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Electro-impedimetric detection of human anti-mycolate antibody biomarkers of TB before, during, and after treatment

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ABSTRACT

Efficient TB management requires rapid and accurate diagnosis of active pulmonary and extrapulmonary TB at the point-of-care. Blood-based antibody biomarker assays may be ideal if unaffected by HIV co-infection and antibody memory from prior TB or vaccination.

Aim: This study assessed electro-impedimetric detection (EIS-MARTI) of anti-mycolate antibodies (AMAb) in TB patients before, during, and after treatment, compared to sputum culture (MGIT) as the gold standard.

Methods: A prospective pilot study enrolled 15 confirmed TB patients and 73 healthy controls at a Pretoria hospital (2016–2017). A prospective monitoring study followed 25 confirmed TB patients over 6 months of treatment at a Pretoria clinic (2019–2020) to evaluate biomarker behavior. Outcomes were analyzed using descriptive statistics, wherein diagnostic accuracy and predictive values were assessed by ROC curve analysis.

Results: EIS-MARTI detected 14/15 true TB-positive cases independent of HIV co-infection and 68/73 true TB-negatives in the pilot study. In the monitoring study, EIS-MARTI correlated with culture in 7/8 cases at treatment end, but not during the first 2 months.

Conclusion: AMAbs arise independently of HIV co-infection in active TB, recede during treatment, and are rapidly detected by a hand-held EIS-MARTI device. While suitability for treatment monitoring remains uncertain, EIS-MARTI shows promise for rapid, accurate TB diagnosis and confirming cure.

PLAIN LANGUAGE SUMMARY

The purpose of this work was to investigate anti-mycolate antibodies as a suitable biomarker for diagnosing tuberculosis, monitoring treatment, and screening people at risk for TB.

ARTICLE HISTORY
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KEYWORDS
Tuberculosis (TB); antibody biomarker for TB; handheld TB diagnostic; TB screening; TB serodiagnosis; TB immunosensor; anti-mycolate antibodies; mycolic acids

1. Introduction

Tuberculosis (TB) is an infection caused by *Mycobacterium tuberculosis (M. tb)* with approximately 7.5 million new incident cases during 2022, the highest number since the World Health Organization (WHO) began global TB monitoring in 1995 [1]. The prevalence of undiagnosed TB remains a global issue with an estimated 3.2 million cases missed during 2023 [2].

Much of the world still relies on sputum sampling using the Ziehl Neelsen smear microscopy method developed during the 1880s [3]. The diagnostic premise is obtaining a sputum sample, staining cell walls with phenol-carbol fuchsin and observation using a light microscope. It has become the backbone of TB diagnosis due to its cost-effectiveness [4]. However, smear microscopy has limitations. It requires a high bacterial load of approximately $5x10^3$-10^4 bacilli per ml [4]. Sampling is often difficult in immunocompromised individuals such as Human Immunodeficiency Virus (HIV)-positive patients [5], TB-asymptomatic children, and patients with extrapulmonary TB who often struggle to expectorate sputum [6]. Smear microscopy's low sensitivity (25–75% compared to culture) makes it difficult to diagnose paucibacillary cases which often result in false negative results [4]. Furthermore, sputum culture is regarded as the reference standard but may not always detect pulmonary TB due to sampling and technical factors and has limitations in extrapulmonary disease [5]. Molecular assays such as the GeneXpert have become the preferred sputum-based method of TB diagnosis, but can still provide false-positive outcomes for some patients previously treated for TB [7]. This is likely due to the stability of nucleic acids in vivo. Due to the fundamental limits of polymerase chain reaction-based technology, it cannot distinguish between nucleic acids from living versus dead bacilli [8]. Cases of false-positive GeneXpert results have been reported up to 5 years post cure [7]. Diagnostic systems like the GeneXpert maintain high diagnostic coverage and effective case management in high-income countries, because they

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Article highlights

- Introduction of a novel handheld TB diagnostic device (EIS-MARTI) for potential rapid screening of TB using minimal volumes of blood samples.
- The device could monitor the behavior of anti-mycolate antibodies as biomarker for active TB before and after anti-TB chemotherapy
- Anti-mycolate antibodies arise independently of HIV co-infection status in active TB patients.
- Anti-mycolate antibodies recede to background levels over the six-month course of anti-TB chemotherapy in active TB patients.
- Detection of anti-mycolate antibodies in blood samples requires high sample dilution to avoid prozone hindrance of antibody binding in both TB patients and healthy controls.
- Anti-mycolate antibody level detection in blood is to be done in parallel with TB-negative human plasma in sequential analyses on single disposable electrodes to achieve acceptable precision.
- The EIS-MARTI device is not useful for monitoring anti-TB chemotherapy, however it showed potential to confirm cure at the end of treatment.
- The behavior of the anti-mycolate antibody biomarker before, during and after anti-TB treatment predicts that the EIS-MARTI test has the potential to fill the need for rapid pediatric and extra-pulmonary TB diagnosis.

are broadly integrated into national TB programs [2], supported by robust healthcare systems, funding, and established laboratory networks [2]. In contrast, access to such sophisticated TB diagnosis is limited in low-income countries, due to the barriers of inadequate infrastructure, maintenance, supply chains, and health system integration [2].

Generating a sputum sample is often problematic in immunocompromised individuals, such as patients infected with HIV [5]. It increases the risk of transmission of the airborne pathogens during the sampling acquisition process. Several challenges are associated with sputum production in children [9], namely an underdeveloped cough reflex, difficulty following instructions, low sputum volume, and contamination with saliva. Young children, especially those under five, have immature respiratory systems and specific musculature which makes it more difficult to generate the forceful cough needed to expel sputum from the lower airways [10]. Young children struggle to follow instructions for sputum expectoration, resulting in inadequate yield of mucus volume for collection [11]. This can lead to insufficient sample volumes for accurate microbiological testing. Samples collected from children often contain excessive saliva rather than deep respiratory secretions, reducing diagnostic sensitivity [11]. Alternative collection methods such as induced sputum or gastric lavage are available, but require specialized equipment and trained personnel, adding logistical challenges [11]. The process of sputum induction, often involving inhalation of hypertonic saline, can be distressing for children, further limiting sample yield [12].

Several non-sputum-based tests have been endorsed by the WHO, each with their own utility and shortcomings. One example is the urine-based lipoarabinomannan (LAM) assay which appears useful when used with other diagnostics in late-stage HIV patients. A meta-analysis indicated the sensitivity of the AlereLAM® test was 45% in people living with HIV (PLHIV) and 56% in patients with CD4 counts equal to or less than 100 cells per µL [13]. The AlereLAM® test reduces mortality when implemented for immunocompromised patients with HIV. However, its sensitivity is suboptimal [13]. Several non-sputum-based tests in development have shortcomings. Detection of cell-free Deoxyribonucleic acid (DNA) in urine requires enrichment steps. Stool-based methods require complex specimen processing. Tongue swabs suffer from low recovery and low sensitivity in paucibacillary TB. Blood-based transcriptomics show low and variable sensitivity with minimal data for PLHIV. Many other blood-based technologies are not yet suitable for use at the point of care. These considerations further illustrate and motivate for the development of rapid, more accurate, highly specific, and sputum-free tests for TB triage and screening [3].

Antibodies to mycolic acids (MA) were first discovered in 1999, when Pan et al. [14] reported that antibodies to cord-factor (a trehalose ester of MAs) could distinguish between different classes of MA, suggesting that this could form a basis for serodiagnosis of TB. In 2002 we reported that the antibodies to MA in human TB patients were elicited independently of HIV co-infection with concomitant immune compromise, but with low sensitivity using standard immunoassay techniques [15]. Technology developments with evanescent field biosensor detection of anti-MA antibodies allowed significant improvement in the sensitivity of biomarker detection, and yet remained untenable for high throughput screening due to prohibitive cost of equipment and complexity of measurement [16]. Electro-impedimetric detection of anti-MA antibodies [17] combined with portable potentiostat devices prepared the way for developing the current handheld Electrical Impedance Spectroscopy (EIS)-MARTI device for TB screening that is assessed in this report. It uses electro-impedimetric detection of Anti-mycolate antibodies (AMAb) on disposable screen-printed carbon electrodes, is affordable for the purpose and shown here to allow for high-throughput analysis of blood samples at a rate of at least 100 sample analyses per week per device.

Several attempts at using the detection of AMAb as biomarker for active TB have been reported since 1999 [16–20], of which the electro-impedimetric, mycolate antibody real-time immunoassay (MARTI) represents one of the most feasible efforts [17,19]. Of these, the MARTI test was revised during 2022, aiming to meet the WHO's Target Product Profiles (TPPs) for a diagnostic device [21], and treatment monitoring [22]. Briefly, the minimum requirements include: identification of active TB at the first encounter; a target population of both adults and children; can be used by persons trained at auxiliary nurse level; sensitivity > 90%; specificity > 70%; non-sputum specimens preferred; < 2 sample preparation steps; time to result of under 30 minutes; battery powered handheld device weighing < 1 kg; simple to navigate user interface and an inclusion of an internal control [21].

The TPPs for treatment monitoring include: a minimum target of ≥75% sensitive; ≥80% specific; detection still possible halfway through treatment; testing frequency of 2; requirement for only basic lab infrastructure; can be conducted by a trained healthcare worker; can target active TB in at least

people with bacteriologically confirmed TB; uses minimally invasive specimens; and has time to result of ≤1 day [22].

A milestone was achieved when electrochemical technology advanced to allow demonstration of the detection of anti-lipid antibody biomarkers in serum samples by means of a handheld device containing a potentiostat linked to a computer that could easily be exchanged by a mobile smart phone. Using such a prototypic device, we attempted a double-blind validation trial on TB patients and healthy controls to investigate whether the AMAb TB biomarker could be detected by electro-impedance in a way that would fill the need for point-of-care TB diagnostic screening of people at risk of contracting TB. The method is minimally invasive – using only a small quantity of blood – and giving a diagnostic outcome within an hour after sampling.

EIS-MARTI provides the first opportunity to study the behavior of the AMAb biomarker to consider its use in a commercial TB screening device. This may contribute to filling the need for point-of-care diagnostic devices toward ending the global TB epidemic. The EIS-MARTI technology is registered on the Foundation for Innovative New Diagnostics (FIND)'s TB test directory pipeline [23].

The aim of this study is to assess EIS-MARTI for its potential to fill the need for a rapid, accurate, and sputum-independent test for TB screening, triage, and monitoring the response to anti-TB chemotherapy, by detection of the AMAb biomarker for active TB.

The first study used the EIS-MARTI technology to detect AMAbs in blood samples from 88 patients to determine its suitability in screening applications as compared to the gold standard Mycobacterial Growth Indicator Tube (MGIT) culture. The second study compares EIS-MARTI to MGIT culture to explore its potential application in the response to anti-TB chemotherapy at baseline, after 1 month, 2 months and at end of treatment, based on the behavior of the AMAb biomarker during treatment.

2. Materials and methods

2.1. Study design and participants

2.1.1. The EIS-MARTI pilot study

Study design, setting, and period: This study was an observational descriptive study with parallel groups, designed to compare the diagnostic outcomes of the gold standard MGIT culture with the EIS-MARTI technique. Presumptive TB patients and healthy controls were enrolled in this study at Steve Biko Academic Hospital and the University of Pretoria from 2016 to 2018, in Pretoria, South Africa.

tudy population: Clinical specimens that met the inclusion criteria were obtained from 88 patients with an age range of 22 to 67 years, with median age of 31, 56% female and 54% male. Statistical modeling revealed that a full validation trial would require samples from 270 participants that meet the inclusion and exclusion criteria. The cohort of 88 patients that were willing to give consent to participate in this study is representative of a pilot study, the results of which may be used in future to calculate sample size for quantitative comparison of EIS-MARTI with existing TB diagnostics. The collection of samples to completion was interrupted during the development of the EIS-MARTI technology to meet precision and reproducibility standards. Once ready to recommence, the South African National protocol for identification of TB patients had changed, making it impractical to complete the collection of samples with the original patient selection criteria. As a result, this study was interrupted. The pilot study obtained far more healthy control samples (73) than TB cases (15).

Sample collection: Figure 1 illustrates the selection process undertaken to collect blood and sputum samples for this study. Light blue blocks represent the steps for MARTI blood collection and processing; gray blocks represent blood samples for comparative analyses and dark blue represents sputum steps for supporting data. Inclusion criteria for the TB-positive group were as follows: willing to provide informed consent; adult presumptive TB patients with newly diagnosed TB according to MGIT culture; not yet on treatment; and a known HIV test result. Exclusion criteria comprised a MGIT-negative culture result. Inclusion criteria for the TB-negative control group: willing to provide informed consent; be ≥18 years of age, be willing to be tested for HIV, and no prior diagnosis with TB. Exclusion criteria for the control groups comprised positive results on an interferon-gamma release assay (IGRA) and HIV-test.

Sputum and blood specimens were collected in parallel prior to patients commencing with TB treatment. Sputum was sent to the National Health Laboratory Services (NHLS) for MGIT culture as part of the standard of care. Blood was collected in serum collection tubes (BD 367,812), centrifuged at 1200xg for 10 minutes using an Eppendorf 5810 R refrigerated centrifuge with a swing-arm rotor. Serum was aliquoted into 100 µl quantities in sealed cryo-vials in a biosafety level 2 cabinet and stored at −80°C until analysis.

Control samples were obtained from participant volunteers from 2016 to 2018 who were apparently healthy, showing no symptoms of TB, and had no prior TB diagnosis. Each participant provided one blood tube that was processed in an identical manner to the TB-positive group, as well as three additional tubes that were sent to an independent pathology lab to rule out active TB by confirmatory testing using the QuantiFERON-gold® interferon-gamma release assay (IGRA).

Data collection: Data was obtained using a uniform procedure for all participants, including external laboratory lab testing and internal lab testing as described. Data was collected by the study principal investigator (PI) using the online electronic health records database of the National Health Library Service in South Africa. The current gold standard test for active infection with *M. tb* is sputum mycobacterial growth analysis. This was done using an automated Becton Dickenson (BD) Mycobacterial Growth Indicator Tubes (MGIT) 960 system in the public pathology labs of the NHLS in Pretoria, South Africa. When compared to GeneXpert, MGIT culture has an overall sensitivity of 91.1% and specificity of 100% when confirmatory identification procedures are applied [24]. Variability is driven by sputum quality, whereas HIV-associated paucibacillary disease may slightly slow detection but does not compromise

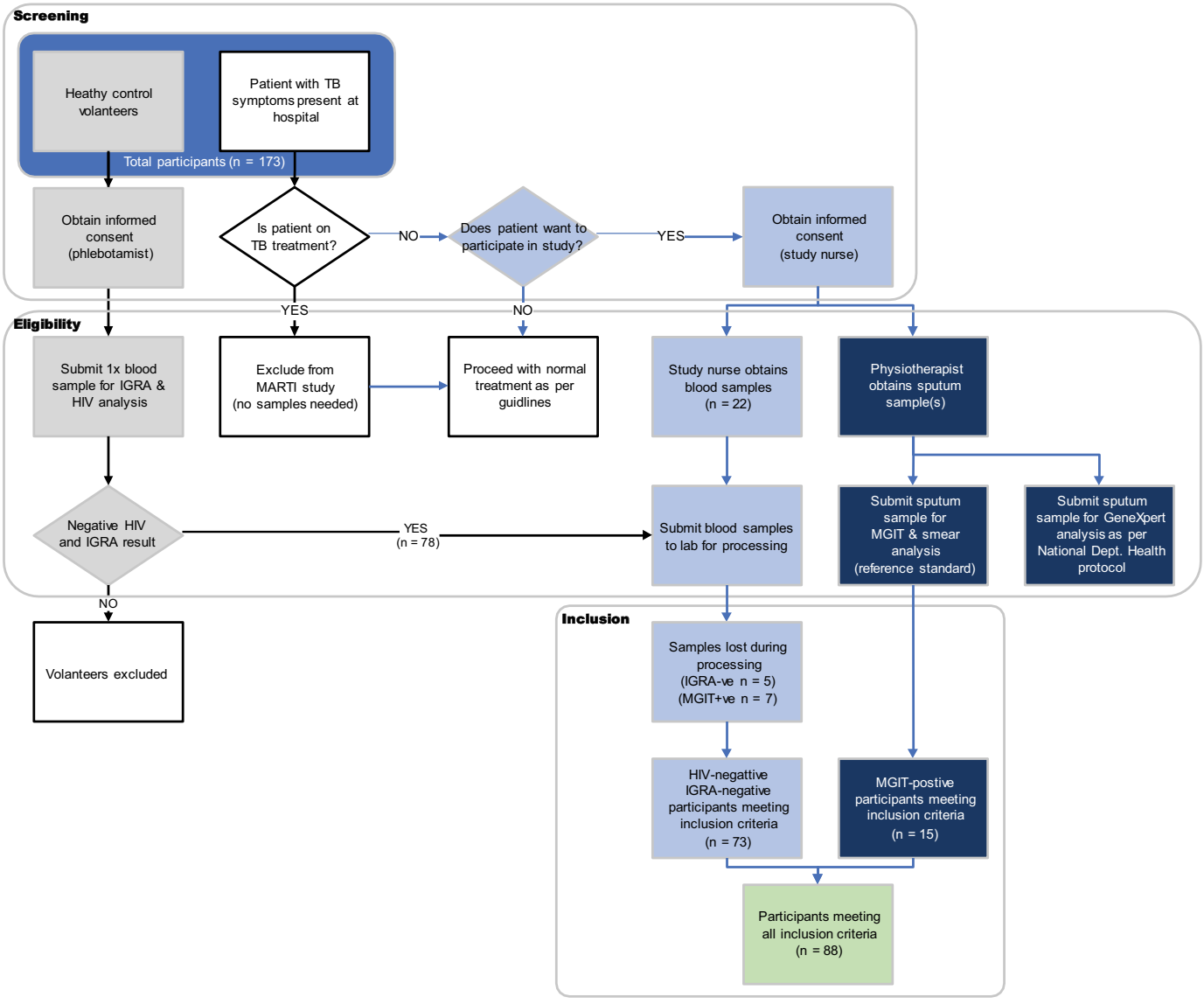

Figure 1. Sample collection protocol for the electro-impedimetric mycolate antibody real-time immunoassay (EIS-MARTI) pilot study with patient inclusion and exclusion criteria. Mycobacterial growth indicator tube (MGIT), interferon-gamma release assay (IGRA), human immunodeficiency virus (HIV), tuberculosis (TB).

specificity [24]. MGIT was used as the gold standard for the statistical comparison to generate the receiver operator characteristic curve.

Laboratory personnel and outcome assessors were blinded to all samples received to minimize detection bias using sequential randomized numbering. After sample analysis, EIS-MARTI results were submitted to the incumbent clinician for unblinding based on access to secondary test information in electronic health records including liquid culture mycobacterial growth indicator tubes (MGIT), GeneXpert and HIV status where available.

Preliminary data indicated that the method yielded poor results at low serum dilutions (1:200). It demanded dilution of at least 1:1000 to overcome typical prozone effects, where the electro-impedance signals of antibody binding were often higher with lower concentrations of serum, even in healthy control sample donors (results not shown). The higher dilution outcome of EIS-MARTI was then used for the pilot validation study reported here.

Descriptive statistics comprised central tendency of the mean and the standard deviation after cleaning by applying the 2.5D rule to rule-out outliers. Mean values were compared to MGIT culture results as TB-negative or TB-positive. Statistical analysis was performed using an online receiver operator characteristic (ROC) curve calculator developed by John Eng at the Johns Hopkins University School of Medicine, Baltimore, Maryland, USA [25]. Statistical significance was set at a p value of ≤ 0.05. Data was codified and inserted into the online calculator. Outputs were then plotted using Microsoft® Excel as ROC curves.

Outcomes: The primary outcome was a correlation of the behavior of EIS-MARTI analyses of the AMAb biomarker with MGIT culture results.

Ethical consideration: Specimen collection, handling of biological specimens and analysis was completed according to a protocol approved by the University of Pretoria Research Ethics Committee, at the Faculty of Health Sciences, reference number 324/2015 which has been

renewed annually until 27 July 2024, with approval number 352/2023.

2.1.2. Monitoring the response to anti-TB chemotherapy by EIS-MARTI

Background and study design: Access to the biorepository of well-characterized human plasma samples collected by the University of Pretoria as a participating site in the Regional Prospective Observational Research for Tuberculosis (RePORT) International common protocol [26] was granted during 2023. During 2019 and 2020, blood samples were consecutively collected at four time points (pre-treatment and at months 1, 2 and 6 of anti-TB chemotherapy) from 25 patients (age range 21–54 years, median 39; female-to-male ratio 9:16). All patients contributed baseline samples, but not equally to all subsequent timepoints. Overall, 61 samples that represented at least a baseline and one later on-treatment or end-of-treatment sample were available for EIS-MARTI analysis. Inclusion criteria: Clinical signs and symptoms suggestive of active pulmonary TB; the ability to provide informed consent; sputum positivity by culture, microscopy, or nucleic acid-based test such as GeneXpert; a willingness to be tested for HIV; agreeing to the collection and storage of blood, urine, saliva, and sputum specimens for use in future research. Exclusion criteria: Exposure to anti-TB drugs for more than 1 week, including any fluroquinolone or other drugs with anti-TB activity. Study diagram flow is shown in Figure 2

Sputum samples were assessed by auramine-O smear, GeneXpert Ultra, and MGIT culture. Blood samples were collected and stored as plasma. All patients received a standard 4-drug first-line anti-TB treatment regimen. Only GeneXpert-confirmed TB patients (19) were recruited.

Sample collection, handling of biological specimens and analysis were completed according to a study protocol based on the RePORT International Common Protocol [26] and approved by the University of Pretoria (UP) Faculty of Health Sciences Research Ethics Committee (Reference number 763/2020; Biomarker approaches for symptomatic, subclinical, and incipient tuberculosis). Data collection was completed using a uniform procedure for all participants, including clinical one assessment, laboratory testing, and longitudinal follow-up.

Outcomes: The primary outcome was a correlation of the AMAb biomarker as detected with EIS-MARTI with the treatment response for 6 months measured with MGIT. The secondary outcome was a correlation of the EIS-MARTI with MGIT start and end of treatment.

Sample size: The cohort of 25 patients that were willing to give consent to participate in this study is representative of

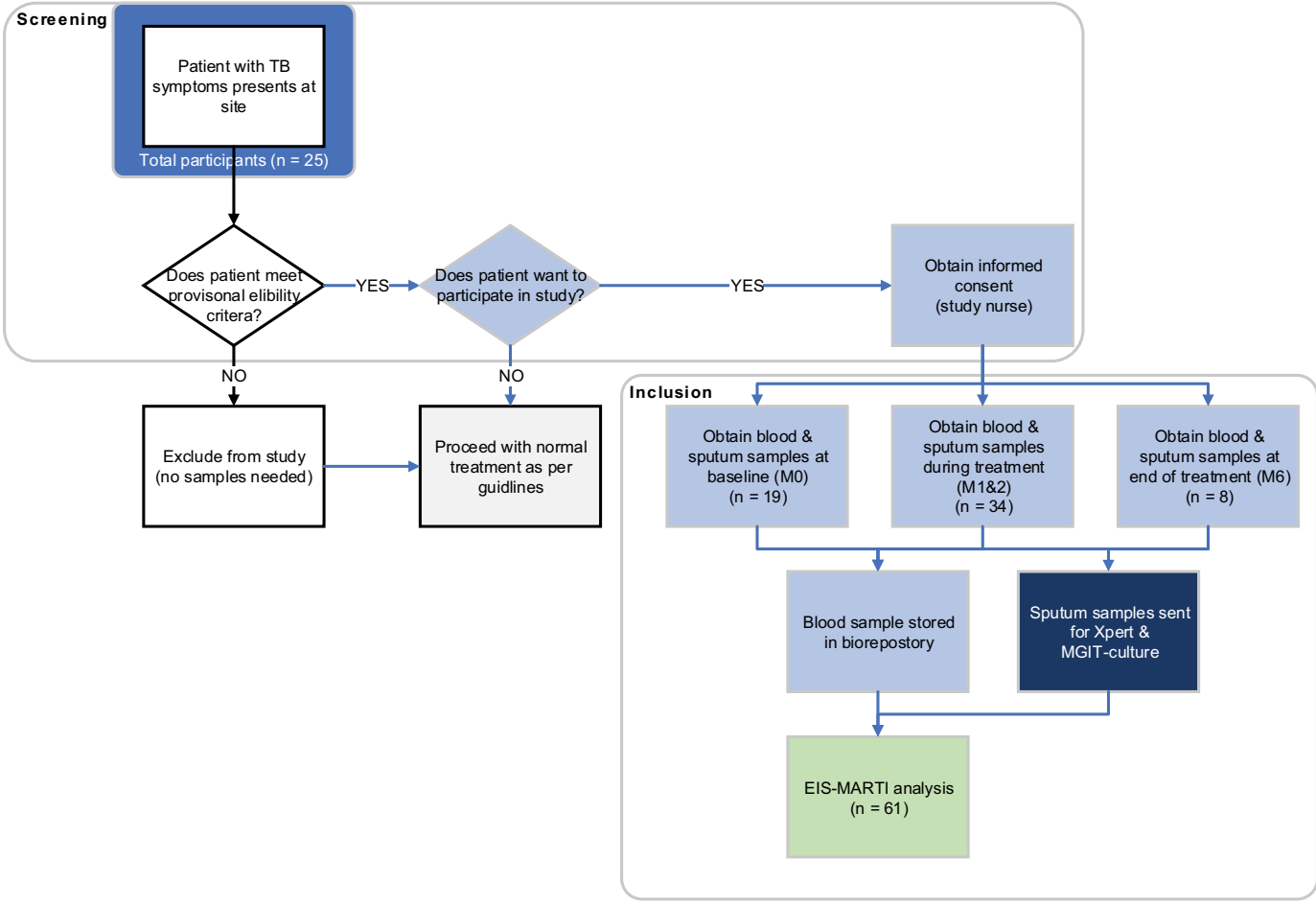

Figure 2. Flow diagram of sample collection protocol for monitoring response to anti-TB chemotherapy by EIS-MARTI study based on the regional prospective observational Research for tuberculosis (RePORT) International Common protocol version 1.1 [24].

a pilot study, the results of which may be used in future to calculate sample size for quantitative comparison of EIS-MARTI with existing TB diagnostics.

Blinding: Laboratory personnel and outcome assessors were blinded to all samples received to minimize detection bias using sequential randomized numbering. After sample analysis, EIS-MARTI results were submitted to the incumbent clinician for unblinding based on access to secondary test information in electronic health records including liquid culture mycobacterial growth indicator tubes (MGIT), GeneXpert and HIV status where available.

Data analysis: Descriptive statistics comprised central tendency of the mean and the standard deviation after cleaning by applying the 2.5D rule to rule-out outliers. Mean values were compared to MGIT culture results as TB-negative or TB-positive. Statistical analysis was performed using an online receiver operator characteristic (ROC) curve calculator developed by John Eng at the Johns Hopkins University School of Medicine, Baltimore, Maryland, USA [24]. Statistical significance was set at a p value of ≤ 0.05. Data was codified and inserted into the online calculator. Outputs were then plotted using Microsoft® Excel as ROC curves.

The current gold standard test for active infection with *M. tb* is sputum mycobacterial growth analysis. This was done using an automated Becton Dickenson (BD) Mycobacterial Growth Indicator Tubes (MGIT) 960 system in the public pathology labs of the National Health Laboratory Services (NHLS) in Pretoria, South Africa. MGIT was used as the gold standard for the statistical comparison to generate the receiver operator characteristic curves in Figure 5

2.2. Laboratory procedures

he MARTI assay is an impedimetric immunoassay, the principle of which is based on detecting a combination of antibody isotypes to mycolate antigens. Mycolate antigens comprise a significant portion of the outermost part of the cell wall of the genus *Mycobacteria* [27]. *Mycobacterium tuberculosis* specifically contains a combination of mycolic acids and lipoarabinomannans that act as antigens.

2.2.1. Electrode preparation and coating

Carbon screen-printed electrodes DS110 (Metrohm-Dropsens, Oviedo, Spain) were submerged for 5 minutes in 99.95% acetone (ACE Chemicals, South Africa), dried for 20 minutes at 80°C in a Labotec Ecotherm oven (Labotec, South Africa) and cooled in a glass desiccator for 5 minutes. Electrodes were then coated with 30 µl of mycolic acids (MA) (Merck Life Sciences, South Africa) in acetone at a concentration of 0.1 mg/ml, using the robotic autosampler of an Autolab ESPRIT biosensor (Autolab, Utrecht, the Netherlands). Trace acetone was removed at 200 mTorr vacuum for 16 hours using a Virtis BenchTop-Pro freeze-drier (SP-scientific, PA, USA). The screen-printed electrodes (SPEs) were subsequently blocked in 1% (w/v) casein hydrolysate in water (Thermofisher Scientific, MA, USA) and rinsed in 18 MΩ doubled-distilled deionized water (ELGA PURELAB, Ultra Bioscience, Buckinghamshire, UK), blotted dry and stored in a glass desiccator.

2.2.2. Electro-impedimetric analysis of serum samples for the EIS-MARTI pilot study

After drying, SPE wells (Metrohm-Dropsens, Oviedo, Spain) were affixed to the electrodes ensuring no overlap on the conductive surfaces. Electrodes were then wetted with redox probe buffer – phosphate buffered saline (PBS) containing 1 mM ferri/ferrocyanide at a pH of 7.44. Baseline cyclic voltammetry (CV) data was recorded between −0.2 V and +0.4 V. Electrical impedance spectroscopy (EIS) data were recorded from 2 kHz to 0.1 Hz at a dc bias of +0.135 V. Electrochemical measurements were completed using a handheld PalmSens 4 potentiostat and Version 5.9 of the PSTrace software (PalmSens, Utrecht, The Netherlands). Baseline impedance data was recorded from the highest inflection point of the Nyquist plot, using the Randle's equivalent circuit.

Each 100 µl aliquot of blood serum was removed from storage at −80°C and thawed at 37°C for 2 minutes prior to use. All fluid handling steps were done using calibrated digital pipettes (Eppendorf, Germany). 30 µl of blood plasma was mixed with commercially available rheumatoid factor blocker (1 mg/ml) (Molecular Depot, CA, USA) and redox probe buffer to effect a 1 in 1000 sample dilution. It was then left to equilibrate for 10 minutes at room temperature. The serum at 1 in 1000 was allowed to bind to the blocked surface of the electrode for 10 minutes, after which impedance data was recorded. The buffer on the electrode was aspirated and discarded. Serum samples at a 1 in 200 dilution were then dispensed onto the electrode and allowed to bind for 10 minutes before impedance data was recorded. Impedance data are measured in ohm (Ω). Data is captured for the three steps being baseline, negative control, and test sample. Test values were subtracted from the negative control and baseline and normalized to a value of between 0 and 100% of the response window in ohm. The threshold for patients considered to have active TB was set at 65% using previously analyzed unblinded patient sera from the MARTI validation trial (Figure S1). All samples were analyzed in quadruplicate.

2.2.3. Electro-impedimetric analysis of plasma samples to monitor the response to anti-TB chemotherapy – a pilot study

Whereas two sample readings were obtained per electrode at 1:200 and 1:1000 serum dilutions of the same sample as discussed in 2.1. Here as an internal negative control, a reading of commercial TB-negative control plasma (Molecular Depot, CA, USA) at 1:1000 dilution preceded the analysis of the trial sample at the same dilution of 1:1000 per electrode. It served the function to compensate for the inter-electrode variation noted during the EIS-MARTI validation trial. Negative plasma at 1:1000 dilution was dispensed onto the electrodes and allowed to bind for 10 minutes. Thereafter the control sample was removed, the test sample was introduced at 1 in 1000 dilution, allowed to bind for 10 minutes and impedance data were recorded. The electro-impedance data

generated for the TB-negative control sample was then subtracted from the diluted trial sample reading to give the EIS-MARTI data that was used to describe the quality of the EIS-MARTI technique. A new cutoff value of 61% of the response window of the EIS-MARTI was established after repeating analyses of several MARTI validation trial samples using this marginally modified method that incorporated a negative control.

2.3. Data analysis

In both the pilot and monitoring studies, biomarker AMAb detection in serum and plasma samples was achieved by measuring impedance values generated using the digital potentiostats with EIS-MARTI. These values were found to vary among the different batches of electrodes provided by the supplier. To standardize the impedimetric data, impedance values were normalized using selected calibration sera analyzed over different batches of electrodes.

2.4. Comparison with the WHO target product profiles

Data generated while monitoring the response to anti-TB chemotherapy using the comparative performance of EIS-MARTI against MGIT were used as an indication to be able to meet, rather than comply with the criteria for TPPs.

3. Results

3.1. EIS-MARTI pilot study

From 2016 to 2018, 173 participants meeting the inclusion criteria were enrolled into this pilot study. 85 participants were excluded based on the exclusion criteria – MGIT culture negative or IGRA positive. 88 presumptive TB patients and negative controls met the inclusion criteria (Figure 1).

Table 1. Study 1 - diagnostic performance of the electrical impedance spectroscopy (EIS)-MARTI in a pilot study against MGIT as a gold standard. Presumptive TB patients and healthy controls were enrolled at Steve Biko Academic hospital and the University of Pretoria from 2016 to 2018, in Pretoria, South Africa. Clinical specimens that met the inclusion criteria were obtained from 88 patients aged 22 to 67 years (median age of 31). 56% were female and 54% were male participants.

Parameter	Value
Number of cases	88
Number correct	82
Pos cases missed	1
Neg cases missed	5
Sensitivity	93.3%
Specificity	93.2%
Accuracy	93.2%
Fitted ROC area	0.973
Empiric ROC area	0.956
Positive predictive value	73,7%
Negative predictive value	98.6%

Receiver operator characteristic (ROC).

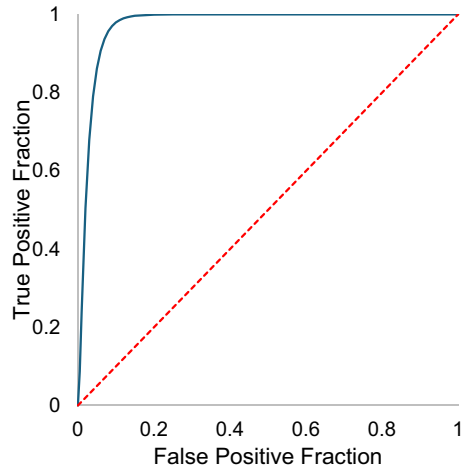

Figure 3. Receiver operator characteristic (ROC) curve of the electrical impedance spectroscopy (EIS)-MARTI data generated from 88 participants in the pilot study (solid blue test line) with an empiric ROC area of 0.956 compared to the gold standard mycobacterial growth indicator tubes (MGIT) culture as the discrimination threshold (dashed red line).

Figure S1 lists the individual results of the EIS-MARTI pilot study and MGIT culture for the 88 participants with age group and site as per the methods section 2.1.1

When compared to MGIT, 14 true positive, 68 true-negative, 5 false-positive and 1 false-negative results were obtained using the EIS-MARTI method. This represents a sensitivity of 93.3%, a specificity of 93.2% with a 93.2% accuracy to detect AMAb, a positive predictive value of 73,7% and a negative predictive value of 98,6% as per Table 1. EIS-MARTI data for each participant is depicted in Figure 1

The ROC curve in Figure 3 depicts the accuracy of the EIS-MARTI test in comparison to MGIT culture with an area under the curve of 0.973. The precision of the method was broad with standard deviations ranging from 1–38% (average of 15,7%) obtained for the 88 samples analyzed.

Outcomes: adherence to the WHO TPPs for screening.

A detailed description of the WHO's target product profiles (TPPs) are summarized and compared to the EIS-MARTI assay in Table 2. The EIS-MARTI appears to meet the minimum requirements to: have a diagnostic sensitivity of >90%; a time to result of < under 30 minutes; be handheld and battery powered; and be simple to navigate with a screen and ability to save results.

The EIS-MARTI appears to meet the WHO TPP optimum requirements for a screening/triage assay by: identifying patients with any symptoms for active TB at the first encounter; work in the target population of adults and children (here data for adults is presented, however due to the nature of the technique being blood-based detection in children is anticipated); be usable by a community health worker as a point-of-care (POC) device; can be used in community, village of higher settings of the healthcare system; has a diagnostic specificity of > 80%; does not use sputum samples; sample preparation can be integrated as a POC device; is disposable with no maintenance as it has no moving parts; and has an internal

Table 2. EIS-MARTI properties that align with the key World Health Organization (WHO) target product profiles (TPPs) for triage or referral test [21].

Characteristic	Optimal requirements	Minimum requirements	Can MARTI meet minimum target?
Goal	Identify patients with any symptoms/risk factors for active TB at 1st encounter	Identify patients with any symptoms of or risk factors for active pulmonary TB at 1st encounter	Yes
Target population	Adults & children with symptoms of active TB	Adults & children with symptoms of active TB	Yes
Target user	Community health worker	Health workers trained to level of auxiliary nurse	Yes (as POC device)
Setting	Community, village, or higher level of the healthcare system	Health posts and primary-care clinics or higher levels of the healthcare system	Yes (as POC device)
Diagnostic sensitivity	>95%	>90%	Yes
Diagnostic specificity	> 80%	> 70%	Yes
Sample type	Non-sputum samples	Sputum/non-sputum preferred	Yes
Manual sample preparation	Sample prep integrated into device	2-steps without timing and measuring	Yes (as POC device)
Time to result	< 5 minutes	<30 minutes	Yes, potentially (as POC device)
Instrument power requirement	None	hand-held device (weighing < 1 kg); battery power or solar power	Yes (as POC device)
Maintenance and calibration	Disposable, no maintenance required	Preventative maintenance after 1-year, minimal expertise or remote calibration	Yes (as POC device)
Operating temp	5–50°C, 90% RH	5–40°C, 70% RH	Not tested
Result capturing and display	Instrument-free with visual readout	Simple-to navigate test menu, LCD screen, ability save results	Yes (as POC device)
Internal quality control	Included for processing the sample and detecting TB	Internal control included for sample processing	Yes

quality control. In this way the EIS-MARTI has significant promise to meet the minimum requirements of and many of the optimum requirements for a screening assay according to the WHO's TPPs.

3.2. Monitoring the response to anti-TB chemotherapy by EIS-MARTI

From 2019 to 2020, 25 participants meeting the inclusion criteria were enrolled into this pilot study. Sixty-one samples were obtained from the participants. Data for one patient was excluded based on a labeling error. All patients were confirmed TB-positive using MGIT culture as per Figure 2

This trial evaluated the behavior of AMAb as a biomarker of active tuberculosis prior to (baseline Month 0), during (Months 1 and 2) and at end of treatment (Month 6). It contained no healthy controls with which to compare the data with.

Supplementary Tables S4A and S4B list the individual results for 61 samples from the 24 patients, including age groups and sites as per the methods section 2.1.2 At baseline, only 15 patients could be confirmed to have active TB based on MGIT outcomes and 8 patients were not confirmed by MGIT. Patient 4 had to be withdrawn due to a labeling error. A list of all the tests completed and their outcomes, together with the sample identity (ID) is included as Table S2A and Table S2B (supplementary information). With the adjusted method of MARTI-analysis (Section 2.2.3), a much-improved precision was obtained with standard deviations ranging from 1–16% (average of 5.9%) for the 61 samples meeting the inclusion criteria and analyzed to monitor the response to anti-TB chemotherapy. Serum samples from the pilot study (Section 3.1) were used as calibration samples to set the new cutoff point of 61% of the response window for distinguishing between TB-positive and TB-negative outcomes when monitoring the response to anti-TB chemotherapy.

3.2.1. Diagnostic assessment of 19 patients at baseline before treatment

Although samples from 25 TB patients were available, data for only 19 participants qualified for inclusion in the final data set. At baseline, only 15 patients could be confirmed to have active TB based on MGIT outcomes and 8 patients were not confirmed by MGIT but by GeneXpert Ultra only. One patient had to be withdrawn due to a labeling error. The data set comprised 15 confirmed MGIT-positive participants and 4 MGIT-negative participants. EIS-MARTI signals matched 11 of the MGIT-positive cases and 4 of the MGIT-negative cases. Therefore, detection of the AMAb biomarker correlated with the MGIT results in 15 of the 19 samples, representing a 73,3% sensitivity, 100% specificity, 78,9% accuracy, positive predictive value of 100% and a negative predictive value of 50% (Column A of Table 3). The ROC curve in Figure 5(A) depicts the accuracy of the AMAb biomarker detected using the EIS-MARTI assay in comparison to MGIT culture at baseline (M0) with an area under the curve of 0.767. Figure 4(A) lists EIS-MARTI signals representative of AMAb binding in descending order for each of the 19 participants at baseline before treatment, for which MGIT data were available. Nine of these patients were HIV-positive. At baseline therefore EIS-MARTI signals gave an accuracy of approximately 79% compared to the 93% scored in the MARTI pilot study (section 4.1). Similarly, the negative predictivity dropped to 50% compared to 99% in the MARTI pilot study, while the positive

Sample ID	Month	MARTI signal	Std. dev	MARTI result	MGIT result
051	M0	97%	3%	TB+	TB+
063	M0	93%	9%	TB+	TB+
006	M0	88%	9%	TB+	TB+
050	M0	88%	3%	TB+	TB+
075	M0	81%	2%	TB+	TB+
068	M0	78%	4%	TB+	TB+
070	M0	75%	9%	TB+	TB+
032	M0	66%	4%	TB+	TB+
061	M0	66%	6%	TB+	TB+
007	M0	63%	4%	TB+	TB+
060	M0	62%	6%	TB+	TB+
049	M0	56%	14%	TB-	TB-
002	M0	56%	1%	TB-	TB-
064	M0	55%	7%	TB-	TB-
048	M0	48%	13%	TB-	TB-
031	M0	48%	3%	TB-	TB+
003	M0	47%	12%	TB-	TB+
001	M0	45%	5%	TB-	TB+
062	M0	40%	5%	TB-	TB+

A

Sample ID	Month	MARTI signal	Std. dev	MARTI result	MGIT result
010	M1	97%	16%	TB+	TB+
042	M1	97%	7%	TB+	TB+
047	M1	82%	8%	TB+	TB-
056	M2	82%	6%	TB+	TB-
038	M1	79%	2%	TB+	TB-
046	M1	78%	3%	TB+	TB-
039	M2	78%	4%	TB+	TB-
065	M1	77%	2%	TB+	TB+
055	M2	74%	4%	TB+	TB-
025	M1	68%	5%	TB+	TB-
036	M2	67%	2%	TB+	TB-
044	M1	63%	7%	TB+	TB-
045	M1	61%	2%	TB+	TB-
035	M1	60%	4%	TB-	TB-
057	M2	59%	16%	TB-	TB-
069	M1	59%	2%	TB-	TB+
054	M2	58%	4%	TB-	TB-
037	M2	58%	7%	TB-	TB-
034	M2	57%	9%	TB-	TB-
033	M2	56%	5%	TB-	TB-
030	M2	53%	3%	TB-	TB-
040	M2	46%	3%	TB-	TB-
041	M2	44%	4%	TB-	TB+
012	M1	43%	6%	TB-	TB-
029	M2	42%	7%	TB-	TB-
053	M1	41%	11%	TB-	TB-
013	M1	39%	3%	TB-	TB+
014	M1	38%	3%	TB-	TB+
052	M1	33%	4%	TB-	TB-
043	M1	32%	6%	TB-	TB+
023	M2	23%	6%	TB-	TB-
066	M1	14%	8%	TB-	TB+
011	M2	12%	5%	TB-	TB-
009	M1	11%	12%	TB-	TB-

B

Sample ID	Month	MARTI signal	Std. dev	MARTI result	MGIT result
017	M6	71%	9%	TB+	TB-
016	M6	45%	4%	TB-	TB-
018	M6	41%	5%	TB-	TB-
020	M6	33%	10%	TB-	TB-
015	M6	33%	3%	TB-	TB-
019	M6	20%	5%	TB-	TB-
022	M6	10%	5%	TB-	TB-
021	M6	6%	9%	TB-	TB-

C

Figure 4. Data representing the anti-mycolate antibody (AMAb) biomarker behavior generated using the electrical impedance spectroscopy (EIS)-MARTI technique, compared to mycobacterial growth indicator tubes (MGIT) as the gold standard for analysis of tuberculosis (TB) patients before and during anti-TB chemotherapy. A: month 0 (baseline), B: month 1–2, and C: end of treatment.

Table 3. Study 2 - summary statistics of electrical impedance spectroscopy (EIS)-MARTI compared to mycobacterial growth indicator tubes (MGIT) result as the gold standard at baseline (M0), during anti-TB treatment (M1–2) and at end of treatment (M6).

Parameter	A: baseline (M0)	B: during treatment (M1–2)	C: end of treatment (M6)
Number of samples (61 samples from 24 patients)	19	34	8
Number correct	15	18	7
MGIT-Pos cases missed	4	6	0
MGIT-Neg cases missed	0	10	1
Sensitivity	73.3%	33.3%	*N/A*
Specificity	100%	60%	87.5%
Accuracy	78.9%	52.9%	87.5%
Fitted ROC area	Degenerate	0.401	*N/A*
Empiric ROC area	0.767	0.44	*N/A*
Positive predictive value	100%	23.1%	0%
Negative predictive value	50%	71.4%	100%

ROC, Receiver operator characteristic.

predictivity increased to 100%, compared to the 74% of the MARTI pilot study.

3.2.2. Assessing EIS-MARTI signal during month 1–2 of treatment

Table 3 column B shows that 18 of 34 plasma samples from 22 patients analyzed using EIS-MARTI matched with MGIT data. From 22 patients contributing samples, 12 had MGIT data available at both month 1 and 2 of treatment, 7 only had month 1 data and 3 only had month 2 data available as per Table S2A and S2B in supplementary information and Figure 4 (B). This represents a 33,3% sensitivity, 60% specificity, 52,9% accuracy, positive predictive value of 23,1% and a negative predictive value of 71,4%. Column B of Table 3 gives the summary of the statistical analysis of the 34 plasma samples from the 22 patients that underwent treatment. The ROC curve in Figure 5(B) depicts the MARTI signals that could be matched with MGIT data for the 34 samples from patients undergoing treatment at months 1 and 2 with an area under the curve of 0,401.

3.2.3. EIS-MARTI outcome at month 6 (end of treatment)

Table 3, column C shows that of eight plasma samples from eight patients analyzed, all tested MGIT negative, which precludes generating an ROC curve. EIS-MARTI signals matched 7 of the 8

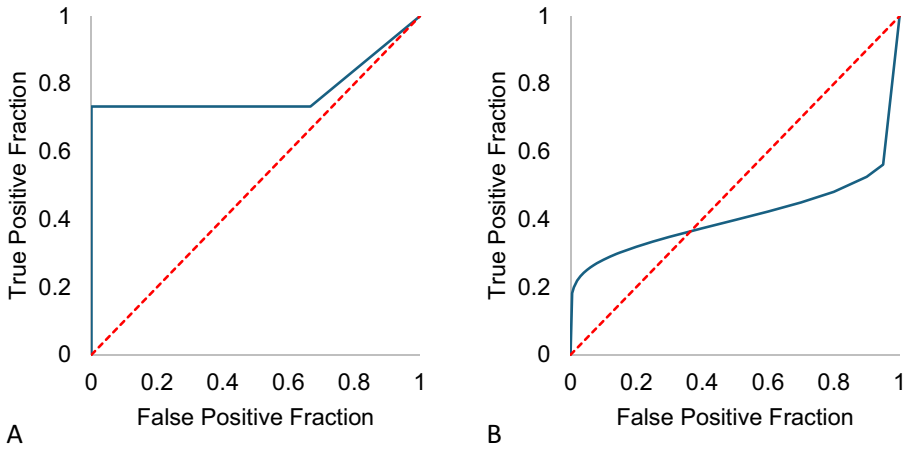

Figure 5. Receiver operator characteristic (ROC) curves (solid blue lines) for the treatment monitoring pilot study of the 19 samples at baseline (A) and 34 samples during treatment months 1–2 (B) compared to the gold standard mycobacterial growth indicator tubes (MGIT) culture as the discrimination threshold (dashed red lines). Empiric areas under the curve are 0.767(A) and 0.44 (B).

Table 4. EIS-MARTI properties that align with the key World Health Organization (WHO) target product profiles (TPPs) for TB treatment monitoring [22].

Characteristic	Min. target	Optimum target	Can MARTI meet minimum target?
Sensitive for poor treatment	≥75%	≥90%	73,3% (at baseline)
Specific for poor treatment	≥80%	≥90%	Yes at baseline & M6, not during treatment
Timing	Halfway through treatment	≤4 weeks	Yes
Frequency	2 points	Follow-up (no baseline)	Yes
Instrument design	Basic lab infrastructure	Point-of-care, portable, battery-operated. Standalone	Yes (as POC device)
Target user	Trained healthcare worker	Healthcare worker min. training	Yes (as POC device)
Target population	People with bacteriologically confirmed TB	All people on TB treatment, incl. HIV+ and children	Yes
Sample type	Sputum	Min. invasive (urine, breath, capillary blood)	Yes
Time to result	≤1 day	≤2 hours	Yes

MGIT outcomes of samples. This yields 87,5% specificity, 87,5% accuracy, a positive predictiveness of 0%, and a negative predictiveness of 100%. EIS-MARTI signals for each of the 8 TB patients at the end of TB treatment for whom MGIT data was available are listed in descending order in Figure 4(C).

Outcomes: adherence to the WHO TPPs and monitoring the response to anti-TB chemotherapy.

Table 4 describes the WHO TPPs minimum and optimum targets to monitor the response to anti-TB chemotherapy relating to the EIS-MARTI technique. With regards to EIS-MARTI, sensitivity of 73.3% does not meet the minimum target of 75%. It meets the minimum target of ≥ 80% at baseline and end of treatment but not during month 1 to 2. It meets the minimum target for frequency of 2 data points, and the optimum timing target of ≤4 weeks; a POC portable battery-operated instrument; can be used by a healthcare worker with minimal training; is suitable for use in all people on TB treatment; uses a minimally invasive blood sample; and can provide a result in ≤2 hours.

4. Discussion

4.1. EIS-MARTI pilot study

The aim of this study was to assess the behavior of biomarker anti-mycolate antibodies detected by using the initial EIS-MARTI method of AMAb detection (section 2.2.2). A surprisingly poor accuracy of detection of AMAb was observed when serum samples were applied at 1:200 dilution, compared to 1:1000 dilution. Lower signals obtained at the higher serum concentration are characteristic of prozone effects that occur when serum concentrations are too high [28]. Of concern was that this effect was observed also with a sizable number of samples from healthy controls, most likely due to the presence of MA cross-reactive anti-cholesterol antibodies that are present in all humans at varying concentrations [29]. At the lower sample concentration of 1:1000 dilution, an acceptable ROC analysis was obtained with accuracy of approximately 93%, albeit with a high degree of variability among the quadruplicate datapoints. The high negative

predictive value of approximately 99% holds promise for potential application of the test to frequently screen people at risk of contracting TB, such as clinical staff in TB hospitals and members of a household where one member is undergoing TB treatment.

Participants were selected based on the inclusion and exclusion criteria as described in Figure 1. The population was representative of both males and females, a broad age range, and those with and without HIV.

4.1.1. Study strengths

The healthy control samples analyzed in the validation trial were all from South Africa, which is considered by the WHO to have a high burden of TB [1] where it is more challenging to ensure with confidence that healthy controls are indeed free of mycobacterial infection. This study made use of the Interferon-gamma release assay (IGRA) because it is known to rule-out TB with high certainty. A meta-analysis of different IGRA tests revealed negative predictive values of greater than 99% [30]. The MARTI-test holds promise to provide a similar negative predictive value to the IGRA test in future fully-fledged clinical trials. An advantage of the MARTI-test over IGRA testing is the improved time to result and minimally invasive sampling associated with EIS-MARTI. IGRA results are typically available within 48 hours, require invasive venipuncture techniques, collection of at least two specimens and transport to a pathology lab. In contrast, the EIS-MARTI, if enabled by a microfluidic cartridge, could give a result in under 30 minutes, would only require 10 µl of capillary blood and has no need for pathology lab infrastructure. This offers significant advantages over the current testing methods that require hazardous and difficult to obtain sputum sampling. It would be of benefit if the EIS-MARTI test could have broader applications than the IGRA test that cannot distinguish between active and latent TB, due to its dependence on active memory T cells to produce the IGRA signal [31]. A positive IGRA test can, besides indicating active TB, also signify mere exposure to TB antigens, such as through vaccination or a previous history of TB disease and cure. The AMAb biomarker should therefore ideally have no memory of previous vaccination or of cured TB disease, an aspect that is discussed under 4.2 below.

Study conclusions: The behavior of the AMAb could serve as suitable biomarker to screen for active TB and does not appear to show memory of previous infection, meeting many of the optimal requirements of the TPPs. If further studies confirm these results, EIS-MARTI could serve as a viable alternative to screen patients for TB resource-limited settings, providing faster and more accurate TB diagnosis.

4.2. EIS-MARTI to monitor the response of TB positive patients to anti-TB chemotherapy

The second aim was to determine the EIS-MARTI's suitability to monitor the response to anti-TB chemotherapy. Here, the data indicated better accuracy at month 0 and month 6 and limited use during months 1 and 2. This study was completed using an adjusted MARTI-method to correct for inter-electrode variations observed with the validation trial discussed in 4.1. Whereas two sample readings were obtained in the MARTI pilot study per electrode at 1:200 and 1:1000 serum dilutions of the same sample as discussed in 4.1, here a reading of commercial TB-negative control plasma at 1:1000 dilution preceded the analysis of the trial sample at the same dilution of 1:1000 per electrode. The electro-impedance data generated for the TB-negative control sample was then subtracted from the trial sample reading to give the MARTI-data that was used to describe the quality of the MARTI-test. A new cutoff value of 61% of the response window of EIS-MARTI was established after repeating analyses of several MARTI validation trial samples using this marginally modified method that incorporated an intrinsic negative control.

Higher sensitivity of 73,3% and specificity of 100% at baseline, compared to sensitivity of 33,3% and specificity of 60% during treatment of months 1–2 imply that the MARTI-test is more accurate and negatively predictive in the early onset of TB, the reason for which becomes clear when considering the performance of the EIS-MARTI test during the first two months of treatment.

The ROC curve for the 22 patients undergoing treatment represented data in which only 12 patients contributed data for both month 1 and month 2, while the remaining 10 patients contributed data for either month 1 or month 2 after treatment. Despite this shortcoming in the cohort tested, the summary statistics of biomarker behavior became especially noteworthy in the peculiar ROC curve obtained with a very low area under the curve (AUC) of 0,401, i.e., a lesser score than would be implied by a completely random outcome that would have been described by an AUC of 0,5. This strange AUC correlated to a sensitivity of approximately 33%, i.e., significantly lower than a random outcome value that would have been implied by a value of 50%. The observation of an initial lower EIS-MARTI score that first increased during treatment before it decreased again at a later stage of treatment, i.e., an active inversion of MGIT and EIS-MARTI outcomes in the initial stages of treatment. The most likely explanation is to be found in the ratio of antibody to antigen concentrations in symptomatic TB patients where initially the MA antigen concentration in the circulation is high, giving a low AMAb: MA antigen ratio and therefore low levels of detectable AMAb. Upon the start of treatment, the MA antigen is reduced, which improves the AMAb:MA antigen ratio and yields a higher detectable AMAb, while the MGIT signal goes down. This implies that the MARTI-test may be unsuitable for monitoring the response to anti-TB chemotherapy in the first few months. The data also explains the drop in sensitivity and negative predictivity at baseline for symptomatic TB patients compared to presumptive TB patients, in the latter of which there is a better likelihood of a lower AMAb:MA ratio that is required for a higher EIS-MARTI signal. The AMAb biomarker signal is therefore dependent on the AMAb:MA antigen ratio, and not a direct quantitative indication of the AMAb concentration in the blood. The EIS-MARTI test is compared to the WHO's TPP's for treatment monitoring in Table 4.

In terms of the effect that co-infection with HIV may have on the outcome of the EIS-MARTI test from Baseline to Month 2, it was noted that only one out of eight HIV-positive patients produced a mismatch with MGIT in terms of the evidence for

AMAb generation, i.e., an indication that the anti-MA antibodies come about in a CD4 T cell independent way in TB patients, thus confirming the initial observation by Schleicher et al. [15] of the independence of the AMAb biomarker detection from HIV co-infection. This is novel in that the EIS-MARTI in its POC guise has good potential to fulfill the knowledge gap in field screening for active TB.

At the end of treatment and within the constraints of the limited dataset, AMAbs were not detected in 7 of 8 MGIT-negative samples using the EIS-MARTI technique (Table 3). A high negative predictive value of 100% in this cohort suggests that the disappearance of the AMAb biomarker within six months of treatment may be useful to confirm the success of treatment. This implies that the AMAb biomarker has no memory of TB disease or vaccination and subsides as TB disease subsides in the patient. This is novel in that it is different from IGRA-based tests that rely on stimulation of memory T-cells [31]. The results suggest that the MARTI-test should be clinically trialed to determine whether it can replace the IGRA-tests to rule-out active TB infection in people at risk of contracting TB, using only a finger prick of blood to obtain a sample for analysis.

4.3. Amenability towards WHO TPPs

The data in Table 4 suggests that the EIS-MARTI does not meet the WHO optimum requirements in terms of sensitivity and specificity to monitor anti-TB chemotherapy progress. However, many other factors including timing; instrument design; target user; target population; sample type and time to result do meet the optimum target. The minimum target is met at baseline and end of treatment, implying that EIS-MARTI may not be suitable for treatment monitoring, but may find application for use in cure confirmation at the end of treatment.

The EIS-MARTI has significant promise to meet the minimum requirements of and many of the optimum requirements for screening according to the WHO's TPPs.

4.4. Study strengths

Working at a higher (1 in 1000) dilution of plasma minimized the prozone effect of cross-reacting antibodies and thus improves the accuracy of the technique. HIV status was included as part of the inclusion criteria and provided evidence that the appearance of the AMAb biomarker in active TB is not affected by HIV co-infection which can have a significant negative impact on existing diagnostic assays. The inclusion of the internal commercial TB-negative human plasma control significantly improved the average standard deviation (5,9%) in this dataset compared to the average of 15,7% from the MARTI pilot study, implying an almost threefold improvement of the precision of the method. The monitoring of the biomarker response to anti-TB chemotherapy was a prospective study that followed patients to the end of treatment. Even though accuracy was not found to be suitable during treatment, it gave good indication of successful chemotherapy at the end of treatment, suggesting an important field of application.

4.5. Study limitations

The limited data limits the interpretations that can be drawn from both these pilot studies. A sample set of 88 participants with only 16% TB-prevalence has the risk of overestimating findings based on an underpowered investigation. Regarding the monitoring anti-TB chemotherapy study, no negative control participants were included other than the inherent TB negative commercial human plasma control against which all patient data was measured. Additional participant sample data is required to obtain the necessary statistical power of internal validation and eventual clinical trials of the EIS-MARTI test in its productized handheld format.

Age is unlikely to have influenced the results as the population seemed representative of a broad age range of adults, but the fact that South Africa ranges among the highest TB burdened countries in the world may significantly impact outcomes of a TB diagnostic test. Socioeconomic status of patient participation can influence TB diagnostic outcomes, as TB is typically a disease of poverty [32]. Co-morbidities such as HIV co-infection, diabetes, malnutrition, or secondary infections may have influenced this study due to the location of sampling, which here was done at health care clinics and a public hospital that mainly draws patients of poverty. Sputum sampling in HIV co-infected participants is known to be difficult [33], which may have been the reason why it was often difficult to obtain sputum samples to allow for MGIT confirmation of GeneXpert TB diagnosis. However, this study design specified an inclusion criterion of known HIV result which would minimize this risk. Diabetes can affect bacterial replication rate, with potential discrepancies in culture yields [34], but was not included as an exclusion criterium in this study. MGIT culture is known to have a high positive predictive value exceeding 90% in high-burdened settings [35], but is no longer generally required by health care providers in South Africa, which caused the inconvenience of having to interrupt sample collection in the validation trial of EIS-MARTI, when the latter was eventually developed to a workable prototype. More TB positive patient samples are needed with adjusted inclusion criteria to allow a proper validation of the test. Among the technical challenges that hindered the validation trial was the inter-electrode variation that manifested as high standard deviations among repetitive MARTI-runs of the same sample. The opportunity for double blind validation ended in the analysis of a skewed population consisting of healthy controls (73) and only 14 TB patient cases. This pilot study was therefore focused on deriving and excluding potential application fields for EIS-MARTI from what could be learned from the AMAb biomarker antibody behavior before, during and after TB treatment.

5. Conclusion

This study provides proof of principle for the detection of AMAbs as a biomarker using minimally invasive blood samples, using a handheld diagnostic device that can in principle be powered by the battery of a mobile phone. Its application fields may be found in situations where sputum samples are

difficult to obtain such as in young children, screening for the initial stages of active pulmonary and extrapulmonary TB rather than confirmation of severely symptomatic patients, and determining the eventual success rather than monitoring of anti-TB chemotherapy.

The pilot study of the EIS-MARTI TB diagnostic method showed that it may be negatively affected by prozone suppression of signal by cross-reactive antibodies in patient and healthy control participants, which can be avoided by applying the serum samples at high dilution. The biomarker and its application as a point-of-care diagnostic seems to meet many of the WHO's target product profiles for a TB screening assay.

The second study, that monitored the behavior of the AMAb in anti-TB chemotherapy, showed that an internal commercial negative human plasma control imbues almost threefold improvement in precision. The decreasing AMAb binding signal during anti-TB chemotherapy indicates that the AMAb biomarker appears to show no memory of previous disease or vaccination against TB. According to the parameters specified in the WHO's target product profiles for treatment monitoring assay, EIS-MARTI does not meet the criteria, however it may be sufficient to confirm cure at end of treatment.

6. Future research

To learn whether the EIS-MARTI test for TB diagnosis can fill the knowledge gap and need for accurate, fast, affordable, high throughput TB screening for active case finding the next steps are to develop a microfluidic cartridge for blood handling to be integrated into the handheld MARTI-test device that will enable external validation of the test at decentralized point-of-care sites to prepare the way for proper clinical trialing. The components of the device are to be tested for rigor, shelf-life and user-friendliness for this purpose.

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Author contribution

Carl Baumeister: conceptualization, methodology, writing – original draft and revised draft, validation, analysis, investigation. **Jan Verschoor**: conceptualization, writing – review and editing, analysis. **Veronica Ueckermann**: sample collection and storage, data curation, final review. **Mosa Molatseli**: validation, analysis, investigation, final review. **Thoriso Sesing**: validation, analysis, investigation, final review. **Nomthandazo Khuboni**: validation, analysis, investigation, final review. **Bernard Fourie**: conceptualization, sample collection storage and data curation, writing – review and editing. **Anton Stoltz**: trial concept, sample collection, and processing – Removed from the list of authors since he passed away.

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No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval and/or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Ethics approval for study to monitor anti-TB chemotherapy was obtained from The Research Ethics Committee, Faculty of Health Sciences, University of Pretoria, and complies with the International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use (ICH) Guideline for Good Clinical Practice (GCP) (ICH-GCP) guidelines and has US Federal-wide Assurance. FWA 00002567, approved May 2002 and expiry by 18 March 2027. IORG# IORG0001762. OMB No. 0990–0279. Approved for use through 28 February 2022 to expiry on 3 April 2023. Renewal was obtained. OMB No. 0990–0278. Approved for use through to 31 August 2023. Ethics reference No. 763/2020. Title: Biomarker Approaches for Symptomatic, Subclinical and Incipient Tuberculosis.

Data availability statement

All data required is included within the text of the manuscript.

AI declaration

No generative AI tools were used in content generation or proofing.

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