Interferon gamma release assays for tuberculosis and their potential as efficacy markers for intervention trials
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CHAPTER 8

8. INTERFERON GAMMA ELISPOT AS A BIOMARKER OF TREATMENT EFFICACY IN LATENT TUBERCULOSIS INFECTION: A RANDOMIZED, BLINDED AND PLACEBO CONTROLLED CLINICAL TRIAL.

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Interferon gamma ELISPOT as a Biomarker of Treatment Efficacy in Latent Tuberculosis Infection: a Randomized, Blinded and Placebo Controlled Clinical Trial

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Abstract

Background
Biomarkers to evaluate new interventions against latent tuberculosis infection (LTBI) and active TB disease are urgently required.

Methods
We undertook a randomized, placebo controlled trial of Isoniazid (INH) in ESAT-6 (E) and CFP-10 (C) ELISPOT and Mantoux positive TB contacts. 211 contacts received a 6-month course of 900mg INH twice weekly or placebo. Primary endpoints were qualitative and quantitative reversion of EC ELISPOT tests. EC ELISPOT testing was done at baseline, 1, 3, 6 and 12 months. INH acetylator genotypes were determined. Qualitative and quantitative reversions were assessed by conditional logistic regression, Wilcoxon signed-rank test and mixed-effect compound Poisson models. This trial was registered with ClinicalTrials.gov (NCT00130325).

Results
The proportions of EC ELISPOT positive subjects reduced over time (p <0.001) but did not differ by treatment allocation (p=0.36). EC spot forming units (SFU) also declined significantly with time (p<0.001) but not by study arm (p=0.74 and 0.71 respectively). No interactions were found between acetylator status, INH treatment and ELISPOT results over time.

Conclusions
In TB contacts with LTBI, INH therapy has no impact on qualitative and quantitative reversion of positive EC ELISPOT tests over time. The EC ELISPOT is probably not a useful biomarker of treatment efficacy in LTBI.

Keywords:
Tuberculosis, Latent Infection, Isoniazid, Chemoprophylaxis, Interferon gamma release assays, Tuberculin Skin Test, Clinical Trial, ELISPOT reversion,
Introduction

Tuberculosis (TB) remains a significant public health problem causing nearly two million deaths from ~ 9 million cases annually.¹ New vaccines, diagnostic and shorter treatment regimens are required to combat TB globally. Development and assessment of the utility of novel vaccines and therapeutic agents are currently limited by absence of appropriate biomarkers that correlate with protection from vaccination; and efficacy of treatment regimens in active disease and latent TB infection (LTBI).²

Interferon gamma (IFN-g) produced by T-Lymphocytes is a key effector molecule associated with protective immunity to M. tuberculosis.³ The IFN-g response to specific M. tuberculosis antigens (ESAT-6, CFP-10) is used in IFN-g release assays (IGRAs) as a biomarker for screening immune responses in TB vaccine trials and for diagnosis of latent⁴ and active tuberculosis⁵. It has been assessed as a prognostic marker for treatment efficacy⁶ ⁷, and progression to disease⁸. There is some evidence IGRA readouts may correlate with M. tuberculosis antigen load. Significant qualitative and quantitative test reversion following effective therapy for active TB disease or chemoprophylaxis for LTBI have been reported.⁶ ⁹ ¹⁰ However, spontaneous conversions and reversions of IGRAs have also been reported from cohort studies of untreated latent TB infection ¹¹ ¹². Therefore we conducted a formal evaluation of an IGRA, as a biomarker for treatment efficacy in LTBI, using a randomized blinded placebo controlled clinical trial.
Methods

Study site and population

This trial was conducted at Medical Research Council (MRC) Unit, Fajara The Gambia from 2004-2010. The study area is mainly urban with a population of 698,581 people (2003 Census, Gambia Bureau of Statistics).

The study was approved by the MRC/Gambia government and the London School of Hygiene and Tropical Medicine ethics committees (registered as SCC 965). It was registered at ClinicalTrials.gov registry with identifier NCT00130325.

Trial design

This was a blinded, parallel group, placebo controlled randomized trial

Participants

Written informed consent was obtained from every participant after they had been given a chance to consider trial information including HIV counselling. Inclusion criteria were; household contacts aged ≥15 years of newly diagnosed sputum smear positive TB cases, residency in study area and most of the time in the same compound as the case; normal physical examination and chest x-ray; evidence of LTBI (defined as a positive tuberculin skin test [TST, ≥10mm size] and positive EC ELISPOT test). They were excluded if they had a clinical diagnosis of TB, significant history of immunosuppressive or allergic disorder; haemoglobin <8g/dl; serum creatinine concentration >130mmol/l, alanine transferase >80IU/L and aspartate transferase >42IU/L and Hepatitis B surface antigen or HIV positive.

Procedures

Clinical

The interventions were Isoniazid (INH) 900 mg, or matching placebo. INH and placebo supplied by Svizera ® (Mumbai, India) were identical in taste, size and appearance. They were delivered packed in separate plastic containers and were re-packed into drug envelopes by staff unconnected to this study.

Enrolled participants received a 6-month course of 900 mg INH or placebo twice a week by Directly Observed Preventive Therapy (DOPT) at home or a location of their choice.
Randomisation and masking:

Sequence generation
Balanced recruitment numbers in each arm of the study was ensured using block randomization to generate the randomization code corresponding to a list of sequential enrolment numbers. The study investigators, subjects and laboratory staff were unaware of block sizes.

Allocation concealment mechanism
The safety monitor held the generated randomization code and study number corresponding to study arm in a safe inaccessible to trial staff.

Blinding
The sequential enrolment numbers corresponding to the randomization code and linked to numbered, and pre-packaged, envelopes containing INH or placebo were used to assign participants to study arms. All study personnel, subjects and laboratory staff responsible for EC ELISPOT testing was blinded to treatment allocations.

Implementation
Sampling
A 20ml blood sample was taken at screening and 15ml at 1, 3, 6 and 12 months after enrolment. At baseline, ELISPOT, HIV and Hepatitis B serology, electrolytes, urea and creatinine (E&U, Cr), Liver Function Tests (LFTs), and full blood count (FBC) were performed. Genomic DNA was extracted from whole blood to determine N-acetytransferase 2 (NAT2) polymorphisms i.e. INH acetylator status. FBC, E&U, Cr and LFTs were also performed at follow-ups. Fresh mid-stream urine specimens were obtained at 3 months 24-36 hours after ingestion of the tablets provided (INH or placebo) to identify INH metabolites. During follow-ups, participants were asked about side effects of the medications and symptoms suggestive of TB disease. Those with symptoms were evaluated for active TB. The trial schema is shown in figure 1.

Laboratory
Sputum smear microscopy was done with Auramine phenol and Ziehl-Neelsen staining. Isolation and identification of *M. tuberculosis* was done using recommended techniques. Drug resistant forms of *M. tuberculosis* are very uncommon in The Gambia.
HIV status was determined by enzyme linked immunosorbent assays (Murex 1.2.0, Abbott-Murex Biotec, Dartford, Kent, UK), Hexagon HIV (Human Diagnostics GmbH, Wiesbaden, Germany) and type specific immunoblotting kit (Pepti-LAV I/II, BIORAD, Marnes-la-Coquette, France) for confirmation.

The ex-vivo IFN-γ ELISPOT assays were performed as previously described. ESAT-6 and CFP-10 peptide pools were used at 5 μg/ml while Phytohaemaglutinin (PHA 5 μg/ml; Sigma, Aldrich, UK) was used as positive control with medium as negative control. EC ELISPOTs were done within 4 hours of sampling: 200,000 peripheral blood mononuclear cell (PBMCs) were incubated in duplicate wells for each *M. tuberculosis* antigen and controls for 16-18 hours. Numbers of spot forming units (SFUs) were counted with an automated ELISPOT reader (AID-GmbH, Strasberg, Germany). Positive test wells were pre-defined as those with ≥ 8 SFUs/well/2x10⁵ PBMCs more than negative control wells. For a positive ESAT-6/CFP-10 result, one or more pools of peptides had to be positive. PHA wells were set to 150 SFUs/well/2x10⁵ PBMCs above negative control wells. Negative control wells had to have less than 20 SFUs/well/2x10⁵ PBMCs. EC ELISPOTs were considered to have failed when specifications for negative control and PHA wells were not met. EC ELISPOT conversion and reversion were defined as newly positive or negative test and change in the combined ESAT-6 and CFP-10 count (above the negative control) of ≥ 6 SFU/well/2x10⁵ compared to the previous result.

Urine samples were tested using Isoscreen® (GFC Diagnostics, Oxfordshire, UK) for presence of INH metabolites, as recommended ([http://www.gfcdiagnostics.co.uk/isoprocedures-print.htm](http://www.gfcdiagnostics.co.uk/isoprocedures-print.htm)). The Isoscreen is a device with an upper 2mL syringe and a lower reaction chamber containing dried chemicals for the Arkansas test method. Unprocessed urine was aspirated, injected onto the dried chemicals, shaken and 5 minutes was allowed for stable colour development. Presence of INH metabolites was denoted by a dark blue/purple colour, green indicated metabolites of INH taken more than 36 hours previously and no colour meant metabolites were absent. Sensitivity of 95-98% has been reported for this method.
Polymerase chain reaction amplification of the entire coding region of the NAT2 gene (870 bp) was performed as previously described. The NAT2 gene sequence from Ensembl public database (ENSG00000156006 transcript) was used as the reference sequence for detecting mutation. Acetylator status was classified according to the NAT2 gene allele found—NAT2*4, NAT2*11A, *12A, *12B, *12C and *13A alleles are fast acetylators; and NAT2*5A, B and C, NAT2*6A, B and C, NAT2*7B, and NAT2*14A and B are slow acetylators. The other NAT2 alleles are also thought to give slow acetylator phenotypes.

**Sample size**
We estimated from previous studies qualitative reversion in the control group would be approximately 40% at 3 months. At 5% significance, a sample size of 300 would give 80-90% power to detect an increase of 20% reversion for the intervention. A 20% difference between the arms was considered the minimum for a clinically significant biomarker. The target sample size at the start of the study was 150 ESAT-6/CFP-10 and Mantoux test positive contacts of tuberculosis patients per study arm. The trial was stopped after recruitment of 211 participants for futility because the data and safety monitoring panel found reversion overall was not different from that seen in our previous uncontrolled longitudinal study of untreated contacts. And it was clear further recruitment to the target sample size was unlikely to change this outcome.

**Outcomes**
The primary outcomes were qualitative (positive/negative) and quantitative reversion (decline in spot forming units) of positive ELISPOT results over time compared between study arms.

**Statistical analyses**
All data were double entered into a database and statistical analyses were performed using Stata 12.1 (Stata Corp LP, College Station, Texas) and R 2.15.0. The main outcomes were differences in qualitative and quantitative reversion of the ESAT-6 and CFP-10 IFN-γ ELISPOT by treatment arm. A mixed effects (random effect for subject) logistic regression model was used to compare the proportion of positive responders for CFP-10 and ESAT-6, between the
treatment arms. The quantitative responses were zero inflated and severely over dispersed. To allow for this, a mixed effects Tweedie (compound Poisson) model was fitted using the R package cplm. All regressions were adjusted for potential confounders such as age, gender, sleeping proximity to index TB case, BCG scar, and sputum smear grade in index case. Statistical significance was measured at the 5% level.
Results

Patient Characteristics
A total of 1659 household contacts were screened with the TST, 643 (38.8%) and 1016 (61.2%) were TST positive and negative respectively. Only 32.8% (211 of 643) were TST and ELISPOT positive and eligible for enrolment into this study (see figure 2). ~ 90% of participants (189 of 211) completed study procedures and were analyzed for outcomes of interest. The baseline characteristics of the enrolled participants were similar (see Table 1). Overall 2% (range 0.5-4.2% across the time points) of all ELISPOT tests failed.

Quantitative results over time

ESAT-6
The medians and interquartile ranges (IQR) SFUs/200,000 PBMCs for ESAT-6 were similar: 28.5 (IQR: 15-60) and 30.5 (IQR: 16-57) for placebo and INH arms at recruitment respectively. There was no significant interaction between time and treatment arm (p=0.91) with a significant decline in SFU’s (p<0.001) during the first month of treatment, but no significant difference in decline between treatment arms (p=0.81). In both study arms, there was ~ a 2-fold decline (a 50% decrease) in SFUs between recruitment and the first month as shown in figure 3A. There were no significant interactions for acetylator status.

CFP-10
The medians and IQRs for CFP-10 were lower than for ESAT-6 but were similar across study arms at recruitment: 12.5 (IQR: 3-26) vs. 9.5 (IQR: 4-20.) for placebo and INH arms respectively (figure 4B). As with ESAT-6, the interaction time and treatment arm (p=0.81) was not significant. Significant decline in SFUs between recruitment and 1 month (p<0.001) and 1 and 3 months (p=0.018) was seen, but no significant differences in decline between treatment arms (p=0.9) were noticed. The fold changes were 1.2 and 2.4 for the INH and placebo arms respectively by the first month of follow-up but these did not differ significantly. There were no significant interactions for acetylator status (p=0.50)
Qualitative results over time

The SFU count data was converted to dichotomous data by considering a cut-off of ≥ 8 SFUs/well/2x10^5 PBMCs above the medium for ESAT-6 and CFP10. For a combined response based on both ESAT-6 and CFP10, one or both antigens had to be positive. At 5% significance level, the qualitative data analysis confirmed the inference of no differences in reversion between the treatment and placebo groups, as seen in the analysis of continuous data.

**ESAT-6**

At recruitment, 95.2% (100 of 105) of subjects in the Isoniazid arm were ESAT-6 positive while 93.4% (99 of 106) of those in the placebo arm were positive. After 1 month of treatment, 68.1% (95%CI: 57.7-77.3%) and 73.3% (95%CI: 63.0-82.1%) remained positive in the placebo and INH arms respectively, while at 6 months, 65.2% (95%CI: 54.3-75.0%) and 66.3% (95%CI: 55.5-76.0%) were positive for both arms respectively (see figure 4). By 12 months, there was 27.4% reversion in the placebo and 30.9% in the INH arm. However, consistent with the analysis of the count data there was no significant difference in qualitative ESAT-6 reversion by study arm (p=0.78).

**CFP-10**

There were 65 of 105 and 58 of 106 subjects with positive CFP-10 results in the placebo and INH study arms respectively (see figure 4). There was more reversion in the placebo arm (25.4%) at 12 months compared to the INH arm (9.4%). This was probably due to a slight increase in CFP-10 positive results at 12-months in the INH arm. Again, the difference in proportion of subjects reverting was not significant at the 5% level.

**Combined ESAT-6 and CFP-10**

At enrolment, all subjects (99.1%) were EC ELISPOT positive except 2 (1 subject in each study arm recruited had negative results and were reported as protocol deviations). Only 56.6% (60 of 106) and 49.5% (52 of 105) were positive for both ESAT-6 and CFP-10 in the placebo and INH arms respectively. There were no differences in the pattern of reversion between study arms while allowing for time at the 5% level.
Urine test for INH Metabolites

The Isoscreen urine test results for INH metabolites were available for 121 subjects (61 in the INH arm and 60 in the placebo group). Positive test results were obtained from 50 and 1 subject(s) from the INH and placebo groups respectively giving 82% [70-90.6%] sensitivity and 98.3% [91.1-100%] specificity. The positive predictive value (PPV) was 98% and negative predictive value was 84.3%. The results of the urine test were unaffected by acetylator status (OR 1.02 [0.60-1.80], p=0.95).

Acetylator Status-specific analysis

The acetylator status was determined for 171 consecutively enrolled subjects i.e. 82 in placebo arm and 81 in the INH arm. Of these, 65 (30.4%) were slow acetylators, 98 (45.8%) fast acetylators and for 8 (3.7%), acetylator status could not be determined.

Within the INH group, reversion and acetylator status were defined for 70 of 106 participants: 25.7% (18 of 70) and 48.1% (25 of 52) were fast acetylators among the reverters and non-reverters respectively (p=0.09). There was no difference in the decline of SFUs/200,000 PBMCs for ESAT-6 and CFP-10 by acetylator status (p=0.44).
Discussion

In this trial, we have shown a significant decline in qualitative and quantitative EC ELISPOT results occurs over in TB contacts with LTBI but unrelated to INH therapy. There was also no evidence of any interaction between acetylator status and INH therapy with respect to ELISPOT reversions. This is the first formal assessment of the EC ELISPOT as an efficacy biomarker for treatment of LTBI that takes into account the likely influence of a genetically determined difference in drug metabolism among participants.

The results have implications for the usefulness of T cell responses in biomarker discovery and evaluation, and the clinical trial methodology utilised can serve as a model for future biomarker evaluations. In particular this study exposes the limitations of uncontrolled studies in the evaluation of biomarkers in LTBI. We acknowledge the importance of confirming our findings in other independent datasets and varied settings.

Interferon gamma release by activated CD4+ T cells is an essential component of the protective immune response to *M. tuberculosis*. Reports of IGRA reversion in active disease and LTBI suggest an association between the magnitude of this immune response and load of infecting *M. tuberculosis*. Spontaneous reversions and conversions of IGRA in LTBI have been seen as well. Our data suggests claims of INH induced reversion in latent tuberculosis infection from uncontrolled before and after studies appear incorrect: the reversion seen is most likely due to natural decline of T-cell frequencies and not therapy.

These results add new insights and raise new questions regarding understanding of LTBI as a spectrum of ongoing host-pathogen interactions. They suggest T-cell frequencies, as measured by IFN-γ ELISPOT response to EC, do not accurately reflect organism load during or after INH treatment for LTBI. An intriguing question, in the presence of persistently elevated T cell frequencies, is whether treatment with INH reduces the bacterial load but does not eliminate the pathogen, or whether INH has a delayed effect. While there is some animal model evidence that INH has a relatively delayed effect, significant organism clearance would be
expected over the course of treatment. It is likely different *M. tuberculosi*s antigens are secreted during the different stages of natural LTBI. A mix of antigens, including those predominantly secreted in quiescence, might be a better predictor of *M. tuberculosi*s bacillary burden in response to therapeutic interventions. However, it is possible the T cell response to LTBI is simply too complex to provide a reliable efficacy biomarker. For example, some individuals maintain an effector response even in the absence of ongoing antigen stimulation.

Since host metabolism may affect the pharmacokinetics of administered INH, we expected qualitative and quantitative reversion in those receiving INH to be greater in slow acetylators. However, we found no evidence to support this.

Since compliance with treatment regimens for LTBI chemoprophylaxis is a well known problem, a DOPT strategy using a twice weekly INH regimen was used. The detection of INH metabolites in urine (Isonoscreen test) added further evidence of compliance and correct allocation. While the urine test suggested that compliance was less than 100% and at least one person in the placebo arm received INH, this may reflect the limitations of the test. If the study allocation is used as the gold standard, the performance of this test is similar to findings in other settings.

Our study has some strengths; it is the first of its kind, employed a robust study design and 90% of participants had analyzable results. It was conducted in a setting of extremely low INH resistance in primary and re-treatment cases of TB. Adherence to intervention was assured by use of simplified treatment regimen administered under direct observation. In addition, adherence was largely confirmed using a urine test and the possible role of genetically determined drug metabolic patterns was investigated.

However, there were some limitations. The subjects studied may not be representative of all TB contacts with LTBI because of the strict ‘double positive’ inclusion criteria. Although these ‘double positive’ subjects are less likely to have spontaneous reversions, it might not have been necessary to have such a stringent inclusion criterion as discordant individuals have
similar rates of progression to active disease as double positives. IGRAs are known to perform differently in low vs. high burden settings so results here are not necessarily generalizable to all settings. As the estimated incidence of TB in The Gambia is 273 per 100,000 population, exogenous re-infection may have had some impact on our results. Taking into account the study design, this would have had the same impact across study arms. For practical purposes we screened with EC ELISPOT only if Mantoux positive. There are some reports of boosting of IGRA results when TST is done first. However, the consensus is TST has little or no effect on IGRA results when done after 72 hours. EC-ELISPOT in this study was done 4 weeks after TST. The reversion rates here are similar to those in our previous studies where EC-ELISPOT was conducted at the same time as the TST. Although a small minority of EC-ELISPOT tests failed, the numbers of failed tests were evenly distributed across study arms. There is a small risk of bias since not all (100%) of the data was available for analysis but the chances of the missing results affecting the conclusions here are extremely low.

For high TB burden settings, current global guidelines recommend INH preventive treatment (IPT) in TB contacts under 5 years old, but this recommendation is rarely implemented by TB programmes. While this study used INH as a tool to evaluate a biomarker, it showed high levels of success in giving IPT in adults. This may be useful to the search for ways to successfully implement WHO policy in under 5 year old children, and for its expansion to older children and adults in the future. It is noteworthy both arms of the study were followed actively for the development of TB disease for a period of 12 months, providing a higher standard of care for all participants than standard practice.

A biomarker is a characteristic that is objectively measured and evaluated as an indicator of a pathogenic processes or responses to therapy. It has correlates with the activity of the disease process and/or provides feedback on treatment efficacy. This study has shown that qualitative and quantitative reversion of the EC-ELISPOT has no utility as an efficacy marker for treatment of LTBI.
Acknowledgements

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Contributors

PCH, RB and RAA obtained funding for and IMOA and HBI initiated the study. PCH and RB conceived the study. IMOA, MDL, AH, NB, PO, MA, PA acquired the data. IMOA and DJS analysed and interpreted the data. IMOA, PCH and MOC drafted the paper and all authors contributed to the critical revision and development of intellectual content. MOC, RW, PA, MA, NB, SD provided administrative, technical, data management and material support. PCH, MOC and RAA were the study supervisors. All authors contributed to and have seen the manuscript. The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication.

Conflicts of interests

The Authors have no conflict of interest to declare
Table 1. Basic Characteristics of the Trial Population

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Figure 1. Trial Schema
ELISPOT-Enzyme Linked Immunospot Assay
IFN-g-Interferon gamma
TST-Tuberculin Skin Test
Figure 2. Trial Profile
Figure 3. Comparison of median SFUs over time for ESAT-6 and CFP-10 by trial arm

A. ESAT-6

B. CFP-10
Error bars represent the interquartile range (IQR)
Figure 4. Changes in proportions of contacts positive for combined and individual antigen ELISPOT assay during TB treatment and follow-up.

A. Combined ESAT-6 and CFP-10 ELISPOT Results

B. Individual ESAT-6 and CFP-10 results by study arm across time periods
References


