite reference standard[33]. All PTB patients were sputum smear positive at baseline. A drug susceptible *Mycobacterium tuberculosis* strain was detected in 5/10 culture positive samples, whereas drug susceptibility testing was not performed in the remaining five cases. No genotypical rifampicin resistance was detected in the 10 Xpert MTB/RIF positive samples. All PTB patients and 32 EPTB patients received standard TB treatment (isoniazid, rifampicin, pyrazinamide and ethambutol) for 6–10 months, whereas four EPTB patients received modified treatment because of previous PTB or active hepatitis. After 2 months of treatment, 2/8 PTB patients remained sputum smear positive, both of whom demonstrated smear conversion at the follow-up sputum

| Patient characteristics | EPTB (n = 36) | PTB (n = 8) |
|---|---|---|
| Age in years, median (range) | 33 (18–67) | 34 (20–52) |
| Female, n (%) | 18 (50) | 2 (25) |
| HIV-infected, n (%) | 9 (26)[a] | 2 (25) |
| **TB case category, n (%)** | | |
| Confirmed TB | 6 (17) | 8 (100) |
| Probable TB | 23 (64) | |
| Possible TB | 7 (19) | |
| **Routine diagnostics (positive/total)** | | |
| Culture (baseline) | 3/32 | 7/7 |
| Xpert (baseline) | 5/23 | 5/5 |
| AFB smear baseline | 6/36 | 8/8 |
| AFB smear 2 months | NA | 2/8 |
| AFB smear 5 months | NA | 0/7[b] |
| **Site of infection, n (%)** | | |
| Lymph node | 16 (44) | |
| Pleura | 9 (25) | |
| Abdomen | 4 (11) | |
| Spine | 1 (3) | |
| Lymph node and PTB | 2 (6) | |
| EPTB ≥2 sites/disseminated TB | 4 (11) | |
| **ART status for HIV-infected patients, n (%)** | | |
| ART before start of TB treatment | 3 (33) | NA |
| ART initiated during TB treatment | 4 (44) | NA |
| Patient died before initiating ART | 2 (22) | NA |

**Table 1.** Characteristics of the study participants. Abbreviations: EPTB, extrapulmonary tuberculosis; PTB, pulmonary tuberculosis; AFB, acid fast bacilli; ART, antiretroviral therapy; NA, not available or performed. [a]HIV status was unknown for one EPTB patient. [b]Sputum smear result at 5 months was missing for one PTB patient. This patient was registered with a negative sputum smear at 2 months.

control one month later. A successful treatment outcome was recorded in the National TB program registers for all the PTB patients. All EPTB patients that were evaluated at 2 months showed clinical improvement, except four patients, three of them HIV-infected, who presented with a transient enlargement of lymph nodes, possibly due to paradoxical immune reactions. At the end of treatment, all EPTB patients that completed therapy demonstrated clinical improvement. Blood samples were taken before start of TB treatment, after 2 months and at the end of treatment. Altogether, four patients died, six patients were lost to follow-up and a final blood sample was not obtained from four patients (Fig. 1). Thus, a complete set of samples at three time points were available for six PTB patients and 24 EPTB patients.

**IP-10 decline corresponds to sputum conversion in pulmonary TB patients.** We first analysed IP-10 levels in the eight patients with confirmed active PTB disease (Fig. 2A). There was a significant decline in IP-10 from baseline to the end of treatment in plasma samples [1081 pg/mL (886–2321) vs. 203 pg/mL (129–427), $p = 0.028$], DPS samples [25 pg/2 discs (15–63) vs. 4 pg/2 discs (3–7), $p = 0.027$] and DBS samples [47 pg/2 discs (31–98) vs. 15 pg/2 discs (9–19), $p = 0.043$]. The decline in IP-10 levels was observed in 8/8 PTB patients already after 2 months of treatment in plasma ($p = 0.012$), DPS ($p = 0.018$) and DBS ($p = 0.018$), and corresponded with sputum conversion for six of the patients.

**Plasma IP-10 decline during TB treatment in extrapulmonary TB.** We then analysed the *plasma* levels of IP-10 in the patients with EPTB. There was a significant decline in IP-10 levels from baseline to the end of treatment [660 pg/mL (269–1224) vs. 337 pg/mL (210–535), $p < 0.001$] (Fig. 2B). As for the PTB patients, the IP-10 decline was observed already after 2 months ($p < 0.001$). Individual changes in IP-10 levels during TB treatment are presented in Fig. 3. In most EPTB patients, plasma IP-10 levels decreased from baseline to each follow-up time point and corresponded to a good clinical response to treatment. However, markedly increasing plasma IP-10 levels towards the end of treatment were seen in four patients despite good clinical response. Two of these patients were pregnant during treatment (patient 3–4 in Fig. 3), the third patient was HIV-infected (patient 1 in Fig. 3) and the last patient with ascites and pleuritis demonstrated pleural thickening on X-ray at the last visit but was otherwise in general good clinical state (patient 2 in Fig. 3). No intercurrent infections were registered in any of these patients, who all finished treatment successfully. In contrast, only one of four patients that presented with increasing or persistent lymph node swelling at 2 months of therapy showed a corresponding increase in IP-10 levels.

**DBS IP-10 and DPS IP-10 decline during TB treatment in extrapulmonary TB.** We found a high positive correlation between IP-10 measured directly in plasma and IP-10 extracted from DPS ($r = 0.809$, $p < 0.001$), and DBS ($r = 0.767$, $p < 0.001$) in all samples. As in the plasma samples, there was a significant decline in IP-10 levels

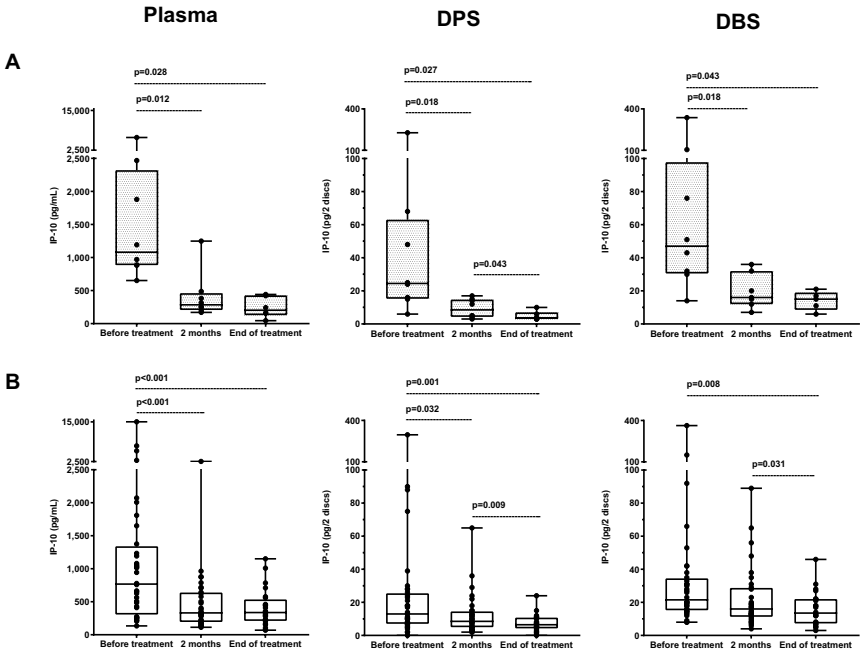


**Figure 2.** Changes in IP-10 levels in plasma, dried plasma spots (DPS) and dried blood spots (DBS) during tuberculosis (TB) treatment. IP-10 concentrations measured using enzyme-linked immunosorbent assay from (**A**) patients with pulmonary TB before treatment (n = 8), at 2 months (n = 6) and at the end of treatment (n = 6) and from (**B**) patients with extrapulmonary TB before treatment (n = 36), at 2 months of treatment (n = 32) and at completion of 6–10 months of treatment (n = 24). Boxes represent the median and interquartile range, and the whisker show min/max values. Dotted lines indicate statistically significant differences. Statistical significance was evaluated by Wilcoxon Signed Ranks test using a significance level of 0.05.

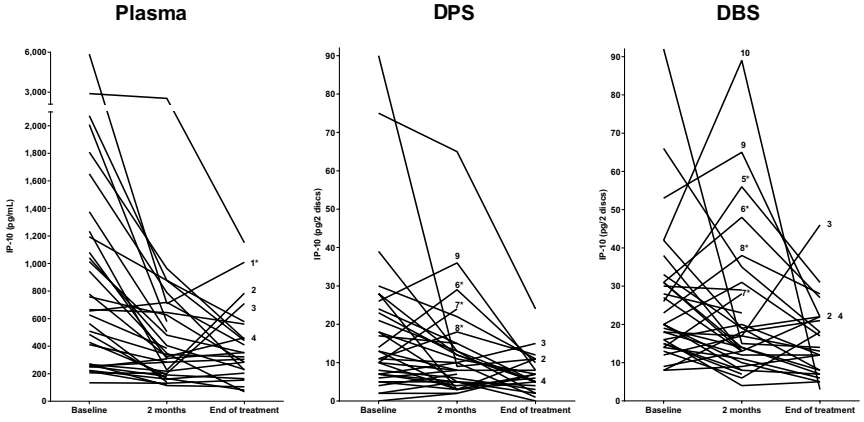


**Figure 3.** Comparison of IP-10 levels in plasma, dried plasma spots (DPS) and dried blood spots (DBS) for individual extrapulmonary tuberculosis (EPTB) patients during TB treatment. IP-10 levels during 6–10 months of TB treatment in **plasma**, **DPS** and **DBS** in patients with extrapulmonary TB at baseline (n = 32), at 2 months of treatment (n = 32) and at the end of treatment (n = 24). IP-10 levels in baseline samples from the four patients who died shortly after start of TB therapy, are not presented in this figure. [1]TB pleuritis, [2]Combined ascites and TB pleuritis, [3–4]Pregnant during treatment, [5–7]Persistant or increasing lymph node swelling at 2 months of treatment, [8–9]Lymph node TB, [10]Abdominal TB. *HIV-infected patient.

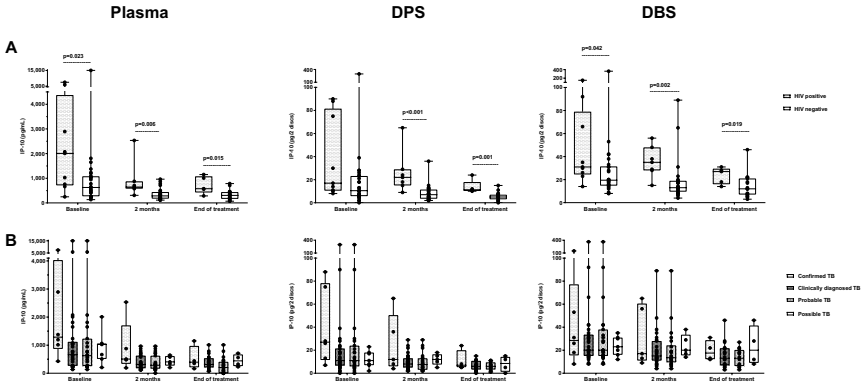


**Figure 4.** IP-10 levels in different clinical groups of extrapulmonary tuberculosis (EPTB) patients during treatment in plasma, dried plasma spots (DPS) and dried blood spots (DBS). IP-10 concentrations were measured using enzyme-linked immunosorbent assay. EPTB sub-grouped according to (**A**) HIV status; before treatment (n = 35), at 2 months of treatment (n = 32) and at the end of treatment (n = 24), and according to (**B**) TB category; before treatment (n = 36), at 2 months (n = 32) and at the end of treatment (n = 24). HIV status was unknown for 1/36 EPTB patients. Boxes represent the median and interquartile range, and the whisker show min/max values. Dotted lines indicate statistically significant differences. Statistical significance was evaluated by Mann-Whitney U test, using a significance level of 0.05.

from baseline to the end of treatment in both DPS [12 pg/2 discs (7–24) vs. 7 pg/2 discs (4–11), p = 0.001] and DBS [20 pg/2 discs (15–31) vs. 14 pg/2 discs (7–22), p = 0.008] (Fig. 2B). However, a significant decrease in IP-10 from baseline to 2 months of treatment was only observed in DPS (p = 0.032) and not in DBS (p = 0.217). In contrast to the plasma samples, a further decline in IP-10 from 2 months to the end of treatment was seen both in DPS (p = 0.009) and DBS (p = 0.031) samples.

Similar patterns of individual changes in IP-10 levels during treatment were observed in the filter paper samples as in the corresponding plasma samples for the majority of patients. However, an apparent IP-10 increase from baseline to 2 months of treatment was seen more often in DPS and DBS filter paper samples compared to plasma samples (Fig. 3). Interestingly, this included three of the four patients who did not demonstrate clinical improvement after 2 months of therapy (patient 5–7 in Fig. 3), as well as two other patients with lymph node TB (patient 8–9 in Fig. 3), one with an intercurrent inflammation, but both with clinical response to TB therapy. Further, a transient and high IP-10 increase in DBS only, was seen in one patient with abdominal TB with good response to treatment (patient 10 in Fig. 3).

**IP-10 levels in various clinical groups of TB patients.** In general, there was a tendency to higher IP-10 levels at baseline in PTB patients compared to EPTB patients both in DPS (p = 0.075) and DBS (p = 0.013), but not in plasma (p = 0.114). When EPTB patients with concurrent PTB infection (n = 2) or disseminated TB (n = 4) were excluded from the analysis, the difference in IP-10 levels between the PTB and EPTB patients became even more apparent both for DPS (p = 0.045) and DBS (p = 0.01).

Since the vast majority of EPTB patients were clinically diagnosed (83%), we also compared IP-10 levels between patient groups according to TB diagnostic criteria. At baseline, there was a tendency to higher IP-10 levels in plasma (p = 0.071) and DPS samples (p = 0.086) from EPTB patients with confirmed TB diagnosis compared to patients with clinical TB diagnosis (Fig. 4). For those groups large enough to analyse we found no significant differences in baseline IP-10 levels between the various extrapulmonary sites of infection.

**The effect of HIV co-infection on IP-10 levels during TB treatment.** In the EPTB group, the IP-10 levels were significantly higher in HIV-infected (n = 9) compared to HIV negative (n = 27) patients at all time points and assessed by all three methods (Fig. 4). In contrast to the joint analyses of HIV-infected and HIV negative EPTB patients, a significant decline in IP-10 at 2 months of treatment was observed not only in plasma (p < 0.001) and DPS (p = 0.011), but also in DBS (p = 0.045) when HIV negative patients were analysed separately. For the HIV-infected EPTB patients, the IP-10 decline at 2 months of treatment was only significant in plasma (p = 0.043), but only few patients were included in these analysis.

## Discussion

In this study, we have evaluated the potential of the DPS/DBS IP-10 assay compared to measuring IP-10 in plasma, for assessing treatment responses in EPTB patients in a clinical setting where diagnostic tools and resources are limited. To our knowledge, this is the first study evaluating IP-10 from DPS/DBS during the first months of TB treatment also in clinically diagnosed EPTB patients. We demonstrate that IP-10 is readily detectable both in unstimulated plasma and DPS/DBS filter paper samples. Further, we show that IP-10 levels decrease upon

completion of successful treatment in plasma, DPS as well as DBS, and that the decline is significant already after 2 months of treatment in HIV negative patients.

IP-10 is a pro-inflammatory chemokine expressed by antigen presenting cells, mainly in response to T-cell derived interferon-γ[34]. Increased levels of IP-10 are found in blood, plasma or urine in infections such as HIV[35,36], hepatitis C[37–40], bacteremia[41,42] and TB[43–50]. It has recently been demonstrated that IP-10 secretion during TB disease originates from infected macrophages and multinucleated giant cells located in the granulomas, and early inactivation of the secreted chemokine by antagonist form of IP-10 which binds to CXCR3 but does not induce signalling, may also play a role in the pathogenesis of TB[51,52]. Several studies show that IP-10 declines during efficient treatment of many diseases including TB[25,38,42,53,54]. Limited data also indicates that IP-10 increases in non-cured TB patients from baseline to 2 months of treatment, and in TB patients who relapse or develop active TB disease[25,55]. This makes IP-10 a strong candidate as a biomarker for treatment response although specificity is an issue. In our study, we found a significant decline in plasma IP-10 after 2 months and upon completion of successful treatment, which is consistent with findings in several other investigations[20,22–26]. However, as all of the TB patients in our cohort responded to treatment, we cannot fully evaluate the potential of IP-10 to detect treatment failure in the present study. Further, a significant decline in plasma IP-10 levels has been reported to occur as early as 1–2 weeks after initiation of TB therapy[24,26,54]. We did not include earlier time points for evaluation due to the low-resource setting limiting closer follow-ups, but the DPS/DBS assay may also have potential for earlier evaluation of diagnosis and treatment efficacy.

High levels of IP-10 are observed in a variety of inflammatory diseases and conditions[56–58]. We found markedly increasing IP-10 levels towards the end of treatment despite clinical improvement in four patients. Two of the patients were pregnant during therapy, which is known to cause physiological changes in cytokine profiles[59], and one patient presented with an unspecified inflammatory swelling in a foot. This is in line with our previous observation that intercurrent disease could cause IP-10 production[24], and emphasises that IP-10 measured in unstimulated blood specimens is not a specific marker for TB disease and must be interpreted within a clinical context. Likewise, a significantly higher level of plasma IP-10 in HIV-infected patients compared to HIV negative TB patients was observed throughout treatment in our study, which may reduce the specificity of IP-10 based tests in HIV-infected patients. However, we and others have shown that IP-10 levels can differentiate patients with latent TB infection from patients with TB disease irrespective of HIV status[23,26]. With regard to the impact of HIV infection on IP-10 responses during TB treatment, discordant results are reported[20,60]. We observed a decrease in plasma IP-10 during treatment in EPTB patients irrespective of HIV status, which is supported by a recent study demonstrating a decline in serum IP-10 levels during the early phases of treatment in HIV-infected patients[54]. For the DPS and DBS samples in our study, the IP-10 decline was only significant in HIV negative patients, but the HIV patient group is too small to conclude.

Evaluation of treatment efficacy in EPTB patients is important for timely and proper management, as many are started empirically on treatment due to low sensitivity of routine confirmatory tests. Further, smear and culture conversion cannot be used to assess response, underlining that EPTB patients would greatly benefit from new methods for treatment monitoring. We found a tendency to higher levels of pre-treatment plasma IP-10 in bacteriologically confirmed EPTB patients compared to clinically diagnosed patients, which supports the assumption that clinically diagnosed EPTB patients in general have a lower bacterial burden associated with less advanced disease. Still, there was a significant decline in IP-10 both after 2 months and at the end of treatment also for patients defined as *probable TB* cases.

DBS provides a minimally invasive method for blood collection using finger prick blood, and requires no centrifugation for sample processing, making DBS particularly user-friendly in low-resource settings. Our data indicate that the DBS IP-10 method can be used in settings where storage and transport of plasma is not feasible. However, discordant results between the three methods were observed for some patients, mostly HIV-infected. We have no clear explanation to this, but due to a limited number of DPS/DBS per patient, all filter paper samples were assayed as singlets in the ELISA. The use of triplicates in further studies would provide important information about whether variance in the measurements is causing discordant results between methods. Further, apparent stable or weakly fluctuating low levels of plasma IP-10 (<300 pg/mL) throughout treatment were observed in a few EPTB patients despite good clinical response. TB patients represent a heterogenous group of patients, both in terms of infection sites and variation in systemic inflammation. Thus, low systemic plasma IP-10 levels in some patients could reflect low disease activity already at baseline, or compartmentalized production and degradation of cytokines at the site of infection, both limiting the use of the assay in this group of TB patients.

There are some limitations to our study. The small sample size gives reduced power to our results. As we did not have any TB patients with unsuccessful treatment and only followed patients until completion of treatment, the association between IP-10 and treatment failure or relapse cannot be assessed. Further, several factors may influence IP-10 levels and lead to variation; firstly, the EPTB group was heterogenous with various TB localisations, secondly, the number of HIV-infected patients was low, CD4 cell counts were not performed and the anti-retroviral therapy coverage during TB treatment varied according to decisions made by local clinicians. Thirdly, a clinical TB diagnosis is uncertain and elevated IP-10 levels could reflect other diagnoses than TB, although we believe that the use of a composite reference standard for TB diagnosis that includes clinical treatment response makes this less likely. Finally, the time points for sample collection during therapy varied somewhat compared to the original study protocol, as many follow-up controls had to be postponed to make it possible for the patients to participate in the study. For a couple of patients, the baseline sample was collected a few days after start of TB treatment and may not represent the true pre-treatment level of IP-10, as changes in immune responses have been reported to occur as early as one week after start of treatment[26,54]. Accordingly, to measure IP-10 levels few weeks after initiation of therapy could also have provided data on the kinetics of IP-10 in a clinical setting.

To conclude, the present study shows that IP-10 declines during successful treatment of a clinically heterogeneous EPTB population in a TB endemic area. The simple and robust DPS/DBS IP-10 method allows for easy and manageable monitoring in low-resource settings. Our data suggest that IP-10 may serve as a biomarker for treatment responses, albeit further studies including larger cohorts of patients with treatment failures and relapses are needed to confirm this.

## Methods

**Study participants.** The study was part of a larger study that was conducted at the tertiary care hospital Mnazi Mmoja Hospital at Zanzibar, Tanzania, from August 2014 to September 2015[33]. Thirty-six patients ≥18 years of age with either clinically diagnosed or bacteriologically confirmed EPTB and 8 smear positive and bacteriologically confirmed PTB patients, were included in the study and longitudinally followed during TB treatment (Table 1). The TB diagnosis and decision to start TB treatment was made by local clinical/medical officers. Exclusion criteria were ongoing or earlier treatment for TB during the last 12 months, or refusal to participate in the study. Clinical assessment and collection of blood specimens for the EPTB group were performed before treatment [median day 0 (5–95 percentile range −7 −+ 8 days)], after the intensive phase of treatment at 2 months [median day 71 (5–95 percentile range 54–120)] and at the end of treatment [median day 175 (5–95 percentile range 149–342)]. Treatment outcome and sputum smear results at baseline, 2 months and 5 months of treatment for the PTB patients were collected from the National TB Program registers.

**Diagnostic TB groups.** Based on a composite reference standard[33], the patients were categorized as confirmed, probable or possible TB cases. Briefly, a confirmed TB case was defined as a culture and/or Xpert positive patient. A culture and Xpert negative patient presenting with one of the following; a positive Ziehl-Neelsen (ZN) smear or radiological findings suggestive of TB or histology/cytology consistent with TB or lymphocytosis on fluid cytology, was defined as a probable TB case if the patient also demonstrated clinical response to treatment or had confirmed concomitant PTB. If clinical response to treatment was unknown (lost to follow-up) and there was no concomitant PTB, the same patient was defined as a possible TB case. A patient presenting with symptoms suggestive of TB, but for whom all tests (culture, Xpert, ZN smear, histo/cytopathology and diagnostic imaging) were negative, was defined as a possible TB case if the patient demonstrated good clinical response to treatment.

**Filter paper sample preparation.** Whole blood was drawn into EDTA or CPT tubes (Vacutainer, BD). $2 \times 25\,\mu L$ whole blood was spotted directly onto Whatman 903 filter paper (GE Healthcare) before the remaining whole blood was centrifuged within 30–40 minutes after collection to obtain plasma (EDTA tubes at $3000 \times g$ for 10 min at room temperature (RT), and CPT tubes at $1800 \times g$ for 20 min at RT). Afterwards, $2 \times 25\,\mu L$ plasma was spotted onto another Whatman 903 filter paper. The remaining plasma was stored at −80 °C. The filter papers were left to dry for 3–4 hours at RT before placed in zip-locked plastic bags with desiccants, and stored at −20 °C. The filter paper cards were transported at room temperature and the plasma samples on dry ice to Bergen, Norway, for analysis.

**Protocol for IP-10 determination in plasma, DPS and DBS samples.** Quantification of IP-10 in plasma and filter paper samples was performed in triplicates and singlets respectively using ELISA-based assays as described elsewhere by Aabye *et al.* and Drabe *et al.* respectively[27,30]. For the DPS and DBS samples, 2 filter paper discs of 5.5 mm were punched from the dried plasma or blood spots (1 disc per spot) and stacked horizontally in a filter microtiter plate (Luminex, Merck Millipore) and 80 μL dilution buffer was added per well before the plate was incubated at RT for 1 hour to allow for diffusion-based extraction of the DPS/DBS sample. After incubation, the filter microtiter plate was stacked on top of a capture mAb coated ELISA plate containing 20 μL dilution buffer with detection mAb per well. The stacked plates were centrifuged at $700 \times g$ for 10 minutes, to filter the extracted sample into the ELISA plate, and incubated at RT for 1 hour. After incubation, plasma, DPS and DBS ELISA plates were washed and revealed for 30 min at RT, before stopping the reaction and reading at 450 nm with 630 nm reference. Plasma levels were corrected for dilutions, DPS and DBS measurements are presented as pg/2 discs.

**Statistical analysis.** All values are presented as median and interquartile range (IQR), unless otherwise stated. Statistical analyses were performed using SPSS Statistics V25 (IBM). Non-parametric statistical methods were applied. Wilcoxon Signed Ranks test was used to evaluate change in IP-10 levels between different time points during treatment in individual patients. For comparison of difference in IP-10 levels in independent groups at baseline, Mann Whitney U test was applied when the groups compared consisted of ≥5 individuals per group. Correlation analyses were performed using Spearman's Rank Correlation Coefficient. A significance level of 0.05 was used. Graphical presentations were made using Prism V7.03. (Graphpad).

**Ethical considerations.** Ethical clearance was obtained from the Regional Committee for Medical and Health Research Ethics, Western-Norway (REK Vest) and the Zanzibar Medical Research and Ethics Committee (ZAMREC) before conducting the study. All methods were carried out in accordance with the relevant guidelines and regulations. Permission to export plasma, DPS and DBS samples out of Tanzania was approved by ZAMREC (Ministry of Health, Zanzibar), Mnazi Mmoja Hospital and the study participants. All participants provided informed written consent.

## Data Availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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## Author Contributions

Conceived and designed the experiments: I.H., M.R., T.M., A.M.D.R. Recruited the patients and collected clinical data: M.J., M.M. Conducted the experiments: I.H. Analysed the results: I.H., M.J., M.R., T.M., A.M.D.R. Contributed reagents/materials/analysis tools: M.R., A.M.D.R., T.M. Drafted the manuscript: I.H., A.M.D.R. All authors have reviewed and approved the manuscript.

## Additional Information

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