

Protection from *Mycobacterium tuberculosis* infection: Learning from exposed but uninfected children

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Submitted in fulfilment of a Doctor of Philosophy Degree

February 2018

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ABSTRACT

Background: Children are more susceptible than adults to progression from infection to tuberculosis and development of severe forms of disease. However, most children exposed to *Mycobacterium tuberculosis* are able to effectively control the pathogen without becoming infected. A better understanding of effective paediatric immunity to tuberculosis infection and discovery of correlates of protection could help to develop a novel vaccine as part of the End TB Strategy.

Methods: A matched case-control study was conducted in The Gambia recruiting pairs of children with discordant tuberculin skin test infection status but the same sleeping proximity to the same adult with sputum smear positive tuberculosis (**H**ighly **E**xposed **I**nfected [HEI] and **H**ighly **E**xposed **U**ninfected [HEU] children). Whole blood samples were used in a novel assay with an autoluminescent strain of *Bacillus Calmette–Guérin* (BCG). Serial luminescence measurements were recorded and supernatants from baseline, 24 hours and 96 hours were analysed with a multiplex assay to detect cytokines and targeted mass spectrometry to detect eicosanoids.

Results: 29 pairs of HEI and HEU children aged between 5 and 15 years old were enrolled. HEU children had higher levels of luminescence at 96 hours than HEI children, on the threshold of statistical significance. HEI children produced significantly more BCG-specific interferon- γ than HEU children at 24 and 96 hours. At 24 hours, HEI children had significantly suppressed BCG-specific levels of 12-oxo-LTB₄, a metabolite of the pro-inflammatory eicosanoid LTB₄. BCG-specific levels of the prostacyclin metabolite, 6ketoPGF-1 α , were significantly higher in HEI children at baseline, but this pattern was reversed at 24 hours.

Conclusions: In the functional luminescence assay, HEU children did not demonstrate superior control of mycobacterial growth than HEI children. BCG-specific interferon- γ levels reflected the infection status of the children. A potential role of prostacyclin in paediatric immunity against *Mycobacterium tuberculosis* infection has been identified with implications for vaccine design and adjuvant host-directed therapy.

DECLARATION

I declare that all of the work presented in this thesis is my own, except where I have referenced or acknowledged the work of others.

Dr Robindra BASU ROY

February 2018

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DISCLAIMER

At the time of submission of the final thesis, a manuscript containing parts of Chapter One has been accepted for publication by The Lancet Infectious Diseases.

ACKNOWLEDGEMENTS

I am extremely grateful for the Clinical Research Training Fellowship that supported this doctoral research project (MR/K023446/1). This fellowship was jointly funded by the UK Medical Research Council (MRC) and the UK Department for International Development (DFID) under the MRC/DFID Concordat agreement and was also part of the EDCTP2 programme supported by the European Union.

The study was further enabled by the Program Grant awarded to Professor Beate Kampmann (MR/K007602/1, MR/K011944/1, MC_UP_A900/1122)



I am indebted to the kindness and generosity of the children and families that agreed to take part in this study. I will always remember the warm welcome, many deep friendships, and wonderful things learnt in The Gambia.

Beate Kampmann has been all that one could want as a PhD supervisor and academic mentor. She has followed the twists and turns of my life for many years and always been a supportive, positive and encouraging influence.

Without Basil Sambou and Muhamed Sissoko, this project would not have been possible. It was a pleasure and privilege to have Basil Sambou as the Trainee

Scientific Officer for this project. He taught me a great deal, and I hope I gave him something back in return. Muhamed's work ethic and charm worked well both with the families and within the team!

There are too many at MRC Unit The Gambia to list individually but I am all too aware of all the small contributions that go towards projects such as these being successful.

The childhood and adult TB teams at the Gambia were always a great source of support, help and laughter. Again, there are too many to name but many thanks to Uzoh, Leo, Toyin, Abdou, Sheriff, Monica, Fatoumata, Fatou, Njilan, Edward, Abigail, Marie, Hadi Jane, and Jayne for their specific help.

I learned a great deal from Clinical Services at MRC Unit The Gambia and am particularly grateful to Suzanne Anderson and Yande Osagiede. Thanks to Sophie Roetynck in the Vaccines & Immunity theme for her patience, kindness, scientific support and resilience-building!

Many thanks to Elaine Holmes for co-supervising my PhD and all at the Centre for Computational and Systems Medicine, especially Arnaud Wolfer, Nick Andreas, Maria Gomez-Romero and Verena Horneffer-van der Sluis for introducing me to the world of metabonomics and mass spectrometry.

Statistical advice and encouragement with STATA came courtesy of Artemis Koukounari, Schadrac Agbla and the Harvard Global Clinical Scholars Research Training Program.

For all things mycobacterial, thanks Brian Robertson and Iria Uhia-Castro!

Team Kampmann – Beth Holder, Nick Andreas, Tom Rice, Anna Battersby, Kirsty Le Doare, Myrsini Kaforou and Vanessa Sancho Shimizu (honorary members) – thanks for the friendship and early lunches!

Thanks Denis Keane - for being so good, so silly, and so distracting.

Thank you Babak Javid and Eric Rubin for mentoring from afar.

Thanks to Seilesh Kadambari, Simon Drysdale, Andy Pollard, Dominic Kelly and Shelley Segal – for patiently waiting for me to arrive, and then patiently waiting for me to finish writing this!

Many thanks to Mike Levin and Jon Friedland for being members of my review panel, and to Mike for his mentorship and encouragement over the years.

Finally, sincere thanks to Professors David Moore and Ajit Lalvani for kindly examining this thesis, the stimulating *viva voce* examination, and helpful suggestions.

Finally, to my family:

A Ana: Porque apostó por la idea de empezar a salir con un inglés que estaba a punto de trasladarse a Gambia, y por todo lo que vino a raíz de su decisión.

To Rono and Raja for always helping me keep my feet on the ground.

To Mum and Dad – who have given everything for the three of us, and to whom I am eternally grateful.

And to life in general, for being fun and interesting!

CONTRIBUTIONS

The immediate study team in The Gambia consisted of myself, Basil Sambou (Trainee Scientific Officer) and Muhamed Sissoko (Field Worker).

All work contained in this thesis was carried out by myself with the following contributions.

- A manuscript co-written with Dr. E. Whittaker, Dr. JA Seddon and Prof B Kampmann containing parts of chapter one has been accepted for publication at The Lancet Infectious Diseases at the time of submission of the final thesis. I took the lead on all parts of that manuscript that have been adapted for inclusion in this thesis.
- Pilot data were provided by Professor Beate Kampmann and the sample size calculation was carried out by Dr. David Jeffries.
- Under the leadership of Dr. Uzoh Egere, Dr. Toyin Togun, and Dr. Abdou Sillah, the childhood TB household contact platform team at MRC Unit The Gambia carried out all initial screening, TST tests, clinic visits, and data entry. This was the basis for evaluating eligibility of families for this study.
- Myself and Muhamed Sissoko screened families for inclusion in this study. Muhamed Sissoko contacted eligible families to inform them of the study and seek informed consent. Where consent was given, he would arrange for transport to bring the children and their guardian to the MRC Unit The Gambia.
- Sheriff Kande and Monica Davies carried out venepuncture.
- The Clinical Services Diagnostic Haematology Laboratory, MRC Unit The Gambia analysed full blood count samples.
- In-house IGRAs were run by Marie Gomez and Basil Sambou.

- The first multiplex cytokine plate with IFN α and IFN β was run by myself. Due to a delay in receipt of reagents for the subsequent plates and equipment malfunction, Beth Holder kindly ran the remaining samples.
- Mass spectrometry was carried out by myself and Arnaud Wolfer with support from Maria Gomez Romero, Verena Horneffer-van der Sluis and Elaine Holmes.
- Principal Component Analysis (Figure 42) and Partial Least Squares-Discriminant Analysis models were carried out by Dr. Arnaud Wolfer.
- All statistical analyses were carried out by myself with advice from Artemis Koukonari.
- Transformation of the BCG-GFP-LuxFO strain was carried out with Iria Uhia-Castro with supervision from Brian Robertson
- The development of the BCG-GFP-LuxFO method was carried out by myself with experimental assistance from Basil Sambou
- All BCG-GFP-LuxFO assays for children recruited to this study were carried out by myself with experimental assistance from Basil Sambou.
- Anna Turkova kindly translated the two papers by VI Petrenko (283 and 284).

ABBREVIATIONS

6ketoPGF1 α – 6-keto-prostaglandin-F1- α

7H9 – Middlebrook 7H9 broth culture medium

7H11– Middlebrook 7H9 broth culture medium

ADC – Albumin Dextrose Catalase

ANOVA – Analysis of Variance

ANCOVA – Analysis of Covariance

APC – Antigen Presenting Cell

BAL – Broncho-alveolar lavage

BCG – Bacillus Calmette–Guérin

BCG-*lux* - Bacillus Calmette–Guérin expressing luciferase AB (*Vibrio harveyii*)

BCG-GFP-LuxFO - Bacillus Calmette–Guérin expressing Green Fluorescent Protein and Luciferase Full Operon (*Photorhabdus luminescens*)

CFU – Colony Forming Unit

CI – Confidence Interval

CL – Containment Level

CFP-10 – Culture Filtrate Protein 10

CONSORT - CONSolidated Standards Of Reporting Trials

CXR – Chest X-ray

ELISA – Enzyme-Linked ImmunoSorbent Assay

ESAT-6 – 6kDa Early Secreted Antigenic Target 6

GFP – Green Fluorescent Protein

HEI – **H**ighly *M. tuberculosis*-**E**xposed **I**nfecte**I**d Children

HEU – **H**ighly *M. tuberculosis*-**E**xposed **U**ninfecte**D** Children

HEPES - 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)

IGRA – Interferon- γ Release Assay

IL-1 α - Interleukin-1 α

IL-1 β - Interleukin-1 β

IL2-RA - Interleukin-2 receptor alpha

IL-10 – Interleukin-10

IFN- γ - Interferon γ

IQR – Interquartile Range

LEU – Lower *M. tuberculosis*-Exposure Uninfected Children

LTBI – Latent TB Infection

Lux – Luciferase AB (*Vibrio harveyi*)

LuxFO – Luciferase Full Operon (*Photorhabdus luminescens*)

M. tuberculosis – *Mycobacterium tuberculosis*

MDR-TB – Multi Drug Resistant tuberculosis

MGIT – Mycobacterial Growth Indicator Tube

MIP – Macrophage Inflammatory Protein

M:L ratio – Monocyte:Lymphocyte ratio

MOI – Multiplicity of Infection

MS/MS – tandem mass spectrometry

MTB - *Mycobacterium tuberculosis*

OADC – Oleic acid Albumin Dextrose Catalase

OD – Optical Density

PBMC – Peripheral Blood Mononuclear Cells

PBS – Phosphate-buffered Saline

PGE2 – Prostaglandin E2

PHA - Phytohaemagglutinin

PPD – Purified Protein Derivative

QC – Quality Control

RCT – Randomised Controlled Trial

RLU – Relative Light Units

RPM – Revolutions Per Minute

RPMI– Roswell Park Memorial Institute

SNP – Single Nucleotide Polymorphisms

SPE – Solid Phase Extraction

TB - tuberculosis

TNF- α - Tumour Necrosis Factor α

UPLC – Ultra Performance Liquid Chromatography

CHAPTER 1: INTRODUCTION

1.1 Paediatric tuberculosis

Traditionally, paediatric tuberculosis has been relatively neglected, although recent years have seen a welcome increase in policy focus including the goal of zero childhood tuberculosis deaths and the explicit inclusion of children in the early detection, treatment, and prevention pillar of the End TB Strategy.^{1,2} To achieve this ambition, significant progress now needs to occur, given that one million children were estimated to develop tuberculosis in 2016, with 250,000 children dying of the disease.³ This represents 10% of the total burden of incident tuberculosis and 15% of the total mortality.³ There are many differences of clinical importance between children and adults with tuberculosis. Young children have the highest risk of disseminated forms of tuberculosis such as miliary tuberculosis and tuberculous meningitis.⁴ Younger children are also the most likely to die, with tuberculosis mortality rates, from the pre-treatment era, of nearly 50% in those less than five years of age, significantly higher than in older children.⁵ It is also difficult to confirm a microbiological diagnosis due to the paucibacillary nature of paediatric tuberculosis and obtaining suitable specimens.^{6–10}

By contrast, certain paediatric subpopulations seem to demonstrate relative protection against tuberculosis. Children of junior school age (five to ten years old) seem to be relatively protected against tuberculosis before there is a second peak in incidence during adolescence and into adulthood.¹¹ In fact, many children control *M tuberculosis* without intervention; studies from the pre-chemotherapy era show that a) the majority of children survive tuberculosis disease without treatment,⁵ b) that pathological appearances on pulmonary radiographs frequently clear

spontaneously,⁶ and c) that *M tuberculosis* can be cultured from asymptomatic children that have been recently infected and do not proceed to becoming unwell.¹² Contemporary data confirms that some children who have culture-confirmed Multi-Drug Resistant (MDR) tuberculosis remain well and symptom free in the absence of drug therapy.¹³

1.2 Paediatric tuberculosis infection

1.2.1 Definition and diagnosis of infection

Latent tuberculosis infection represents an asymptomatic state with potential to develop symptomatic tuberculosis disease.¹⁴ It is defined by evidence of immunological sensitization by mycobacterial proteins. Currently available diagnostic tests for tuberculosis infection in children are the tuberculin skin test (TST) or Interferon- γ Release Assays (IGRAs). The TST involves intradermal injection of either 5 tuberculin units (TU) of tuberculin purified protein derivative (PPD-S) or 2 TU of tuberculin PPD RT23, followed by measurement of surrounding induration 48-72 hours afterwards.¹⁵ Interferon Gamma Release Assays, which rely on identification of T-cell production of interferon-gamma following incubation of blood with MTB-specific antigens (ESAT-6 [6kDa early secreted antigenic target] + CFP-10 [Culture Filtrate Protein 10] $\pm$ TB7.7), are thought to be as sensitive but more specific than TST as they are not confounded by *Bacillus Calmette–Guérin* (BCG) vaccination status and environmental mycobacteria.^{16–20} There is no gold standard test for latent tuberculosis infection.

The definition of immunological evidence of *M. tuberculosis* infection within the latest consensus definitions for childhood tuberculosis research is: ²¹

- A positive tuberculin skin test (using 5TU PPD or 2TU PPD RT23) defined as:
 - ≥ 10 mm if HIV uninfected
 - ≥ 5 mm if HIV infected or severely malnourished
- or
- A positive IGRA test

1.2.3 Progression from infection to disease

The clinical relevance of this immunophenotype is based upon the potential to progress to active tuberculosis disease. Data from the pre-chemotherapy era established that age plays one of the most significant roles in determining which children will progress to disease. In the absence of preventive therapy, infected infants have a 50% risk of progression to disease. However, this risk reduces with age, before then rising again as children enter adolescence.^{6,22–24} Contemporary data show progression rates of 43% for untreated children under five years of age with IGRA-confirmed infection, significantly higher than the rate of 10.3% for adults.²⁵ A recent Dutch study of contacts of patients with tuberculosis again showed a significant age-related effect with a 5-year risk of prevalent and incident tuberculosis of 33.3% in contacts less than 5 years old compared to 6.7% in those older than 15 years.²⁴

Beyond age-related differences, the ability to distinguish children with high risk of progressing from infection to disease on the basis of currently available clinical tests remains poor. In a multi-site study in Europe of 5,020 tuberculosis contacts, where 10% of contacts were <14 years, the positive predictive values for IGRAs were

below 2%.²⁶ A recent systematic review and meta-analysis, where 8/15 of the included trials involved paediatric participants, showed similar incidence rate ratios for tuberculosis disease progression for a TST>10mm (1.60 [0.94–2.72]) or a positive IGRA (2.11 [95% CI 1.29–3.46]).²⁷ Prospective follow-up of a South African Adolescent Cohort study showed that IGRA conversion from a baseline negative test result to a positive result had a positive predictive value for the development of tuberculosis disease within 2 years of 2.6%, representing an 8-fold higher risk than those who did not convert.²⁸ An increased relative risk remained even in cases where a positive IGRA reverted to negative.²⁹

Whether the magnitude of TST reactions or interferon gamma responses can further stratify risk is debatable.^{25,27,30,31} A study of nearly 20,000 children in Hong Kong who were administered a TST aged 6-10 years showed that those with a result >15mm had a 12-fold increased risk of developing tuberculosis during adolescence compared to those with a result between 10mm and 14mm.³⁰ The odds of developing tuberculosis nearly doubled with every unit of IFN γ measured per millilitre in one study,²⁵ but did not significantly affect risk of tuberculosis in other populations.^{26,27} A recently published analysis of the infant cohort from the MVA85A vaccine trial shows that in this population, infants with conversion to a positive IGRA with conventional thresholds showed no greater risk of developing tuberculosis than those with a persistent negative IGRA. However, those who had a quantitative result ten times greater than the conventional threshold for positivity had significantly higher risk than both IGRA negative, and lower magnitude IGRA positive infants.³¹

Negative predictive values for developing tuberculosis for both IGRAs and TST are consistently high at >98%, reinforcing that many tuberculosis-exposed children are

able to effectively control the pathogen.^{25,26}

1.2.3 Risk of infection

Following exposure, the risk that a child will develop *M tuberculosis* infection is influenced by the infectiousness of the source case, the duration and intensity of the interaction, the infectivity of the organism and the immune status of the child.^{32–38}

Source cases are more infectious if they are sputum smear-positive or have multiple affected zones on a chest radiograph.^{33,34} The duration of cough increases the risk of transmission, with children more likely to be infected if the source case has been coughing for a longer period of time.³⁴ Cultural and socioeconomic factors also determine sleeping and living arrangements, and therefore degree of exposure to the index case.^{34–39}

Most contact studies demonstrate that children are more likely to have a positive TST with increasing age.^{34,40,41} Given that these tests reflect infection at any point in their lives, these results are not surprising as older children are more likely to have been exposed to infectious cases of tuberculosis in the community in addition to the identified household source case. Whether uninfected older children are more likely than uninfected younger children to develop a positive TST following known exposure is unclear. Outbreak investigations in low prevalence settings suggest that perhaps younger children are more vulnerable, but this could be explained by increased intensity of exposure.⁴²

Whether certain mycobacterial strains are more infectious is unclear with conflicting findings regarding an increased infectiousness and virulence of the Beijing strain, as compared to other strains.^{43,44} A large Indian study showed that isoniazid-resistant strains were associated with higher rates of infection but similar rates of disease compared to isoniazid-susceptible strains,⁴⁵ whilst a recent study from Peru suggested that contacts of MDR-tuberculosis cases were less likely to develop tuberculosis than contacts of drug-susceptible cases.⁴⁶

1.2.3 Protection against infection – host factors

A Colombian household contact study demonstrated approximately 65% of the variability in TST results is attributable to genetic contributions.⁴⁷ Specifically, a genome-wide linkage study of 128 families including 350 children in Cape Town, South Africa studied 6,000 single-nucleotide polymorphisms and identified a major locus for TST positivity (TST=0 vs >0mm) at chromosome region 11p14, and a locus for quantitative TST reaction at chromosome region 5p15.⁴⁸ The 11p14 result was recently replicated in a prospective household contact study of 97 families in Paris, a low tuberculosis prevalence region,⁴⁹ and the locus overlaps a region involved in TNF- α production.⁵⁰ In a Ugandan context, genetically determined TNF α responses have also been found,^{51,52} as have polymorphisms associated with resistance against infection on chromosome 5p and 5q, the latter in HIV-positive individuals.^{53–}

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Investigation of the persistently TST negative phenotype in both children and adults in this Ugandan cohort suggests that persistent TST negativity is multifactorial incorporating genetic, clinical, epidemiological and immunological components.^{39,52–}

⁵⁹ There were significant differences in age when comparing persistently TST- individuals to those with LTBI or who converted to a positive TST.³⁹ Of note, there was a high proportion of children in the 5-15 year age range amongst those with persistently negative TST results.³⁹ Interferon- γ levels in response to mycobacterial stimulation are lower in persistently TST negative individuals compared to those with LTBI or who convert to positive TST results in two distinct cohorts in Uganda and another in Pakistan.^{56,60,61} Hypothesis-generating approaches have also been employed with gene expression profiles of *in vitro* MTB-infected monocytes from individuals with persistently negative TST compared to those with LTBI showed differences related to histone deacetylases,⁵⁸ and broad-ranging plasma proteomic changes.⁵⁹ These and other key studies are summarised in Table 1.

1.2.4 Protection against infection – vaccine inducible effects

Children are the only group for whom there is strong evidence of inducible protection against tuberculosis through vaccination.^{62–64} Bacillus Calmette-Guérin (BCG), a live, attenuated vaccine was first given to humans in 1921 and has since been administered to more people than any other vaccine in history. Neonatal BCG vaccination appears to provide 60% protection against pulmonary tuberculosis in childhood (95% CI:42-71%),^{64–67} and protects young children from tuberculous meningitis and miliary tuberculosis with an efficacy of 75-85%.^{62,64} With the advent of IGRAs as tests of latent infection, and therefore greater specificity for detecting infection, there is mounting evidence that BCG also protects children against LTBI, with a recent systematic review and meta-analysis showing an estimated 19% efficacy (95% CI 8-29%) to protect against infection.^{63,68–71}

1.2.5 Experimental findings in tuberculosis infection

Key studies that have investigated experimental associations with tuberculosis infection and their relevant results are summarised in Table 1. For clarity, and in keeping with the focus of this thesis, where studies address multiple areas the table focuses on findings related to comparing persistent TST negative to TST positive individuals. Where studies recruited patients with and without HIV infection, findings are summarised for the study participants without HIV infection. Studies exclusively addressing tuberculosis infection in HIV-positive individuals are not included.^{54,55} A study published since the thesis was first submitted to the examiners has also been included.⁷² It is worthy of note that there is significant variability amongst the studies even beyond what is summarised in the table. The definitions of tuberculosis infection used vary, with some studies using TST (with different thresholds for a positive result), some IGRA, and some combinations of both tests. There is also considerable variability in the focus of the research question, with comparisons between TST negative individuals, those who convert from TST negative to positive, those with a positive test of infection at baseline, and those with tuberculosis disease. Study factors such as duration of follow-up, protocols for repeat testing for infection, provision of isoniazid prophylaxis, and adjustment for index case characteristics also differ between publications. Experimental variables including the antigens or mycobacteria used, duration of assays, use of whole blood or Peripheral Blood Mononuclear Cells (PBMCs), and time-points again vary considerably.

Table 1: Summary of key studies investigating immunological factors in mycobacterial infection

The alternating shading in the first column groups together articles in chronological order published by the same broad research group, although there is some overlap in authors. Matching refers to matching of cases and controls based upon exposure to MTB.

Country	Article	Study design	Age	Matching	Findings
Uganda	Stein <i>et al.</i> (2005) ⁵¹	817 Household contacts of TB	Mean 18-19y SD (14-15)	Kinship-based genetic analysis	TNF- α levels at 24H following stimulation of whole blood with MTB culture filtrate had a high heritability (34–66%)
Uganda	Whalen <i>et al.</i> (2006) ⁶⁰	222 household contacts of TB who had baseline TST<5mm	179 <15y	No	IFN- γ levels at baseline following 120 hours incubation with MTB culture filtrate protein were greater among TST converters compared with those who did not convert. Baseline levels were similar between converters and non-converters for TNF- α (18hours) and transforming growth factor- β
Uganda	Lewinsohn <i>et al.</i> (2008) ⁷³	1,267 household contacts of TB	739 children <15 years	No	IFN- γ responses in a 5-7-day whole blood assay with MTB culture filtrate protein were higher in TST+ children (>5mm) compared to TST- children in all age groups except for BCG unvaccinated children <2 years old.

Country	Article	Study design	Age	Matching	Findings
Uganda	Stein <i>et al.</i> (2008) ⁵³	803 household contacts of TB	Median age 50 (range 1-80)	Kinship-based genetic analysis	Two genetic regions linked to persistent TST-, attained suggestive significance, one on chromosome 2q21-2q24 and another on chromosome 5p13-5q22 TNF- α levels at 24H following stimulation with MTB culture filtrate did not differ significantly between PTST- and LTBI
Uganda	Mahan <i>et al.</i> (2012) ⁵⁶	Household contacts of TB: persistent TST- persons (19) TST converters (42 TSTC) TST+ at baseline (42 TST+)	Age >15	No	No difference between persistent TST- and baseline TST+ in TNF- α levels at 18hours in response to stimulation with MTB culture filtrate, MTB 19kDa protein Rv3673 19 kDa (TLR2 ligand), or lipopolysaccharide (TLR4 ligand) +/- pre-treatment with IFN- γ . V δ 2 $\gamma\delta$ T cell responses (IFN- γ levels in whole blood at 7 days following stimulation with bromohydrin pyrophosphate) did not differ between the groups. IFN- γ levels in whole blood at 7 days following stimulation with MTB H37Ra, MTB culture filtrate, and Antigen85B were higher in TST+ compared to persistent TST-

	Country	Article	Study design	Age	Matching	Findings
	Uganda	Hall <i>et al.</i> (2015) ⁵⁷	718 household contacts of whom 75 persistently TST-	<10years – 303 of whom 55 persistently TST-	Kinship-based genetic analysis	None of the SNPs from 29 candidate immune-related genes met experiment-wide significance but with p<0.05 SNPs associated with persistent TST- in NOD1, NOD2, SLC6A3 and TLR4
	Uganda	Bark <i>et al.</i> (2017) ⁵⁹	Household contacts of TB: Discovery cohort: Non-infected n=8 LTBI n=38	Median age Non-Infected= 20y Median age TST+:25 y	No	67 proteins identified by tandem proteomic mass spectrometry differentially expressed between TST+ and persistent TST- including complement factors, oxidative stress enzymes, immunoglobulin receptors and a Danger Associated Molecular Pattern molecule.
	Uganda	Seshadri <i>et al.</i> (2017) ⁵⁸	Persistently TST- (n = 10) and TST+ (n = 18) household contacts of TB	N=5 less than 15 years old in this analysis	No	24 gene sets associated with TST+ and 27 gene sets with persistently TST- in genome-wide transcription profiles following 6 hours infection of archived PBMCs with MTB including those associated with inhibition of histone deacetylase function
	Pakistan	Hussain R, <i>et al.</i> (2009) ⁶¹	93 Household Contacts of TB = 93 from 20 families	Age of TST+: mean 25.8y (SD 15.9) Age of TST-: mean 20.0 y (SD 10.7)	No	No difference at baseline in day 5 IFN- γ or day 2 IL-10 levels following MTB culture filtrate stimulation of whole blood between TST- and TST+ household contacts. At 6 months follow-up, but not at 12 or 24 months, TST+ individuals had higher day 5 IFN- γ levels and lower day 2 IL-10 levels.

Country	Article	Study design	Age	Matching	Findings
Western Cape, South Africa	Cobat <i>et al</i> (2009) ⁴⁸	350 children from 128 families living in high TB-endemicity area	Age not specified but children	N/A	Absence of TST reactivity (0mm vs non-zero) maps to TST1 locus on chromosome region 11p14 & Quantitative TST reactivity maps to major locus (TST2) on chromosomal region 5p15
Colombia	Cobat <i>et al.</i> (2012) ⁴⁷	371 healthy household contacts of TB	mean age 27.8 y (range 1–83 y)	Kinship-based genetic analysis	Heritability of 0.72 for a co-dominant major gene controlling residual TST variance
Western Cape, South Africa	Cobat <i>et al</i> (2013) ⁵⁰	392 children from 135 families living in high TB-endemicity area	Age not specified but children	N/A	TNF- α production in whole blood at 24H after incubation with BCG +/- IFN- γ is linked to chromosomal region 11p15 TNF locus 1 (TNF1) and in vicinity of TST1 locus
France	Cobat <i>et al</i> (2015) ⁴⁹	237 household contacts of TB	102: < 10y 66: 10-20y	Kinship-based genetic analysis	Absence of TST reactivity (0mm vs non-zero) maps to chromosomal region 11p15, overlapping the TNF1 locus and in vicinity of TST1 locus

	Country	Article	Study design	Age	Matching	Findings
	South Africa	Maertzdorf <i>et al.</i> (2011) ⁷⁴	34 LTBI and 9 non-infected TB contacts	>18 years	No	No difference in genome-wide transcription profiles between LTBI and non-infected donors.
	Germany	Herzmann <i>et al.</i> (2017) ⁷⁵	25 IGRA- vs 25 IGRA+ household contacts of TB	Presumed adults	No	Higher percentage of lymphocytes and subset of regulatory T-lymphocytes in BAL samples from blood IGRA-positive donors Higher IL-2 response following MTB infection of BAL cells from blood IGRA-positive donors
	The Gambia	Sutherland <i>et al.</i> (2010) ⁷⁶	19 TST- contact vs 20 TST+ contact	TST- IQR: 18-31 TST+ IQR: 19-39	No	↑IFN- γ , IL-13, IL-17 in TST+ vs TST- in 7-day whole blood assay with PPD. Also ↑IFN- γ & IL-13 with ESAT-6/CFP-10.
	South Africa, The Gambia, Ethiopia, Malawi and Uganda	Sutherland <i>et al.</i> (2013) ⁷⁷	454 HIV- TST+ vs 204 HIV - TST- (household contacts of TB or random community selection)	≥18 years of age	No	IFN- γ responses in a 7-day whole blood assay to ESAT-6/CFP-10 fusion protein, PPD, MTB antigen Rv2029, TB10.3, TB10.4 and PHA were significantly higher in HIV- TST+ (LTBI) compared to HIV-TST- subjects
	The Gambia	Buchwald <i>et al.</i> (2014) ⁷⁸	64 TST+ at baseline, 26 TST converters, 39 persistent TST-	>18y	No but household adjusted for in statistical analysis.	IL-2 was lower in persistent TST- as compared to TST+ after one day of stimulation of PBMCs with ESAT-6/CFP-10. After 6 days of stimulation with ESAT-6/CFP-10, persistent TST- had lower levels of IFN- γ , MIP1- β , IL2-RA and IL-13 compared to TST+. No differences in TNF- α at day one or six.

	Country	Article	Study design	Age	Matching	Findings
	The Gambia	Elliot <i>et al</i> (2015) ⁷⁹	17 TST+ and 20 TST- household contacts of TB	TST+ median age 34y (IQR 21–42) TST- median age 31y (IQR 23–48)	No	Within CD3+ T cell population, following ESAT–6/CFP-10 stimulation, there were more early apoptotic cells from TST+ compared to TST-
	The Gambia	Owolabi <i>et al</i> (2016) ⁸⁰	83 TB cases, 46 TST+ and 52 TST- household contacts including those who progressed to TB	TST+ median age 24y (IQR 22-33) TST- median age 23 (IQR 20-35)	No	No difference between 25(OH) vitamin D or Vitamin D binding protein levels in TST+ and TST- household contacts. 25(OH) D level at recruitment higher at recruitment for the incident cases (progressors) compared to non-progressors
	Mongolia	Ganmaa <i>et al</i> (2012) ⁸¹	RCT of 6 months 800IU vitamin D in healthy school-age children with initial TST-.	Aged 12-15 years old. Intervention (n=45) vs placebo (n=41)	N/A	Borderline significant reduction in TST conversion rate from negative to positive in the vitamin D supplementation group compared to placebo group (P = 0.06)

	Country	Article	Study design	Age	Matching	Findings
	Spain	Arnedo-Pena <i>et al</i> (2011) ⁸²	Cross-sectional study of household TB contacts: TST+ (n=50), TST- (n=152)	TST- mean 34.8, SD 14.3 vs TST+ mean age 38, SD 16.9	No	No significant association with baseline Vitamin D levels
			Case-control study of conversion from negative TST to positive TST at two months (n=11) vs persistent TST negative (n=82)	Conversion to TST+ (mean age 39.6, SD 13.1) vs persistent TST negative (34.7, SD 14.5)	No	Significantly higher levels of vitamin D at baseline in those with persistent negative TST compared to those who converted to TB infection.
	Spain	Arnedo-Pena <i>et al</i> (2015) ⁸³	household contacts of pulmonary TB with conversion to positive TST/IGRA at 8 weeks (n=18) vs persistent negative TST (n=180)	Excluded children <10y. Conversion mean age 32.8 (SD17.2) and persistent TST negative 38.1 (SD 12.4)	No	Significantly higher levels of vitamin D at baseline in those with persistent negative TST compared to those who converted to TB infection.

	Country	Article	Study design	Age	Matching	Findings
	South Africa	Baguma <i>et al</i> (2017) ⁷²	Cross-sectional study of IGRA+ vs IGRA- healthy HIV-negative individuals living in a high TB burden area.	Adults: 19-51y 29 IGRA+ vs 26 IGRA- Young adults: 18y 37 IGRA+ vs 21 IGRA- Children: 8y old 20 IGRA+ vs 28 IGRA-	N/A as not recruited based on exposure.	No difference in whole blood Mycobacterial Growth Inhibition Assays with MTB H37Rv, MTB HN878, or MTB CDC1551 strains between IGRA+ vs IGRA- children; IGRA+ vs IGRA- young adults; nor between children vs young adults. Growth of MTB H37Rv, MTB HN878 and MTB CDC1551 correlated in children and young adults. Growth of H37Rv and BCG correlated in adults. No association between neutrophils, monocytes or myeloid dendritic cells (mDCs) and MTB H37Rv growth in adults. No difference in BCG-GFP-positive neutrophils, monocytes or mDCs (including by IL-6, IL-12 and/or TNF α expression) between IGRA+ and IGRA- adults, nor correlations with MTB H37Rv growth. No difference in IL-2, IFN- γ , TNF- α , and/or IL-17-expressing subsets of CD4, CD8 and $\gamma\delta$ T-cells between IGRA+ and IGRA- adults following 12 hours of BCG stimulation, nor correlations with MTB growth.

1.2.6 Discussion of immunophenotype of paediatric tuberculosis infection.

Paediatric tuberculosis infection is a complex and heterogeneous immunophenotype with numerous host, epidemiological, and microbiological contributory factors. Having a positive or negative test for *M. tuberculosis* infection encompasses a range of possible outcomes within the host.^{14,71,84} There is no gold standard test for latent tuberculosis infection. A negative test could simply represent that the individual has not inhaled any bacilli in a stochastic manner, which would relate to the infectiousness of the source case and epidemiological factors that relate to the degree and intensity of exposure.^{32–39} A negative test could also occur where the host is unable to mount a robust cell-mediated immune response as occurs with malnutrition and HIV infection with low CD4 counts.^{85–88} Without serial testing for infection it is difficult to establish whether a positive result relates to a recent defined exposure or preceded it. Both conversion from a negative test of infection to a positive result, and reversion from a positive to a negative test result can occur in children.^{28,29,89} Due to the complex mixture of antigens in PPD, false positive TST results can occur due to BCG vaccination and exposure to environmental mycobacteria. Although more specific than the TST, the antigens contained in IGRA tests are also found in several other mycobacteria.^{10,20,90} Positive test results are associated with an increased risk of developing tuberculosis in children, although most children with a positive test of infection remain healthy.^{6,22–25,91} It could therefore be argued that most children with a positive test of infection have also effectively controlled the pathogen, through a combination of adaptive cell-mediated immunity and other components of the immune response. Existing tests are unable to distinguish these children from those who will go on to develop disease. It is

unknown whether viable mycobacteria are present in the lung (and within host cells) in children with either a positive or a negative test of infection.

Nevertheless, despite this complexity and heterogeneity within patient groups, infection status is an important immunophenotype to study in children for two principal reasons. Firstly, the increased relative risk of developing tuberculosis in children with a positive test of infection compared to those with a negative result suggests that on a population level developing infection reflects a host response that carries a greater associated risk of disease (Section 1.2.3). Secondly, many children have a negative test of infection despite defined exposure, which cannot be explained by relative immunocompromise.³⁵ Therefore, a negative test of infection could also represent an effective immune response with bacilli being cleared without triggering development of an adaptive immune response. Mediators of this early clearance could include the physical and chemical properties of the oropharyngeal mucosa and respiratory epithelium, antimicrobial peptides, recognition of pathogen- or damage-associated molecular patterns, neutrophils, dendritic cells, alveolar macrophages, Natural Killer (NK) cells, mucosal-associated invariant T (MAIT) cells, $\gamma\delta$ T cells, vitamin-D mediated mechanisms, and the inflammatory milieu of cytokines and eicosanoids, and the notion of trained immunity.^{8,84,92–99}

1.3 Potential immunological mechanisms for different paediatric outcomes following exposure

1.3.1 Cytokine responses

There is an extensive literature base for the key roles played by cytokines in human-mycobacterial interactions.^{8,100–108} The importance of CD4⁺ Th₁-cytokine producing

T-cells is evident through the significantly increased risk of tuberculosis in infants with HIV infection,¹⁰⁹ those on anti-TNF α antibody treatment,¹¹⁰ and primary immunodeficiencies.^{103,111,112} However, recent paediatric studies did not identify cytokine-specific T-cells as correlates of protection against disease.^{113,114} The optimal balance between effective pathogen control and tissue-damaging inflammation with pro-inflammatory cytokines such as IFN- γ , TNF- α , IL-12, IL-1 α and IL-1 β , and anti-inflammatory cytokines such as IL-10 remains the focus of intense investigation.^{105,115–127} Impaired TNF α and IL-1 β production are associated with poor outcome in adults with tuberculosis,^{128,129} whilst higher IL-1 β responses to BCG were associated with recurrence of TB.¹³⁰ Polymorphisms in IL-1 β are associated with both pulmonary and extrapulmonary TB.^{57,131} The relevance of Type I interferons (IFN α and IFN β) to host control of mycobacteria has been a more recent addition to the field, with Type I interferon predominance associated with poor host control and disease progression.^{126,132–136}

1.3.2 Eicosanoid responses

The role of eicosanoid inflammatory mediators in host-mycobacterial interactions has also been a relatively recent discovery.^{104,105,116,137–147} Studies in zebrafish and humans have shown *Ita4h*, an enzyme which determines the balance between anti-inflammatory lipoxin A₄ (LXA₄) and pro-inflammatory leukotriene B₄ (LTB₄) is linked to susceptibility to mycobacterial disease.^{116,142} Mice with prostaglandin E synthase knockout macrophages have low prostaglandin E₂ (PGE₂) levels that are associated with significantly higher mycobacterial burden.¹⁴⁸ There are concentration-specific effects of PGE₂ upon T-cell activation and differentiation, together with spatially organized levels of eicosanoids and their enzymes in human granulomas.^{146,149}

PGE2 is broadly considered a pro-inflammatory eicosanoid mediator.¹⁵⁰

Furthermore, there are dynamic interactions with cytokines. For instance, IL-1 induces eicosanoids that then limit excessive type I interferon production and promote mycobacterial control.¹³³ The balance between effective inflammation to control infection versus causing immunopathogenesis is particularly nuanced in TB meningitis.^{116,145,151} Exploration of the role of eicosanoids in paediatric tuberculosis so far has been restricted to genetic association studies of 5-lipoxygenase, an enzyme which when knocked out in mice confers resistance to mycobacterial infection and enhanced T-cell responses.^{152–154} The eicosanoid literature highlights an optimal balance for host control of mycobacteria and immunopathology in the disease state between pro- and anti-inflammatory mediators.^{116,142,143,145,151} In the context of children with *M tuberculosis* exposure but without infection it is hypothesized that an effective host response against mycobacteria would be weighted towards an early robust pro-inflammatory profile enabling clearance prior to development of an adaptive immune response.

1.3.3 Trained immunity.

Traditionally the immune response has been considered to have two pathways: the non-specific rapid innate immune response and the specific memory-based adaptive T- and B-cell mediated response. Trained immunity is the concept that “innate immunity can be influenced by previous encounters with pathogens or their products”⁹⁴ that lies between these two arms.^{84,94–96,155,156} Supporting evidence includes memory responses in plants and invertebrates that had been thought to lack an adaptive response, and increased responsiveness upon secondary stimulation of mammalian cells such as monocytes, macrophages and natural killers

cells. The underlying mechanisms are felt to be epigenetic reprogramming through DNA modification (histone modification, methylation) and the influence of micro- and long-non-coding-RNA. Trained immunity is known to mediate some effects of BCG, including non-specific effects unrelated to mycobacteria and could well play a role in absence of infection despite exposure.^{95,97,155–157}

1.3.4 Monocyte: Lymphocyte ratio

In contrast to the complex network of interactions between different cytokine and eicosanoid inflammatory mediators and regulators, an elevated monocyte:lymphocyte ratio, an easily measured parameter, has also been implicated in poor human control of mycobacteria.^{158–161}

1.4 The research landscape in tuberculosis immunology and vaccine development

The need for a better vaccine is widely recognised in the field of tuberculosis, and development of a protective vaccine by 2025 is a cornerstone of the World Health Organization (WHO) End TB Strategy.^{2,162–165} Infants were the target population for

the first phase II randomized placebo-controlled clinical trial of a new tuberculosis vaccine, the modified Vaccinia Ankara virus expressing antigen 85A (MVA85A).

Unfortunately, the MVA85A vaccine showed no additional efficacy against tuberculosis disease or infection beyond that of BCG in South African infants.¹⁶⁶

These results, despite prior animal models and human immunogenicity data, prompted much reflection in the field of tuberculosis vaccine development.^{164,167,168}

The same trial showed no additional efficacy against *M tuberculosis* infection than BCG (–3·8%, 95% confidence interval: –28·1 to 15·9), although it was not powered for this endpoint.¹⁶⁶

There are at least 14 novel vaccines in clinical trials up to Phase IIb,¹⁶⁹ but to date it has proven difficult to exceed the effectiveness of BCG,¹⁶⁶ which is approaching 100 years since it was first used in man. Furthermore, despite failing to prevent adolescent and adult tuberculosis that drive the ongoing epidemic, BCG not only protects children against infection, pulmonary disease, and disseminated tuberculosis, it also protects against leprosy with an estimated efficacy of 26%,¹⁷⁰ and appears to have heterologous protective effects.^{171–173} Thus, even though BCG vaccination is clearly not halting the epidemic, BCG is proving to be a hard act to follow. A range of approaches are currently being explored including modified recombinant BCG strains for an improved safety profile in infants living with HIV, combinations of novel vaccines with BCG in prime-boost strategies, and post-exposure vaccination.^{71,162,164} A vaccine that specifically targets prevention of infection, thereby optimising an effective immune response at the first encounter with the pathogen and preventing both primary and reactivation disease and accompanying transmission, would be a key contributor in the goal to eliminate tuberculosis.^{71,84}

However, one of the fundamental barriers to being able to implement a new tuberculosis vaccine by 2025 is the absence of a correlate of human immunity against tuberculosis, which could be used as an endpoint in future vaccine trials.^{162,164,174–176} Despite many years of substantial research, through combinations of hypothesis-based and hypothesis-generating methodologies, with the evaluation of unstimulated and mycobacteria-stimulated samples, and with more than 15,000 infants and adolescents enrolled into trials that enabled case-control comparisons, true correlates of protective paediatric immunity remain elusive. Although some

correlates of risk have now been identified (e.g. HLA-DR+CD4+ T-cells,¹¹³ higher monocyte: lymphocyte ratios,^{160,161} and a 16-gene RNA signature¹⁷⁷) the underlying biology still remains to be elucidated and these parameters are not easily tractable to inform vaccine design.

1.5 Summary

The outcomes of children who have been exposed to *M tuberculosis* (Figure 1) is a product of complex interactions between child factors, index case factors, epidemiological factors, and pathogen factors.

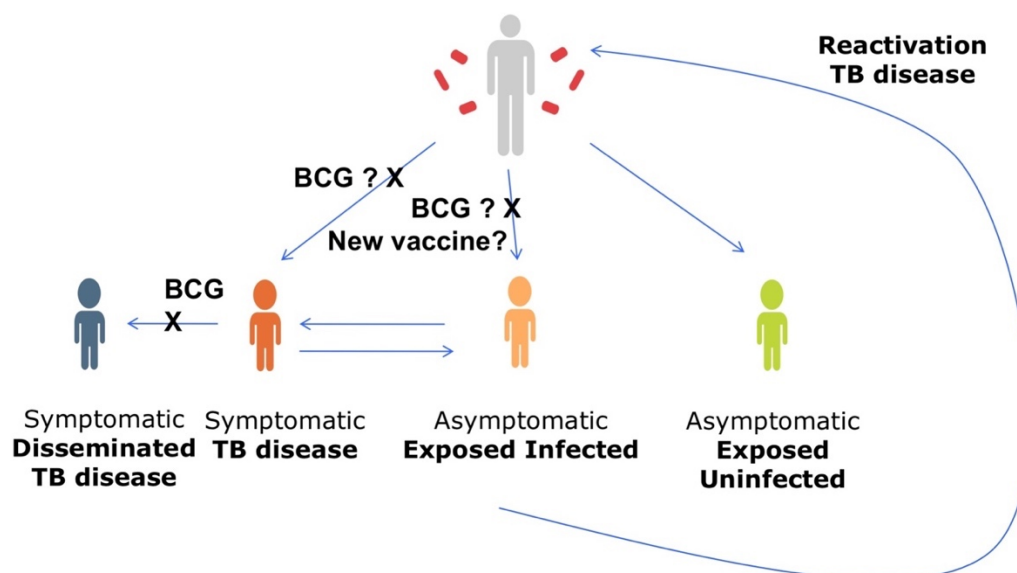


Figure 1: Schematic of the paediatric outcomes following exposure to *M tuberculosis* and intervention points for BCG and novel vaccines.

Although animal models and experimental biology can be informative, we have not yet approached sufficient understanding of human immunity to tuberculosis in endemic settings to establish correlates of protection. In particular, a better understanding of effective paediatric immunity to infection with *M tuberculosis* would make a meaningful contribution to the daunting task of developing a novel vaccine that could contribute to tuberculosis elimination.

CHAPTER 2: STUDY AIM AND HYPOTHESES

2.1 Study aim:

To explore immune mechanisms underlying the observation that some children exposed to *Mycobacterium tuberculosis* show no signs of infection, whilst others in the same house become latently infected

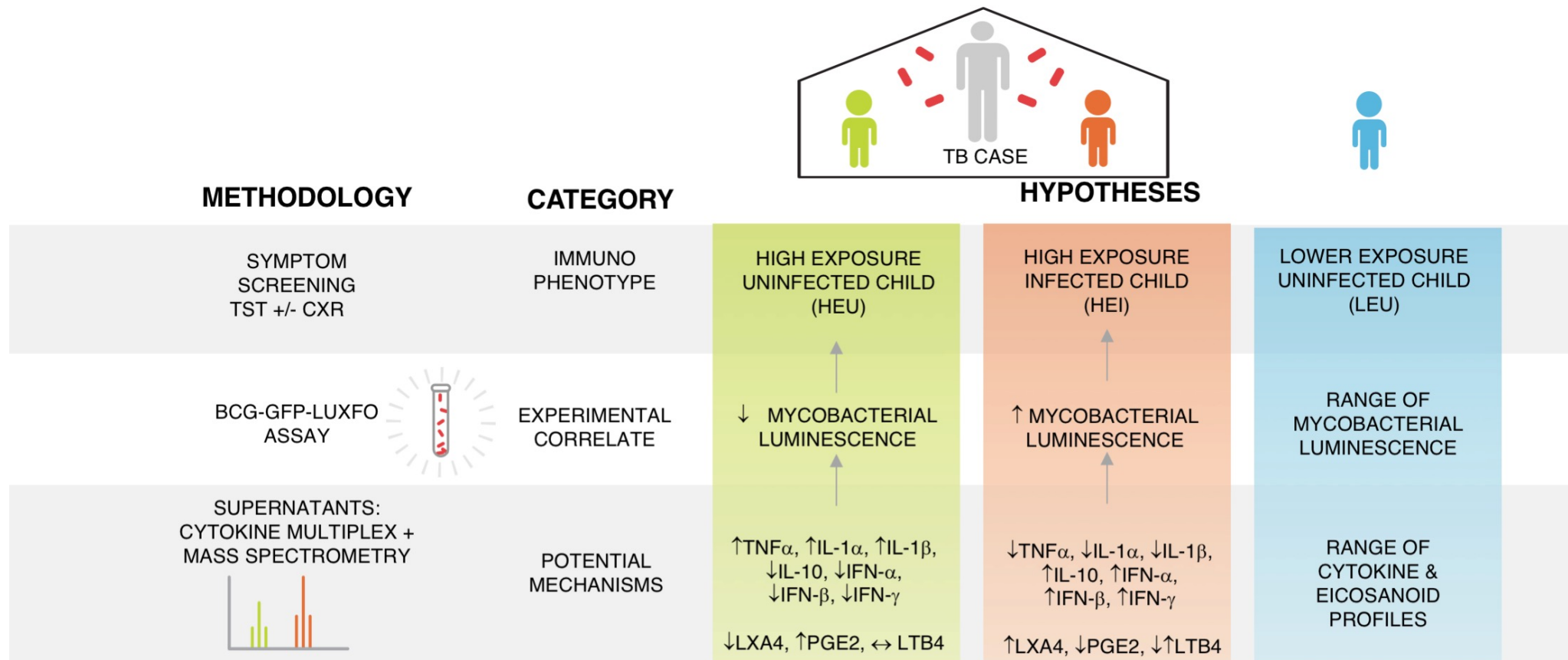
2.2 Hypotheses:

Hypothesis 1: **H**ighly TB-**E**xposed **U**ninfected (HEU) children are better able to restrict mycobacterial growth in an *in vitro* whole blood assay than their matched **H**ighly TB-**E**xposed **I**nfected (HEI) peers.

Hypothesis 2: The cytokine response to mycobacterial infection of whole blood *in vitro* differs between HEU and HEI children. HEU children are predicted to have an early pro-inflammatory profile (with high levels of TNF α , IL-12, IL-1 α , and IL-1 β , and low levels of IL-10, IFN- α , and IFN- β) whilst the adaptive response of HEI children will lead to late elevated levels of IFN- γ .

Hypothesis 3: The eicosanoid response to mycobacterial infection of whole blood *in vitro* differs between HEU and HEI children, with HEU children having an early pro-inflammatory profile (low levels of LXA4 and high levels of PGE2 and LTB4).

Hypothesis 4: The responses of a control group of **L**ower **E**xposure **U**ninfected (LEU) children reflect those of the general population in a high-TB-endemicity setting and encompass *both* HEU and HEI responses. This would suggest that any differences between HEU and HEI children in hypotheses 1→3 are not solely a consequence of infection in the HEI child but may represent intrinsic differences in the immune response of HEU individuals.



LXA4=lipoxin A4, LTB4=leukotriene B4, PGE2=prostaglandin E2, TB=Tuberculosis, TST=tuberculin skin test, IL=Interleukin, IFN=Interferon
 CXR= chest radiograph, BCG-GFP-luxFO assay=Recombinant BCG expressing luciferase full operon and GFP

Figure 2: Schematic of study hypotheses

CHAPTER 3: STUDY CONTEXT, MATERIALS, AND METHODS

3.1 Introduction

Here I summarize the clinical and epidemiological context in which the study took place, followed by the study-specific recruitment and consent procedures, the laboratory methodology employed, and finally the statistical analysis plan. Novel methodological development of the BCG-GFP-LuxFO assay as part of the thesis is described in Chapter 4.

3.2 Study context

3.2.1 The Gambia and tuberculosis

The Gambia is a tropical country in West Africa that encompasses both banks of the western section of the River Gambia. The Gross Domestic Product per capita is estimated at \$1,700. The Gambia has an estimated population of 2,009,648 in 2016 with 37.88% under the age of 14 years old.¹⁷⁸ The capital is Banjul, and the Greater Banjul Area has a population of approximately 700,000.³⁵ Neonatal mortality rate is estimated at 30 per 1,000 live births and the infant mortality rate is estimated at 48 per 1,000 live births.¹⁷⁹ Tuberculosis prevalence is estimated at 90 smear-positive cases per 100,000 population (53-127) and 212 bacteriologically confirmed cases per 100,000 population (152-272).¹⁸⁰ Tuberculosis incidence is estimated at 3,500 (2,600-4,400) per year with an incidence rate of 174 (131-223) per 100,000 population. The incidence of MDR/Rifampicin Resistant-TB is estimated at 120 per year (0-250) with an incidence rate of 6 (0-13) per 100,000 population. The total mortality from TB is 540 (370-760) per year with a mortality rate of 27 (18-38) per 100,000 population.¹⁸¹ Childhood tuberculosis incidence is estimated at 31 per 100,000 population less than 15 years old.¹⁸² The prevalence rate of HIV in adults aged 15-49 is 1.8% (1.5-2.2).¹⁸³ Neonatal BCG immunization coverage is estimated

at 98%.¹⁸⁴ 38% of tuberculosis cases in the Gambia are due to *M. africanum* type 2 (MAF2) rather than *M. tuberculosis sensu stricto*.^{185,186} Although there are differences in T-cell responses between TB patients infected with MAF2 compared to MTB¹⁸⁷ and an increased likelihood of being older, HIV infected and malnourished in MAF2 patients, transmission of infection¹⁸⁸ and on-treatment mortality (3%) are similar in countries where it is a significant circulating strain.¹⁸⁵ In the Netherlands, amongst foreign-born individuals only, *M africanum* was associated with less transmission of infection than other strains, but similar progression to disease.¹⁸⁹ Tuberculin skin test results are similar between individuals exposed to *M africanum* and *M tuberculosis*, although IFN- γ production appears to be decreased in *M africanum*-infected subjects.¹⁹⁰ Protection related to variations in Mannose-Binding Lectin appears to be strain-selective, protecting against *M. africanum* disease but not *M tuberculosis* disease.¹⁹¹ In 7 day whole blood assays with PPD or ESAT6/CFP-10 stimulation, there were no differences in IL-10, IL-12(p40), IL-13, IL-17, IL-18, IFN- γ or TNF- α production between *M. africanum* and *M. tuberculosis*-infected patients.⁷⁶ Given these circulating strains in The Gambia, matching on exposure to the same index case in the study design would account for any differences that might be attributable to the strain.

3.2.2 Household contact platform at MRC Unit The Gambia

A community-based tuberculosis household contact tracing platform has been established for more than 14 years at MRC Unit The Gambia.^{34,35,192–197} During the period in which this study was conducted, paediatric contact tracing was undertaken as part of the MRC Program Grant awarded to Professor Beate Kampmann (MR/K011944/1).

The methods have been reported in detail in several recent publications from the MRC Childhood TB program team and here I summarize only features of the parent study that are relevant to this thesis.^{35,194–196} Adults with smear-positive pulmonary tuberculosis at peripheral health centres or the MRC tuberculosis clinic are identified by field workers from the tuberculosis notifications at each centre. These adult patients are then approached for consent to visit their compounds. A compound is defined as a cluster of buildings often owned by members of the same family.³⁵ If consent is provided, the paediatric tuberculosis fieldwork team visit the compound and approach the parents/guardians of children living in the compound for consent to screen the children. Screening is carried out within the compound and involves a symptom questionnaire to identify children with presumptive tuberculosis (appendix A.3.1) and placement of a tuberculin skin test (intradermal injection by trained personnel of 2 tuberculin units, Purified Protein Derivative RT23 Statens Serum Institut, Copenhagen, Denmark). Fieldworkers return to the compound after 48-72 hours to record the size of transverse induration in millimetres. All children with symptoms suggestive of tuberculosis and/or with a tuberculin skin test result of $\geq 10\text{mm}$ were referred to the Childhood TB clinic at MRC Unit The Gambia where active TB was excluded. All children seen in clinic had an HIV-test and a chest radiograph. Additional blood samples were taken for research including in-house interferon-gamma-release assay.

Children not diagnosed with tuberculosis disease were followed up with symptom surveys every 3 months for one year. All children without tuberculosis disease under the age of five years, regardless of tuberculin skin test results, were commenced on

isoniazid preventive therapy as per WHO guidelines as part of an implementation research program.¹⁹⁶

In a recently published article by the MRC Childhood TB program team, 82.6% (3339/4042) of contacts were classified as exposed but not infected, 15.8% (639/4042) had latent infection and 1.6% (64/4042) had TB disease. Three of the 909 children seen in clinic (0.3%) were positive for HIV infection.³⁵

3.3 Specific considerations for recruiting to this study

The study described in this thesis was nested in the overall research program but differed in four key regards from the parent study:

- The primary research question requires recruitment of pairs of children with discordant infection status but matched exposure to the same index case (HEI and HEU children). A secondary research question to explore whether differing responses between HEI and HEU children were a consequence of LTBI or due to intrinsic differences, required recruitment of TST negative children from the same compound with lower exposure to the TB index case (LEU). Therefore, evaluation of HEU and LEU asymptomatic children with negative tuberculin skin tests was needed. Such children would not otherwise attend the MRC Unit nor have blood samples taken.
- A repeat tuberculin skin test a minimum of three months after the screening of the children with initial negative tuberculin skin test results was required in order to identify and exclude any children who might have converted to a positive tuberculin skin test and confirm the phenotype of HEU and LEU children.

- It was critical that children were not taking anti-tubercular medications to avoid interference with the whole blood BCG GFP LuxFO assay. Therefore, all children under the age of 5 years old were excluded due to the provision of isoniazid prophylaxis in keeping with WHO recommendations.¹⁹⁶
- HIV testing in asymptomatic children in a region with such low prevalence of paediatric HIV infection³⁵ raises potentially challenging issues of study acceptability in The Gambian context and therefore information sharing with the National Leprosy and Tuberculosis Program was sought to ascertain the HIV status of the adult index case as a proxy of risk for the HEU and LEU children.

Therefore, specific scientific and ethical permissions were required for the conduct of this study and were made by myself. Applications were made to the Scientific Coordinating Committee of MRC Unit The Gambia and The Gambia Government/MRC Joint Ethics Committee (Reference number: SCC1405) and the Imperial College Healthcare Tissue Bank (Reference number: R13071). The approvals are included in Appendix 1.

3.2.4 Pilot data and sample size calculation

Unpublished pilot data were provided by Professor Beate Kampmann from a study of TB-exposed siblings in Cape Town and provided the basis for a sample size calculation carried out by Dr David Jeffries, Senior Statistician at the MRC Unit The Gambia.

Using the BCG-LuxAB assay (see section 4.1) a statistically significant difference in mycobacterial growth ratio between TB-exposed infected children (as defined by positive TST, n=18) and TB-exposed but uninfected children (as defined by negative

TST, n=24) was detected (median growth ratio for exposed infected children: 5.2, IQR 3.125→6.9; median growth ratio for exposed uninfected children: 2.9, IQR 1.725-4.25; two-tailed Mann-Whitney U test $p=0.0281$) (Figure 3). Matched statistical analysis was not possible. Age did not significantly differ between the groups (Children with infection: n=14, median: 10.5 years, IQR:3.75→13.25; Children without infection: n=23, median: 6 years, IQR: 4→10; $p=0.252$).

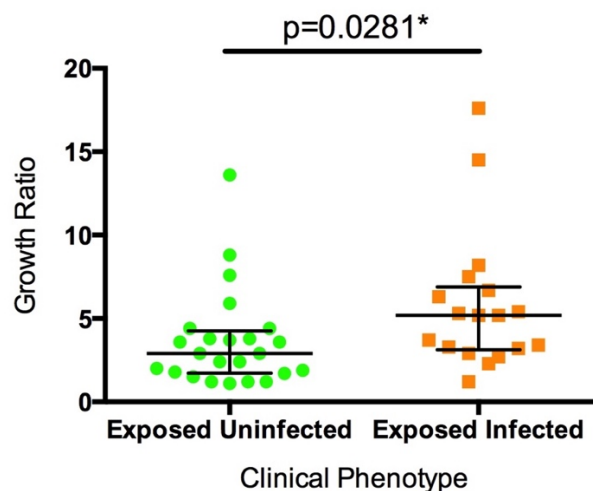


Figure 3: Inhibition of growth of BCG LuxAB by TB-Exposed Uninfected children compared to TB-Exposed Infected children from a pilot household contact study in Cape Town.

Whole blood was incubated with BCG-luxAB and metabolically active bacteria were measured at time 0 and 96 hours by bioluminescence. A growth ratio was calculated (T96/T0). Horizontal lines=median, error bars=interquartile range. (Unpublished data B Kampmann)

On the basis of this pilot data, there were sufficient grounds to support testing the hypothesis that children who have been exposed but remain uninfected are better able to control mycobacteria than those who have LTBI and investigating underlying mechanisms.

Following statistical advice from Dr. David Jeffries, Senior Statistician at MRC Unit The Gambia, a sample size of 30 was estimated to provide 80% power, with a sample size of 40 providing 90% power to detect a significant difference at the 5% level. This was based upon the assumption that the BCG-LuxAB assay would be used (see Chapter 4 for development of BCG-GFP-LuxFO Assay)

3.2.5 Inclusion and exclusion criteria for entry of a compound into this study

In order to be recruited to this study, children needed to have already been recruited to the parent study. Using the information gathered at the initial screening and evaluation carried out by the MRC Childhood TB Program Grant team, potential recruits to this study were identified by myself and the dedicated study fieldworker, using the criteria outlined below (see appendix A.3.2).

Inclusion criteria for a compound to be considered eligible for this study:

- Are there more than two child contacts (age 5 years→15 years) of the index case?
- Does the index case have smear positive tuberculosis?
- Does the family live in the Greater Banjul Area?
- Have the child contacts had Tuberculin Skin Test (TST) placed, read, and recorded?
- Is there at least one child contact with TST greater than or equal to 10mm?
- Is there at least one child with TST less than or equal to 5mm?
- Is there a parent or guardian who can give consent?

Exclusion criteria for this study:

- Is the family planning to leave Greater Banjul Area in next 12 months?
- Do all the children with TST greater than or equal to 10mm have symptoms of TB?
- Do all the children with TST less than or equal to 5mm have symptoms of TB?
- Have the children being considered for this study previously received treatment for TB infection or disease?

3.2.6 *Case definitions*

Case definitions are summarized in Table 2. A schematic of the flow and classification of cases into this study is shown in Figure 4.

Child classification	High Exposure Infected (HEI)	High Exposure Uninfected (HEU)	Lower Exposure Uninfected (LEU)
Age	5 years ≤ Age < 15 years	5 years ≤ Age < 15 years	5 years ≤ Age <15 years
Exposure to adult index case with smear-positive tuberculosis	Sleeps in same bed, same room, or same house	Same sleeping proximity as HEI child.	Sleeps in different building but in same compound
Infection status	Initial TST ≥10mm	Initial TST ≤5mm <i>and</i> repeat TST >3 months later ≤5mm	Initial TST ≤5mm <i>and</i> repeat TST >3 months later ≤5mm
Disease status	Asymptomatic on symptom screening and clinical evaluation including normal chest radiograph	Asymptomatic on symptom screening	Asymptomatic on symptom screening

Table 2: Case definitions for children recruited to the study

TST=Tuberculin Skin Test. See appendix A.3.1 for further details on symptom screening.

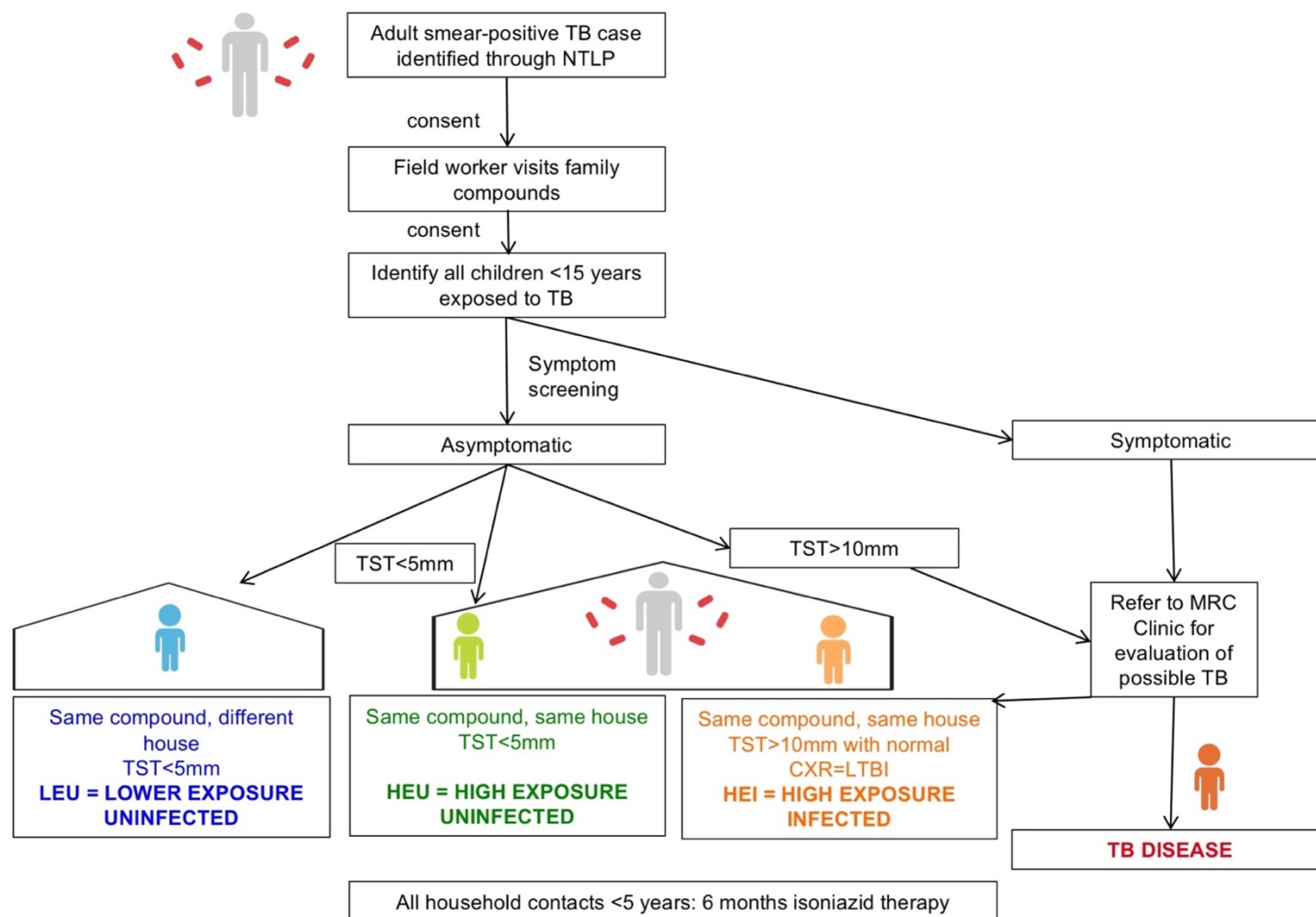


Figure 4: Schematic of study procedures and recruitment

NTLP=National Tuberculosis and Leprosy Program, TB=tuberculosis, TST=tuberculin skin test, CXR=chest radiograph, LTBI=Latent *M tuberculosis* infection, MRC=Medical Research Council

3.2.7 Identification of suitable compounds and consent

The database of the household contact platform was interrogated with the inclusion and exclusion criteria to identify potential compounds, whose eligibility was then verified (See appendix A.3.3). All eligible compounds were approached by a dedicated study fieldworker and those that gave informed consent were enrolled a minimum of three months after initial screening. If an LEU child at the same compound also met the inclusion and exclusion criteria, they were also approached for recruitment following a protocol amendment approval in August 2015. Where more than one child in the compound met the criteria for HEU/HEI/LEU, a single child from those eligible was selected at random.

Consenting participants attended clinic on a scheduled Tuesday or Thursday morning and blood samples were taken for the BCG-GFP-LuxFO assay and other specimens detailed below (See appendix A.3.4). A repeat TST was performed at this point on HEU and LEU children who had previously had a result of <5mm. Transport and breakfast were provided for the children and accompanying adults.

Demographic and epidemiological characteristics of participants were recorded (see appendix A.3.5). Other epidemiological factors related to tuberculosis such as socioeconomic status, smoke exposure (tobacco and solid fuel), household ventilation, and anthropometric measurements including weight, height, and mid-upper arm circumference are not available.^{85,198–203}

In parallel to this study and over the same time period, data was collected on characteristics of the index cases in the household contact platform in order to

evaluate an exposure score for paediatric infection in a Gambian context.³⁶ The self-reported duration of cough is summarised in Table 3. Therefore, 63% of children in the household contact study will have been exposed for >3 weeks to an untreated adult case of smear-positive pulmonary tuberculosis and 95% will have been exposed for >1 week.

Cough duration	Frequency	%
< 1 week	11	5.12
1-2 weeks	68	31.63
3-4 weeks	82	38.14
> 4 weeks	54	25.12
Total	215	100

Table 3: Cough duration of index cases in household study

The consent process and ethical approvals included permission for MRC Unit research staff to request the results of the HIV tests carried out by the National Leprosy and Tuberculosis Program upon the adult with smear-positive tuberculosis index case.

3.2.8 Sample procedures

A maximum of 10ml of blood was collected from each child by nursing staff trained in venepuncture at the Childhood TB clinic at the MRC Unit (appendix A.3.4).

- A total of 7ml of whole blood in 10ml Lithium Heparin Vacutainer tube (BD, Oxford, UK) for the BCG GFP-LuxFO assay and in-house interferon gamma release assay
- 0.5ml in an EDTA Microtainer tube (BD, Oxford, UK) for a full blood count.

Where sufficient blood was available, serum and RNA samples were also taken for future research.

Supernatants from each replicate of the BCG-GFP-LuxFO assay (see Chapter 4) were split into two aliquots (one for cytokine quantification and one for eicosanoid quantification) and stored at -70°C. Samples were shipped on dry ice following the end of recruitment to -70°C freezer facilities at Imperial College London. Prior to cytokine and eicosanoid quantification, triplicate aliquots were thawed, pooled, and sterile filtered by spinning for 10 mins at 5000 RPM with 0.22µm centrifuge Spin-X tube filters (Corning B.V Life Sciences, Amsterdam, The Netherlands). The pooled filtered samples were transferred to 96 well plates and stored at -70°C until the day of analysis.

3.2.9 Data management

Study documentation was stored in a locked drawer in an office accessible by security smart card in MRC Unit The Gambia. Data about the children from the parent household contact platform was stored in the existing bespoke database by the MRC Gambia data storage team. Following data transfer, anonymised participant data was entered into Microsoft Excel files stored using BoxSync (Redwood City,

CA, USA), the recommended secured cloud storage platform of Imperial College London Information Governance Steering Group.

3.4 Immunology assays methodology

3.4.1 In-house interferon gamma release assay (IGRA)

An established in-house IGRA has been in use at MRC Unit The Gambia for several years,^{77,204,205} and its application to children in the contemporary household Contact Tracing Platform at MRC Unit The Gambia has recently been reported.¹⁹⁴ The same method with samples collected under the same procedures and carried out by the same laboratory staff was used to evaluate the status of HEI children included in this study at the time of their initial screening visit to the Childhood TB Clinic.

Quantification of interferon- γ in these samples was carried out in a single batch after recruitment to this study had concluded.

In summary, 100 μ L of undiluted heparinised whole blood was incubated in duplicates with *M. tuberculosis* antigens ESAT-6/CFP-10 fusion protein (EC) (10 μ g/mL final concentration; kindly provided by Tom Ottenhoff, Leiden University Medical Center, Leiden, the Netherlands), and positive (Phytohaemagglutinin [PHA]-L; Sigma-Aldrich, Gillingham, UK; 10 μ g/mL final concentration); & Purified Protein Derivative (PPD; Statens Serum Institute, Copenhagen, Denmark; 10 μ g/mL final concentration) and negative (RPMI 1640 medium; Sigma Aldrich, Gillingham, UK) controls. Plates were incubated at 37°C overnight (maximum 20 hours) with 5% CO₂. Supernatants were harvested and then stored at -20 °C until quantification of the IFN- γ concentrations.

96-well plates were coated with mouse anti-human IFN- γ monoclonal antibody (final concentration 2 $\mu\text{g/mL}$; Pharmingen, Oxford, UK) and incubated overnight at 4°C. Following washing with PBS-5% Tween-20, wells were blocked using PBS/fetal calf serum (Sigma-Aldrich, St Louis, MO, USA) for 2 h. Samples, controls and standards were then added in duplicate and the plates incubated overnight at 4°C. After washing on day 3, biotinylated mouse anti-human IFN- γ detection antibody (final concentration 1 $\mu\text{g/mL}$; Pharmingen) was added, the plates incubated for 45 min at room temperature and a final colorimetric step performed by addition of avidin–peroxidase followed by OPD Fast (both from Sigma-Aldrich, USA). The reaction was stopped using 2 M H_2SO_4 and the plates were read within 45 min at 492 nm with a four-parameter curve-fit. The level of IFN- γ measured in the negative control wells (medium only) was subtracted from that measured in the antigen-stimulated wells to calculate the *M. tuberculosis* antigen-specific IFN- γ values. We defined a positive IFN- γ response as ESAT-6/CFP-10 fusion protein-specific IFN- γ values ≥ 155.0 pg/mL.¹⁹⁴ An assay was considered indeterminate if there was no measurable IFN- γ response to either of PHA or PPD.

The assay was also run with samples taken as part of this study but quality control standards were not met and therefore the data is not presented.

3.4.2 Multiplex cytokine quantification

A standardised commercial multiplex magnetic bead-based immunoassay, Bio-Plex Pro (Bio-Rad, Hercules, CA, USA), was used to quantify cytokine levels.

Two panels were tested:

- Bio-Plex Pro Human Inflammation 5-plex: IFN- α 2, IFN- β , IFN- γ , IL-10, IL-12 (p40).
- Bio-Plex Pro Human Cytokine 4-plex (IL-1 β , IL-10, IFN- γ , TNF- α) and Bio-Plex Pro Human Cytokine IL-1 α Set

All samples from children from a matched compound were run on the same plate. Each matched group was randomly allocated across plates. Two individual quality control aliquots were run on each plate pooled from supernatants from two subjects not included in this study: “early unstim QC” was pooled from medium only supernatants at baseline and 24 hours and the baseline sample with BCG; “late stim QC” was pooled from the 24-hour and 96-hour samples with BCG and the 96 hour medium only sample.

The manufacturer’s assay guidance was followed. In summary, a fresh dilution series of reconstituted standards for each plate was used to generate an eight-point standard curve with a fourfold dilution between each point. Sample plates were thawed immediately prior to the experiment. Coupled magnetic beads were vortexed and diluted to 1x with Bio-Plex assay buffer. 50 μ l of 1x beads was added to each well of the assay plate. Using a Bio-Rad magnetic handheld plate washer, the plate was washed two times with 100 μ l Bio-Plex wash buffer. Samples, the standard curve dilutions and blanks were vortexed and 50 μ l added to the wells. The plate was protected from light and incubated at room temperature on a rocking platform at 850 revolutions per minute (RPM) for 30 minutes. The plate was washed three times with

100µl wash buffer. 25µl of 1x diluted detection antibodies were added to each well and the plate returned to the rocking platform for 30 minutes. The plate was washed three times with 100µl wash buffer. 50µl of 1x streptavidin-phycoerythrin conjugate was added to each well and the plate was returned to the rocking platform for 10 minutes. The plate was washed three times with 100µl wash buffer. Samples were resuspended in 125µl of assay buffer and returned to the rocking platform for 30 seconds before being read using a Luminex 200 plate reader (LuminexCorp, Austin, Tx, USA) with Bio-Plex Manager software (Bio-Rad, Hercules, CA, USA). Where low values were evaluated as “out of range” they were replaced with ¼ of the lowest detected value.

3.5 Haematology procedures

Full blood count samples taken at the time of phlebotomy for this study were analysed by the Clinical Services Diagnostic Haematology Laboratory at MRC Unit The Gambia using the Cell Dyn 3700 Haematology Analyser (Abbott Diagnostics, Illinois). If this was not available, full blood count samples were run using Medonics M-series (Boule Medical AB, Spånga, Sweden) in the Immunology Research Laboratory at MRC Unit The Gambia.

3.6 Mass spectrometry methodology

A novel targeted Ultra Performance Liquid Chromatography tandem mass spectrometry (UPLC MS/MS) method was used to quantify eicosanoid mediators in this study.²⁰⁶ Quantification of each eicosanoid was obtained by the back-calculation of the ratio of the analyte to a pure internal analytical standard. The use of internal standards in this targeted assay correct for variation in extraction efficiency, instrument variability, ionization efficiency, and sample variability.²⁰⁶

Cell culture supernatants prepared according to the medium only condition of the BCG-GFP-LuxFO assay were used in the validation process for this method and showed similar matrix properties to serum and plasma.²⁰⁶ The effect of 0.22 μ m filtration upon eicosanoid levels was also evaluated through comparison of values derived from samples spiked with internal standards before or after filtration. Relative standard deviations were <5% for all analytes except for PGD2-d4 (17%). Based on the hypotheses and pilot data using the BCG-GFP-LuxFO assay generated with this method, the compounds shown in Table 4 were selected for analysis.

All samples from children from a matched compound were run on the same plate, but each matched group was randomly allocated between plates, and sample order was randomized on each plate. Plates were run on consecutive days.

Abbreviation	Chemical Name	Abbreviation	Chemical Name	Abbreviation	Chemical Name	Abbreviation	Chemical Name
C20:4-d8	Arachidonic Acid – deuterated standard	15(S)-HEPE	15(S)-Hydroxypentaenoic Acid	LXA4	Lipoxin A4	9(S)-HODE	9(S)-Hydroxyoctadecadienoic Acid
5-oxo-ETE-d7	5-oxo-eicosatetranoic Acid – deuterated standard	5(S)-HETE	5(S)-Hydroxyeicosatetranoic Acid	Lipoxin B4	Lipoxin B4	13(S)-HODE	13(S)-Hydroxyoctadecadienoic Acid
5(S)-HETE-d8	5(S)-Hydroxyeicosatetranoic Acid – deuterated standard	15(S)-HETE	15(S)-Hydroxyeicosatetranoic Acid	8(S)-HETE	8(S)-Hydroxyeicosatetranoic Acid	12(S)-HEPE	12(S)-Hydroxypentaenoic Acid
14,15-DHET-d11	14,15-Dihydroxyeicosatrienoic Acid – deuterated standard	16(R)-HETE	16(R)-Hydroxyeicosatetranoic Acid	11(R)-HETE	11(R)-Hydroxyeicosatetranoic Acid	LTB4	Leukotriene B4
PGD2-d4	Prostaglandin D2-deuterated standard	5,6-DHET	5,6-Dihydroxyeicosatrienoic Acid	12(R)-HETE	12(R)-Hydroxyeicosatetranoic Acid	12-oxo-LTB4	12-oxo-leukotriene B4
LTE4-d5	Leukotriene E4-deuterated standard	8,9-DHET	8,9-Dihydroxyeicosatrienoic Acid	6-keto-PGF1alpha	6-keto-Prostaglandin-F1alpha	LTE4	Leukotriene E4
C18:2 (LA)	Linoleic Acid (C18:2)	11,12-DHET	11,12-Dihydroxyeicosatrienoic Acid	PGF2alpha	Prostaglandin-F2alpha	PGE2	Prostaglandin E2
C20:3 (DGLA)	Dihomo-γ-Linolenic Acid	14,15-DHET	14,15-Dihydroxyeicosatrienoic Acid	8-iso-PGF2alpha	8-iso-Prostaglandin-F2alpha		
C20:4 (AA)	Arachidonic Acid	12-oxo-ETE	12-oxo-eicosatetranoic Acid	TXB2	Thromboxane		
C20:5 (EPA)	Eicosapentaenoic Acid	14-HDoHE	14-Hydroxydocosahexaenoic Acid	11-dehydro TXB2	11-dehydro-thromboxane		
C22:6 (DHA)	Docosahexaenoic Acid	17(S)-HDoHE	17(S)-Hydroxydocosahexaenoic Acid	PGD2	Prostaglandin D2		

Table 4: Eicosanoids selected for analysis in this study

All solvents used were high purity LC/MS grade Chromasolv reagents (Sigma-Aldrich, Gillingham, UK). Internal standards and deuterated standards were supplied by Cayman Chemical Company (Ann Arbor, MI, USA). 350µL sample collection plates were used for preparation and collection of samples (Waters Corporation, Milford, MA, USA). Mixed-mode Solid Phase Extraction (SPE) with Strong Anion Exchanger was carried out using an Oasis MAX µElution Plate (Waters Corporation).

The published methodology was followed.²⁰⁶ Briefly, stock mixtures of internal and deuterated standards, at 2 ng/µL, were prepared in methanol and stored at -20°C. For each plate, a working mixture at 300pg/µl was made with a final solvent composition of 1:1 methanol: water. 100µl of sample was transferred into each well of a collection plate and 20µl of internal standards working mix was added. 100 µL of a 2% formic acid solution in water was added to each well and the plate was capped and gently mixed. The SPE plate was conditioned with 200 µL of methanol and evaporated using a vacuum manifold at 3mmHg. The sorbent was equilibrated with 200µl of water and again evaporated to dryness. Samples were transferred from the preparation plate to the SPE plate. Each well of the preparation plate was washed with 50µl of 1:1 water: methanol and this was transferred to the SPE plate. The plate was evaporated to dryness with the vacuum manifold at 3-5mmHg. The plate was then washed with 200µl of water with 2% ammonium hydroxide and evaporated to dryness. The plate was then washed with 200µl of 1:1 water: acetonitrile and evaporated to dryness. A collection plate was then placed beneath the SPE plate and the analytes were eluted four times, each with 25µl of methanol and 2% formic acid and the vacuum manifold. The collection plate was then removed and evaporated to dryness before being reconstituted in 100µl of 1:1 water: methanol,

mixed and placed in the sample manager of the Acquity UPLC at 4°C. A 13-point calibration curve of the internal standards with concentrations ranging from 0.1→1000pg/μl for oxylipins and 1→10,000 pg/μl for free fatty acids was prepared in 1:1 water: methanol solvent.

Liquid chromatography was performed with a Waters HSS T3 UPLC column (100 mm x 1mm, 1.8 μm) column maintained at 40°C on a Waters Acquity UPLC system (Waters Corporation, Milford, MA, USA). Mobile phase A was H₂O + 0.1% formic acid and mobile phase B was acetonitrile +0.1% formic acid (B), with a 0.14 mL/min flow rate. The injection volume was 5 μ L and all analytes were eluted in a linear gradient from 30 to 70% B between 0 and 12 min; 100% B was held between 12.1 and 13.1 min and the column was re-equilibrated to starting conditions between 13.1 and 15 min. A post-column infusion of acetonitrile + 37% formaldehyde 3:1 at a 5μL/min flow rate was added.

The UHPLC system was connected to a Waters Xevo TQ-S triple quadrupole mass spectrometer with an electrospray ionization (ESI) source (Waters Corporation, Milford, MA, USA). In negative ion mode, the source parameters were: capillary voltage 2.5 kV; cone voltage was optimized between 10 and 40 V depending on the analyte; source temperature 150°C, desolvation temperature 500°C; desolvation gas flow at 900 L/h; and cone gas flow at 150 L/h. Analyses were performed in negative Multiple Reaction Monitoring mode, with dwell time, cone voltage and collision energy optimized for each analyte. Peak detection, integration and quantification were realized with Masslynx and TargetLynx software (Waters) and the data exported as comma separated values spreadsheets.

3.7 Statistical analysis

3.7.1 Descriptive statistics

Given the matched study design, appropriate matched statistical analyses were employed. For approximately normally distributed data, means and 95% confidence intervals are used to describe the data and paired t-tests to test for statistically significant differences between groups. For non-normally distributed data, medians and interquartile ranges are used to describe the data and Wilcoxon's matched pairs signed rank test was used to evaluate for statistical significance. For binary variables, McNemar's χ^2 test was used. These analyses were carried out with STATA Statistical Software: Release 12.1. (StataCorp LP, College Station, TX) or Prism 7 for MacOS X (Graphpad Software Inc). Where bivariate analyses were used to assess for interactions between variables, an unmatched two-way ANCOVA was employed using STATA.

3.7.2 Mixed effects modelling for matched data over time

Given that luminescence, cytokine, and eicosanoid experimental data were collected from matched groups of children and involved a minimum of three longitudinal data points for each child, multi-level mixed effects linear regression (or mixed effects modelling) was used to analyse the data. Mixed effects models contain both fixed effects that are equivalent to standard regression coefficients, but in addition random effects to account for variance attributable to clustering or matching within the data.^{207–209} Therefore, in this study design, the between-replicates, between-children and between-matched group variability can be considered as random effects contributing to variance and covariance of the data, whilst group, age of the child,

and time point can be considered fixed effects, the combination of all of which can be used to predict the experimental result of interest (dependent variable).

Histograms and quantile-normal plots by transformation were used for experimental data to evaluate whether there was a suitable transformation that approximated a normal distribution. If this condition was met, mixed effects modelling was carried out with age (divided into 1st [younger]/ 2nd/3rd [older] tertiles), time (hours), group (HEI=2/HEU=3/LEU=4), and the interaction between group and time as categorical fixed effects, and compound and child as random effects. The models were run with the unstructured covariance option, so that all variances and covariances were distinctly estimated and models were fitted via restricted maximum likelihood.

Restricted maximum likelihood forms a set of linear contrasts of the response that do not depend on the fixed effect, but depend only on the variance components.²⁰⁸ The models are presented in Appendix 4. Estimated marginal means and 95% confidence intervals by group and time were predicted from the model treating all fixed effects including age as balanced and these are presented graphically in the relevant results sections. Statistical interpretation of differences between groups at each time point was carried out through pairwise comparisons of adjusted predictions. The distribution of residuals was checked by histograms and quantile-normal plots. All mixed effects modelling was carried out with STATA.

3.8 Reporting of results for experimental data

Where there are figures comparing groups, the following colour code will be maintained throughout the thesis: HEI – orange /HEU – green /LEU – blue. The threshold for statistical significance is considered to be *p* values less than 0.05.

Correction for multiple testing has not been applied and this is discussed in Section 9.4. Where p values meet the threshold for statistical significance they are shown at the relevant time point on the graph and discussed in the text. In view of the study design, p values reflect matched statistical analyses and therefore may not necessarily correlate visually with the non-matched display of data by participant group in the conventional column scatterplots that are used for clarity. For Chapters 7 (cytokines) and 8 (eicosanoids), analyses were carried out for the medium only experimental condition, presence of BCG experimental condition, and BCG-specific responses (values from BCG-experimental condition with medium only values subtracted). For clarity and consistency the following structure has been adopted for reporting results for luminescence data (Chapter 6), cytokine data (Chapter 7), and eicosanoid data (Chapter 8):

- Consideration of whether any adjustment of data was required for reasons such as variability between experimental runs
- Non-parametric analysis of the whole HEI vs HEU dataset
- Where appropriate, mixed effects modelling of HEI vs HEU for the whole dataset
- Subgroup analysis - between HEI children with a positive IGRA and HEI children with discordant IGRA. Non-parametric unmatched statistical testing was carried out with the Mann Whitney U test. Sub-analysis of the HEI vs HEU data following exclusion of compounds where the HEI child had a discordant IGRA are presented if it differs from the main analysis or is of particular biological relevance.

- Subgroup analysis - of data from compounds where a LEU child was recruited, comparing LEU to HEI children and LEU to HEU children. Where appropriate, mixed effects modelling of the compounds where a LEU child was recruited to the study.

CHAPTER 4: DEVELOPMENT OF BCG-GFP-LUXFO WHOLE BLOOD ASSAY

4.1 Introduction to assays of human immunity to tuberculosis

One of the fundamental hurdles in tuberculosis vaccine research is the absence of an immunological correlate of protection in humans.^{163,164} Animal models, such as mouse, guinea pig, rabbit and macaque, although informative, have not always had their findings corroborated in humans.^{167,168} Therefore a range of functional approaches to evaluate human immunity have been employed as summarized in Figure 5 and recently reviewed.^{210,211}

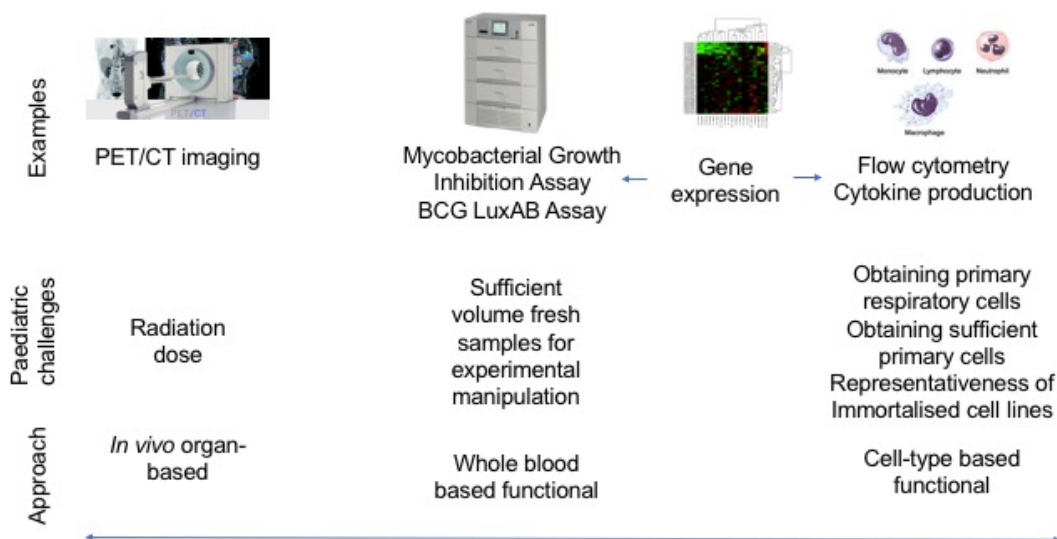


Figure 5: Experimental approaches to study human immunity to *M tuberculosis*

The broad category of mycobacterial growth inhibition assays seek to evaluate the human response to mycobacteria through quantifying mycobacterial growth in either whole blood or peripheral blood mononuclear cells.^{158,211,212} Although using a cell-type-based approach has its advantages, we know for instance that neutrophils are the most commonly infected phagocyte in human tuberculosis,²¹³ that they augment macrophage mycobacterial responses,²¹⁴ and that they drive a type I interferon-

inducible transcript signature in adult whole blood that correlates with clinical severity.¹³² Therefore, whole blood approaches in studying the host mycobacterial interaction have also been adopted.²¹⁵ Two principal approaches have been used to evaluate mycobacterial growth in whole blood beyond counting of colony forming units – Time to Detection in the Mycobacterial Growth Inhibition Assay and luminescence of recombinant mycobacterial strains in primary human samples.

^{72,99,113,211,212,216–228} Each assay has its experimental advantages and disadvantages.

The direct Mycobacterial Growth Inhibition Assay has been extensively developed for use as a relatively standardised tool in vaccine trials.^{72,210–212,216} It involves the addition of an inoculum of 150-600 Colony Forming Units (CFU) of mycobacteria into either fresh whole blood or thawed frozen PBMCs followed by rotating incubation at 37°C for 96 hours.¹¹³ The samples are then lysed and transferred to the commercial Bactec Mycobacterial Growth Indicator Tube 960 system (BD, Franklin Lakes, NJ, USA). The result of the assay is the length of incubation time until growth is detected (time to positivity), with a longer time to positivity representing greater inhibition of mycobacterial growth. The assay has substantial appeal as it is relatively simple, and involves a commercialised mycobacterial incubation system to facilitate comparison of data between study sites.²¹² It was amongst the assays selected to evaluate paediatric TB cases and controls detected in the MVA85A phase II vaccine study, although it was not able to distinguish between cases and controls using frozen PBMCs.^{113,166} Since this thesis was first submitted, data have been published with the Mycobacterial Growth Inhibition Assay in IGRA positive and IGRA negative 8-year-old children without known MTB exposure, showing no difference in growth inhibition between groups (see table 1, page 35).⁷²

The BCG-*lux* assay involves a 96-hour incubation of whole blood in culture medium with BCG-*lux*, a strain of BCG constitutively expressing *luxAB* genes coding for a luciferase enzyme from *Vibrio harveyi* on a plasmid together with a gene encoding hygromycin resistance to enable selection and prevent loss of the plasmid.^{99,219–227,229} Luminescence of samples is measured using a luminometer at baseline and 96 hours following centrifugation and lysis of samples, dilution and addition of the luciferase substrate. The luminescence at 96 hours divided by that at baseline provides a growth ratio. The assay has the advantage of enabling supernatants to be taken prior to luminescence quantification thereby enabling a given volume of blood to generate both functional and biochemical data. A schematic of the assay is shown in Figure 6.

4.2 Rationale for developing a new BCG-GFP-LuxFO whole blood assay

Both the direct Mycobacterial Growth Inhibition Assay and the BCG-*lux* assay give a single value as the functional outcome – either time to positivity or the 96-hour luminescence growth ratio. Although the latter enables supernatant collection for subsequent biochemical evaluation, measuring luminescence is a destructive process with lysis of cells and vortexing with glass beads. Given the constraints on volumes of blood obtainable in paediatric immunology research, an assay that is able to yield even more experimental data from a finite sample would be a useful addition to the methodological options to evaluate human immunity to TB.

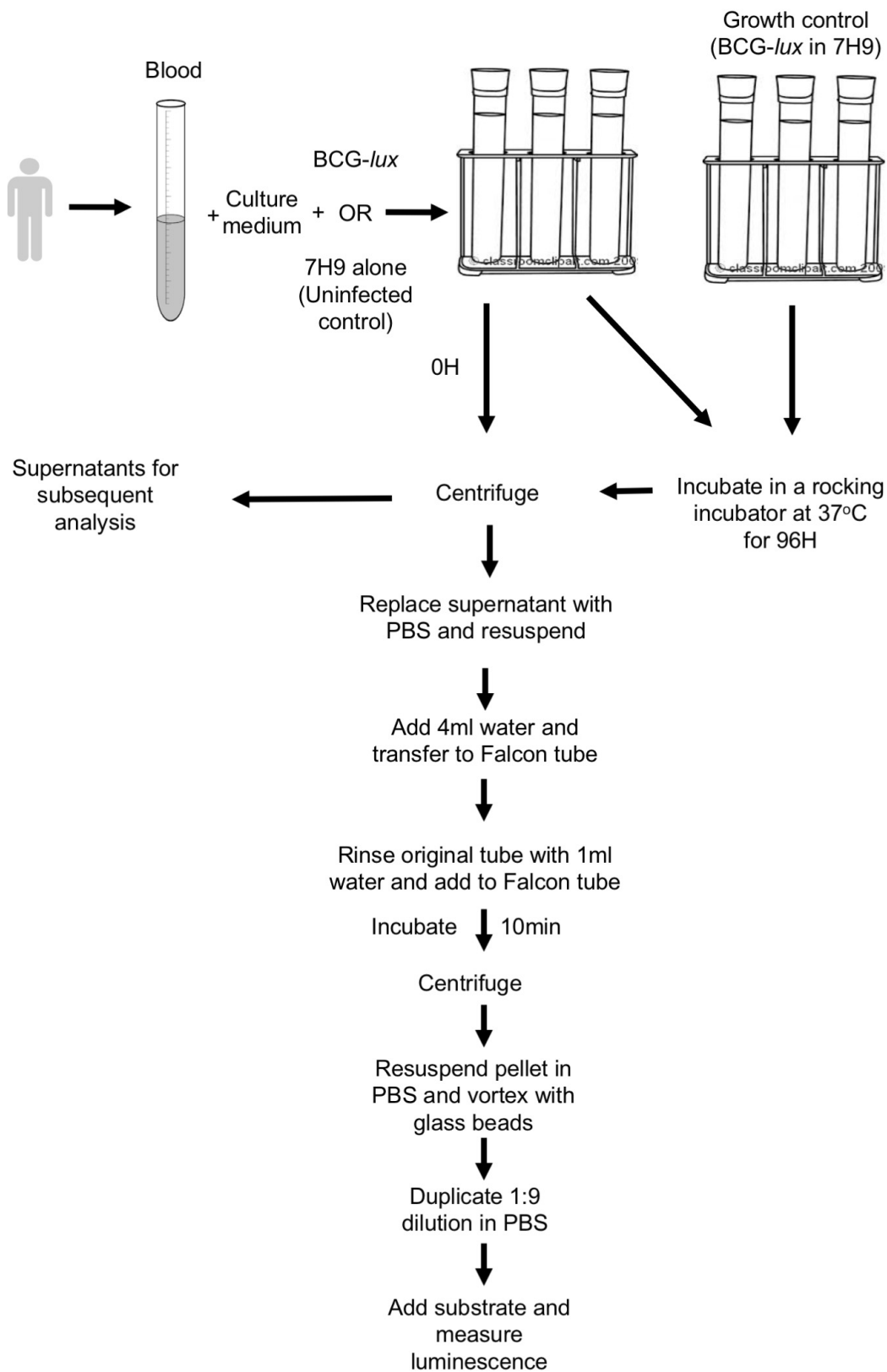


Figure 6: Schematic of BCG-lux assay method

An autoluminescent recombinant BCG strain has recently been published, expressing the luciferase full operon from *Photorhabdus luminescens*, which includes the genes encoding the bacterial luciferase enzyme and the genes for the synthesis of the substrate for the luminescence reaction, thereby enabling quantification of luminescence in a non-destructive manner without needing to add the substrate or lyse samples.^{230–232} This therefore raises the possibility of serial non-destructive luminescence measurements in human-derived samples enabling greater characterisation of the kinetics of host-mycobacterial interactions than existing assays. Furthermore, combination of this with a fluorescent protein would enable cellular assays such as flow cytometry to be carried out on cell pellets as well as biochemical assays on supernatants therefore maximising the data yield from a single sample. In comparison to the BCG-*lux* assay, it could also substantially decrease experimental time, thereby enabling a greater volume of samples to be analysed in a single experiment.

Therefore, as part of this thesis, I developed a recombinant dual reporter BCG-GFP-LuxFO strain for use with minimal volumes of paediatric whole blood.

4.3 Summary of conventional microbiology methods applied in this chapter

4.3.1 Biosafety precautions

BCG is a hazard group 2 organism. Prior to commencing experimental work at both Imperial College London and MRC Unit The Gambia biosafety permissions were applied for and approved. All work was conducted in suitable Category Level 2 laboratories and safety hoods by registered staff trained and approved to work in

these facilities. All work with genetically modified organisms was carried out under existing permissions.

4.3.2 Preparation of liquid media

7H9 Middlebrook broth was prepared with 4.7g of dried broth (Fluka/Sigma-Aldrich, Gillingham, UK), 2.5ml of 20% Tween 80 (Acros Organics/Thermo Fisher Scientific, Waltham, MA, USA), 4ml of 50% glycerol (VWR, Radnor, PA, USA) and dissolved in 893.5ml of dH₂O. This was then autoclaved for 15 minutes at 100kPa at 121°C.

Once cooled 100ml of Albumin Dextrose Catalase (ADC) supplement (BD, Franklin Lakes, NJ, USA) was added and selection antibiotics at the specified concentrations (Hygromycin, Sigma-Aldrich, Gillingham, UK; Kanamycin, Glyco/Life Technologies, Carlsbad, CA, USA). When starting fresh cultures or additional medium was added, 40µL of 20% Tween 80 was added for each 15ml of medium.

4.3.3 Preparation of solid media and CFU counting

21g of 7H11 agar (Fluka/Sigma-Aldrich, Gillingham, UK) and 10ml 50% glycerol was made up to a final volume of 900ml with dH₂O in a 1L bottle. This was then autoclaved for 15 minutes at 100kPa at 121°C. Once cooled, 100ml of Oleic acid Albumin Dextrose Catalase (OADC) supplement (BD, Franklin Lakes, NJ, USA) was added and selection antibiotics at the specified concentrations. This was poured into plates under sterile conditions and cooled before storage at 2-8°C. Prior to use, plates were placed in Containment Level 2 (CL2) Safety Cabinets an hour before use to enable any condensation to dry. 10µL of sample was taken and serial dilutions made in sterile PBS with the 1:100, 1:1,000 and 1:10,000 dilutions plated.

Plates were inspected every 48 hours for 21 days to check for contamination and avoid colonies merging before counting.

4.3.4 Measurement of optical density

Optical density (OD₆₀₀) of 0.5ml volumes of stock was measured using a Biophotometer Plus spectrophotometer (Eppendorf, Hamburg, Germany) with liquid medium only as a blank.

4.4 Transformation of the parent strain

Transformations were carried out as per the protocol of Goude & Parish.²³³

Assistance was provided by Iria Uhia Castro from the group of Brian Robertson. The parent strain used was BCG Danish (Staten Serum Institute, Copenhagen, Denmark).

The following plasmids were used:

- pMV306DIhsp+LuxG13 (Figure 7a): Kanamycin-resistant integrase-free integrative reporter plasmid harbouring the Luciferase Full Operon (*Photorhabdus luminescens*) modified for expression in Gram positive bacteria by replacement of the ribosome binding sites and reorganisation of the genes.^{230,232,234} The *Mycobacterium marinum* promoter G13 is cloned in front of *luxC* in order to increase the amount of substrate synthesised by the Lux operon. (pMV306DIhsp+LuxG13 was a gift from Brian Robertson, Addgene plasmid # 49999)²³²
- pBS-Int (Figure 7b): A suicide plasmid that expresses the integrase gene necessary for the integration of pMV306DIhsp+LuxG13 into the chromosome

antibiotics for 16 hours. Cells were harvested by centrifugation at 3000xg for 10 minutes and dilutions were plated out on 7H11 plates with kanamycin at 20µg/mL and hygromycin at 50µg/mL. Plates were incubated at 37°C until discrete visible colonies appeared. Colonies were then picked and incubated in 7H9 liquid media at 37°C. Those that demonstrated logarithmic growth were frozen down in 15% glycerol. Growth of the frozen stock was then verified, and the most reliable stock was shipped to The Gambia and expanded to make new frozen stocks for use in this assay.

4.5 Serial luminescence measurements and identification of temperature sensitivity

Luminescence was quantified in Relative Light Units/second (RLU/s) using the Sirius Tube Luminometer (Berthold Detection Systems GmbH, Pforzheim, Germany) with the substrate injector switched off. Following an adapted protocol from BCG-*lux*, luminescence was initially measured after dilution in Phosphate-buffered Saline (PBS) and an unexpected variability in measurements was noted. To investigate whether this variability related to time or temperature, 1:10 dilutions of BCG-GFP-LuxFO in logarithmic growth phase were made with PBS at 4°C, room temperature, and 37°C and sequential measurements were made over time. Results are shown in Figure 8.

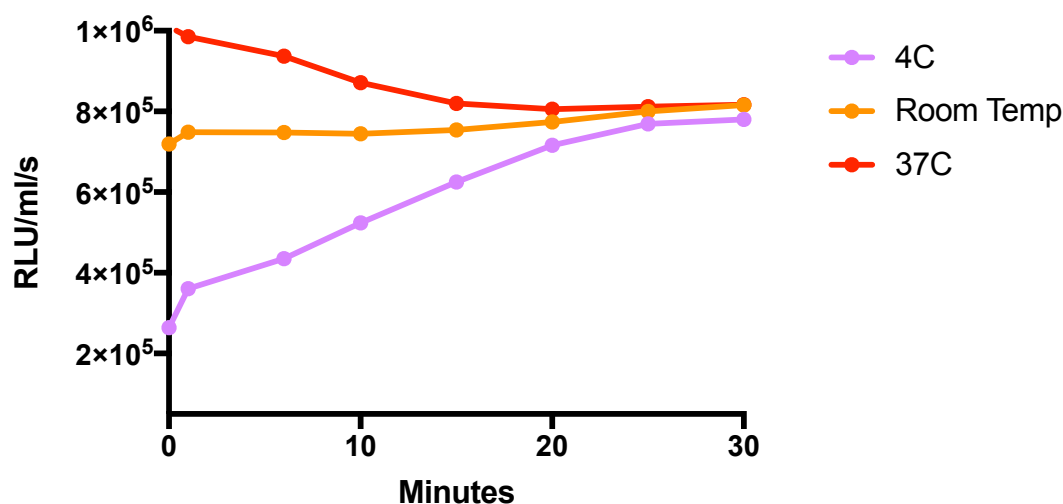


Figure 8: Effect of temperature on luminescence of BCG-GFP-LuxFO.

1:9 dilutions of BCG-GFP-LuxFO were made with Phosphate Buffered Solution at 4C, room temperature, and 37C and serial luminescence measurements were made over time. RLU=Relative Light Units

A clear relationship between temperature of the diluent was demonstrated with convergence on room temperature values over time. Thereafter, the luminescence measurement protocol was adapted to minimise handling and temperature disturbances to samples. 0.5mL of undiluted stock was taken from the Erlenmeyer flask (Corning B.V Life Sciences, Amsterdam, The Netherlands) immediately upon removal from the 37°C incubator and transferred into a sterile 5ml round-bottomed 75x12mm tube with lid (Corning) and luminescence quantified immediately.

4.6 Growth characteristics of BCG-GFP-LuxFO

4.6.1 Liquid media

Growth of the BCG-GFP-LuxFO stocks was measured by optical density using a spectrophotometer, luminescence as described above, and plating of CFUs. The growth characteristics in 7H9 liquid medium and correlations are shown in Figures 9, 10, and 11.

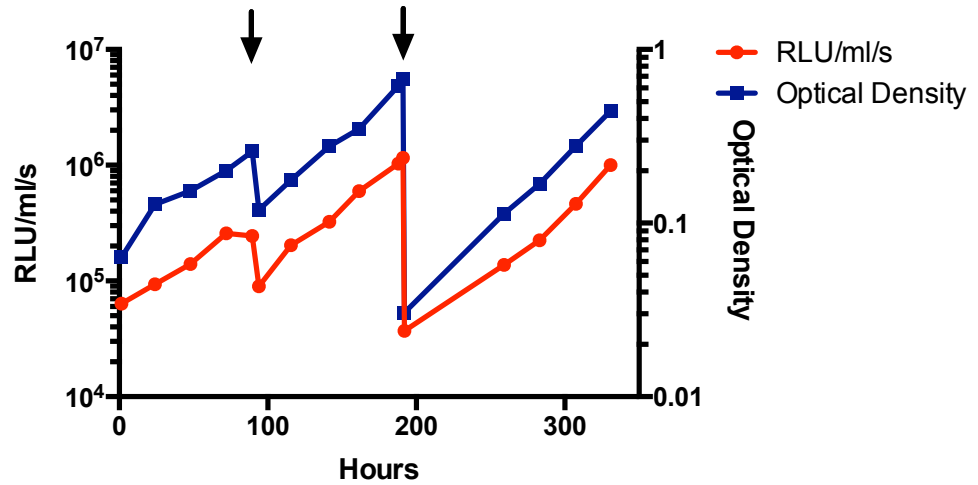


Figure 9: Luminescence and optical density growth curves of BCG-GFP-LuxFO.

Luminescence is shown on the left y axis, indicated in red and optical density on the right y axis indicated in blue. Arrowheads denote addition of media to maintain bacteria in logarithmic growth phase. RLU=Relative Light Units

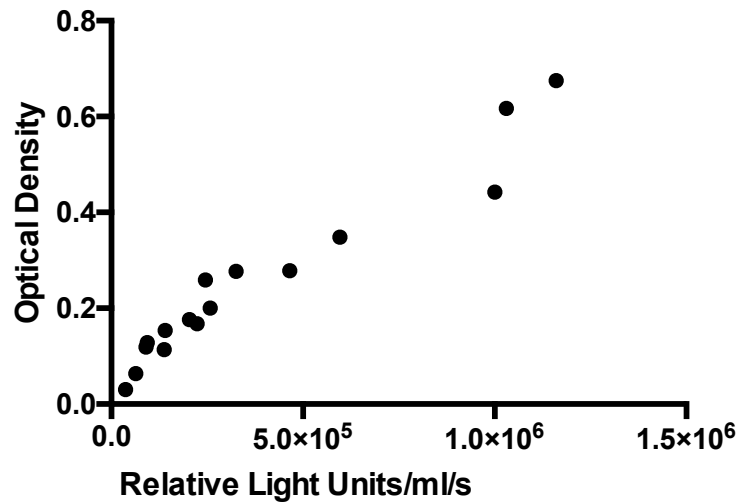


Figure 10: Correlation between optical density and luminescence of BCG-GFP-LuxFO in 7H9 liquid medium.

$n=16$ pairs of observations. Spearman rank correlation coefficient, $r=0.9853$ (95% CI: $0.9556 \rightarrow 0.9952$; $p < 0.0001$)

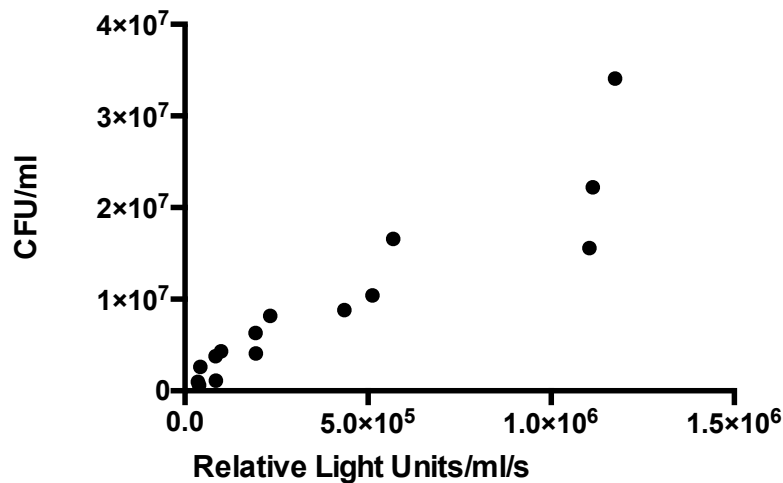


Figure 11: Correlation between colony forming units and luminescence of BCG-GFP-LuxFO in liquid 7H9 medium.

$n=15$ pairs of observations. Spearman rank correlation coefficient $r=0.9714$ (95% CI: 0.9112 to 0.9910; $p<0.0001$)

The data in Figure 11 could be interpreted as showing a threshold effect of luminescence above 1×10^6 RLU/s. However, when maintained in logarithmic growth phase without dilution with additional media, luminescence levels of up to 1×10^8 were reached (data not shown). The RLU: CFU ratio for BCG-GFP-LuxFO is 0.05 RLU: 1 CFU. This is in comparison to a ratio of 3-5 RLU : 1 CFU for BCG LuxAB. The difference in the RLU:CFU ratio between the Lux AB and the GFP-LuxFO strains is likely to reflect the constitutive expression of the whole luciferase operon in GFP-LuxFO and the associated metabolic costs.^{230–232} Furthermore, measuring luminescence in LuxAB involves lysis of red blood cells, vortexing with glass beads, and addition of doses of the luciferase enzyme substrate likely to exceed those produced constitutively by the bacteria.²²⁰

4.6.2 Luminescence and CFU correlation in paediatric whole blood.

10 μ L aliquots were removed from paediatric whole blood BCG-GFP-LuxFO assays every 24 hours and 1:100 dilutions were plated with CFUs counted after 21 days. (Figure 12: full methodology described in section 4.10).

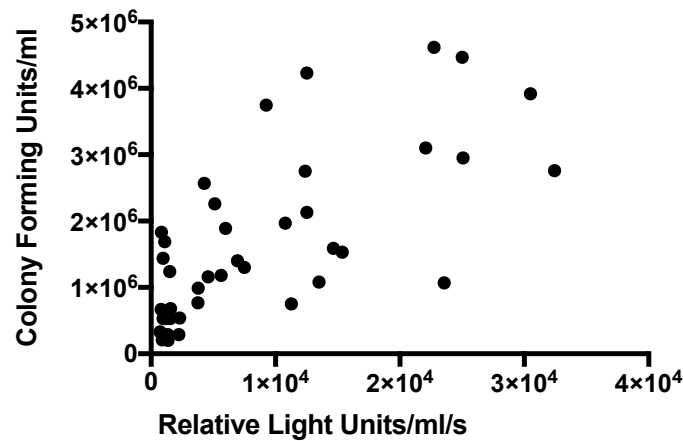


Figure 12: Correlation between luminescence and colony forming units in BCG-GFP-LuxFO paediatric whole blood assay.

$n=45$ pairs of observations (samples from 3 experiments, from 9 children at 5 time points). Spearman rank correlation coefficient $r=0.7123$ (95%CI: 0.5230 to 0.8346; $p<0.0001$)

For each child, there are three replicates, each of which is measured three times at each timepoint. A dataset of three separate experiments, each with three different children, was used to assess for variability within the assay in paediatric whole blood. Mean coefficients of variation were 7.65% for a) the three measurements of each tube and 11.5% for b) the three replicates for each child. The data shows that inter-tube variability is greater than intra-tube variability as would be expected in a biological assay using primary human samples. Replicates were included as a variable in preliminary mixed effects models but did not significantly contribute to variance in the data.

4.7 Confirmation of fluorescence

GFP expression of BCG-GFP-LuxFO was confirmed by flow cytometry (Figure 13)

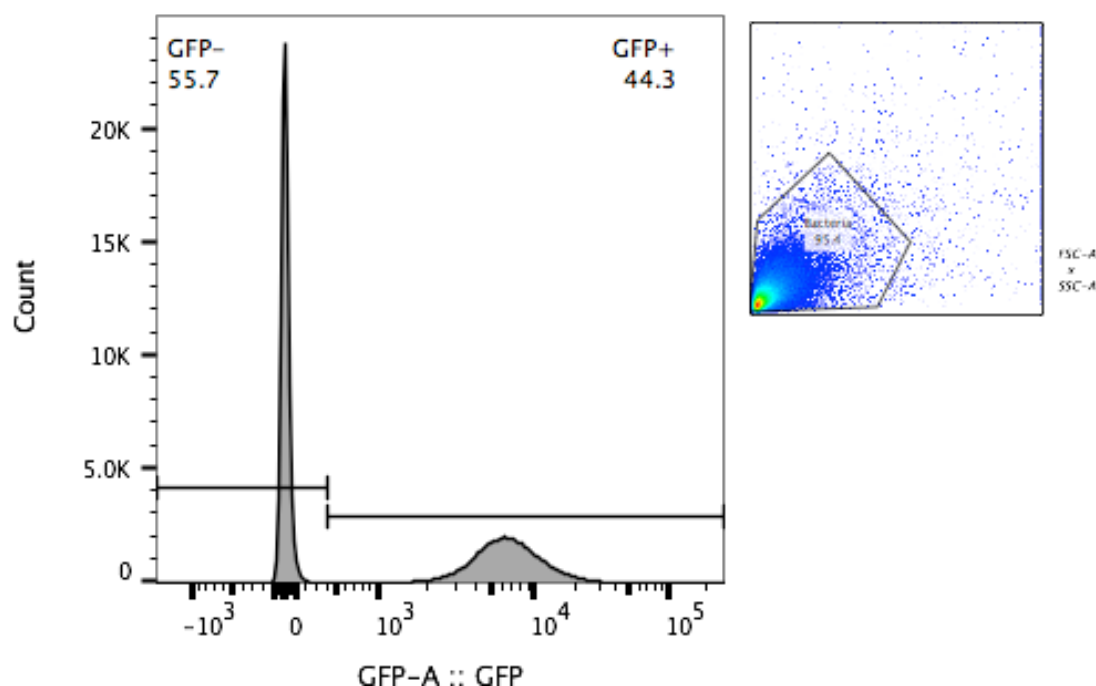


Figure 13: Flow cytometric confirmation of fluorescence of BCG-GFP-LuxFO.

A mixture of non-fluorescent BCG and BCG-GFP-LuxFO in a 1:1 ratio fixed in 2% paraformaldehyde was analysed using the BD Biosciences Fortessa and confirmed fluorescence in the GFP channel in a 1:1 ratio.

Flow cytometry with cell-specific surface markers combined with apoptosis markers could enable investigation of the kinetics of which cell types are infected in the assay over time, and also mode of cell death.^{79,99,139,236} Exploration of this aspect did not prove possible as part of this study. The literature using whole blood mycobacterial assays shows that monocyte/macrophages and neutrophils are the principal infected cell types, with a smaller number of myeloid dendritic cells also infected.^{72,99,237}

4.8 Establishing assay sensitivity

In view of the relative low RLU: CFU ratio of BCG-GFP-LuxFO, it was important to establish assay sensitivity under field conditions in The Gambia. Therefore 20 measures of triplicate tubes of control bacterial media and blood with RPMI culture media were carried out over 5 days to quantify background luminescence in the absence of luminescent bacteria. The threshold of detection following background

subtraction was set at 3 times the standard deviation of the background light levels (Table 5).

	Mean background luminescence (RLU/s)	Standard Deviation (SD) (RLU/ml/s)	Detection threshold (3 x SD) (RLU/ml/s)
Bacterial media	102.6	77.4	232.2
Whole blood: RPMI 1640 culture media	41.4	12.4	37.1

Table 5: Background luminescence of bacterial media and whole blood and calculation of detection thresholds.

RPMI=Roswell Park Memorial Institute

To establish the sensitivity of the luminometer in The Gambia, serial dilutions in 7H9 bacterial media of a neat BCG-GFP-LuxFO culture in logarithmic growth phase were made down to 1: 20,000. Dilutions were replaced in the incubator at 37°C for 1 hour before measurement. Results are shown in Figure 14 and demonstrate a linear relationship to the detection threshold of the assay for bacterial media.

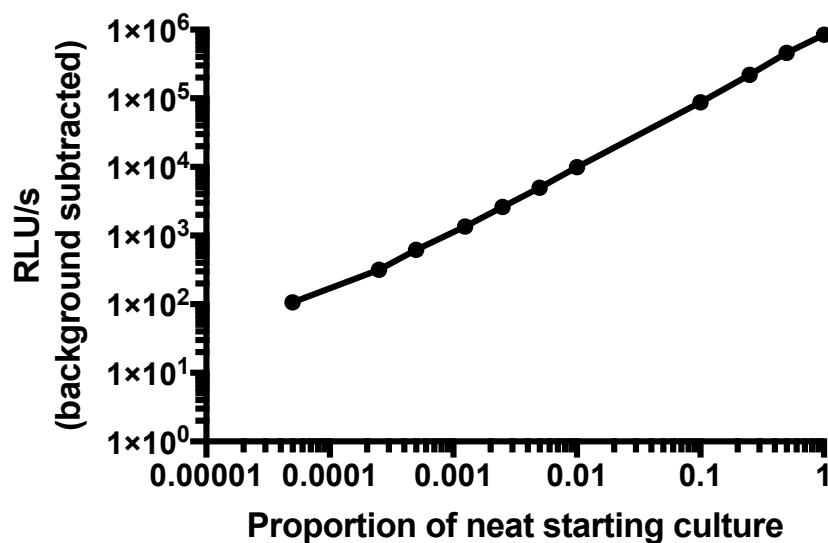


Figure 14: Sensitivity of the luminometer at low absolute luminescence levels.

4.9 Choice of starting inoculum

A starting inoculum for the BCG-GFP-LuxFO whole blood assay was chosen by trying to minimise the equivalent of Multiplicity of Infection (MOI) whilst maintaining the baseline luminescence above the threshold of detection for the assay, so that if luminescence was to decrease from baseline it would still be well within the dynamic range of the luminometer. An inoculum taken from a stock diluted down to a concentration 3.3×10^5 RLU/ml/s BCG-GFP-LuxFO and added to blood:RPMI mixture in a ratio of 1:9 led to luminescence in whole blood samples of approximately 250-350 RLU/s after one hour of incubation and this was therefore chosen as it would enable detection of a log decrease in luminescence from baseline.

4.10 Summary of BCG-GFP-LuxFO whole blood assay methodology

A schematic of the assay is shown in Figure 15. A total of 5ml of whole blood taken in lithium-heparin tubes was required from each child for the BCG-GFP-LuxFO assay. This is sufficient to have triplicate tubes for each condition (i.e. with BCG-GFP-LuxFO added and control uninfected samples with medium only added) for each of the three time points (18 tubes) and a further tube for taking aliquots for CFU measurement.

A vial of BCG-GFP-LuxFO stored in 15% glycerol at -70°C was thawed a minimum of five days prior to a whole blood assay and added to 15ml 7H9 Middlebrook broth supplemented with ADC and kanamycin (final concentration $20\mu\text{g/ml}$) and hygromycin (final concentration $50\mu\text{g/ml}$) + $40\mu\text{l}$ 20% Tween 80 in a 250ml Erlenmeyer flask. This was incubated in an orbital shaking incubator at 37°C with

daily measurements of 0.5ml samples for luminescence and optical density. Further liquid medium was added as required to maintain the bacteria in logarithmic growth phase until the day of the assay. Immediately prior to the blood samples being taken, an aliquot of the stock in logarithmic phase was diluted down to approximately 3.3×10^5 RLU/ml/s BCG-GFP-LuxFO.

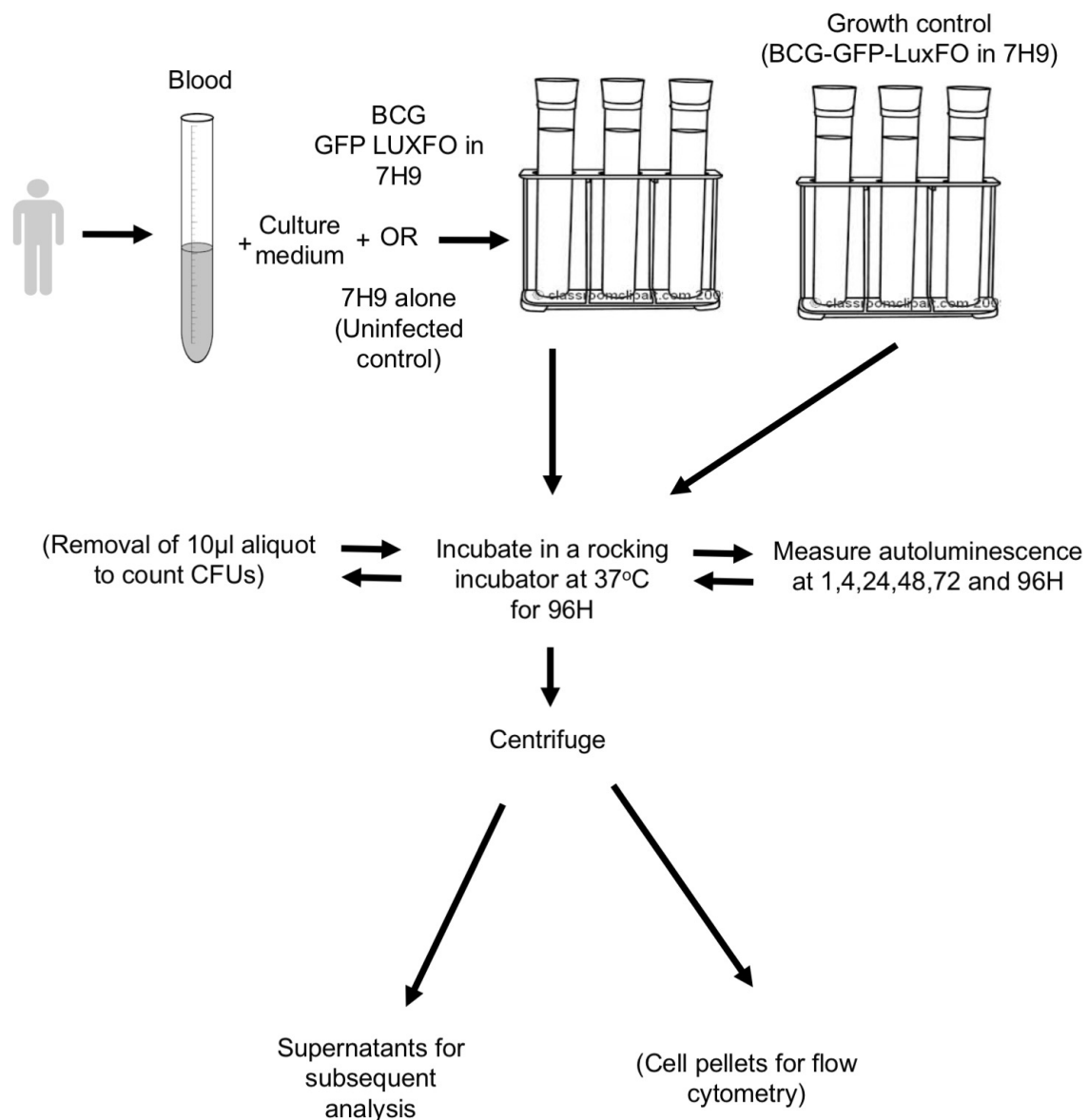


Figure 15: Schematic of novel BCG-GFP-LuxFO whole blood assay method

Whole blood was mixed in a ratio of 1:1 with RPMI 1640 culture medium (Sigma-Aldrich, Gillingham, UK) containing 2.5% 1M HEPES buffer and 1% L-glutamine (both Sigma-Aldrich). For the bacteria-containing samples, the recently diluted BCG-GFP-LuxFO in 7H9-0.05% Tween 80-ADC with kanamycin at 20µg/mL and hygromycin at 50µg/mL was added at a ratio of 1 part BCG-GFP-LuxFO : 9 parts whole blood in culture medium. For the medium only control samples, bacterial medium alone was added in the same ratio of 1:9. Triplicate 0.5ml aliquots of each experimental condition for each time point were placed into sterile lidded 75x12mm standard tubes (Corning B.V Life Sciences, Amsterdam, The Netherlands). For the samples containing BCG-GFP-LuxFO, this equates to approximately 3×10^5 CFU in each 500µL replicate, each of which contain 225µL whole blood. Triplicate growth controls of the recently diluted BCG-GFP-LuxFO in 1:9 ratio with further liquid medium were prepared in parallel to confirm that the bacteria remained in logarithmic growth phase at the time of inoculation.

The baseline blood-containing samples were centrifuged at 2000 x g for ten minutes whilst the remaining tubes were transferred to a rocking incubator at 37°C.

Supernatants were split into two aliquots and stored at -70°C. Luminescence measurements of all remaining bacteria-containing tubes were measured 1 hour after the bacteria were added to ensure that they had equilibrated to 37°C prior to measurement. To minimize temperature disruption, each tube was removed one-by-one from the rocking incubator, luminescence measured immediately, and then returned to the incubator. Luminescence measurements were repeated at 4 hours, 24 hours, 48 hours, 72 hours and 96 hours. After the 24 hour and 96 hour measurements, a set of experimental replicates was centrifuged and supernatants

stored for subsequent analysis. Three measurements of each tube at each time point were recorded, and the mean of these three measurements is used in subsequent analysis. At each time point, background luminescence in three control uninfected samples and the three medium-only tubes was also measured and this was subtracted in all subsequently reported results.

4.11 Discussion and conclusion

The BCG-GFP-LuxFO assay uses 225µL of whole blood per sample from which serial measurements of autoluminescence can be made, enabling the kinetics of host-mycobacterial interactions to be characterized in real-time. Following measurement of luminescence, the same samples can be centrifuged to generate a cell pellet with potential applications for flow cytometry or fluorescent microscopy, and supernatants for biochemical analysis.

In comparison, the direct Mycobacterial Growth Inhibition Assay uses 300µL of whole blood per sample to generate a time to positivity,^{72,217,238} whilst the BCG-*lux* assay requires 450µL whole blood to generate a 96 hour growth ratio.²²³ There is also a considerable decrease in experimental time to measure luminescence compared to BCG-*lux* (Figure 15 compared to Figure 6).

The main disadvantage of the BCG-GFP-LuxFO assay is the low RLU: CFU ratio of 0.05. Therefore, with the methodology outlined above, approximately 3.3×10^5 CFU are present in each 500µL volume tube containing 225µL whole blood in the BCG-GFP-LuxFO assay. In comparison, BCG-*lux* with an RLU: CFU ratio of 3-5 has

approximately 1.2×10^5 CFU present in each 500 μ L volume tube containing 225 μ L whole blood. The whole blood direct Mycobacterial Growth Inhibition Assay involves adding approximately 150CFU of BCG to a total volume of 600 μ L, of which 300 μ L is whole blood.^{217,238} The PBMC-adapted direct Mycobacterial Growth Inhibition Assay adds approximately 600CFU to 1×10^6 PBMCs in 600 μ L total volume. Therefore, the BCG-GFP-LuxFO assay uses two to three times the inoculum of the BCG-*lux* assay and approximately 2,000 times the inoculum of the direct Mycobacterial Growth Inhibition Assay.

Also of note is that the BCG-GFP-LuxFO assay is derived from the BCG Danish vaccine strain and not pathogenic *M. tuberculosis* complex. It had been intended to create an MTB-GFP-LuxFO for this thesis but it proved impractical under the time constraints. However, in assays using human whole blood, BCG and *M. tuberculosis* show similar characteristics with both Mycobacterial Growth Inhibition Assays and luminescent strains.^{72,212,237}

The BCG-GFP-LuxFO assay, in common with all of the mycobacterial growth inhibition assays discussed in Section 4.1, is based upon cells circulating in the blood, rather than pulmonary cells where the initial encounter with *Mycobacterium tuberculosis* occurs. The importance of studying human cells from the relevant tissue cannot be overstated, and bronchoscopy has been employed successfully in tuberculosis research in adults.^{75,239} However, bronchoscopy for research purposes in children is complicated by narrower diameter airways, anaesthetic risk, requirement for highly skilled operators, intensive care support, and issues of consent. Although clinical paediatric bronchoscopy is available in some settings

where there is high prevalence of tuberculosis, it is not available in The Gambia.²⁴⁰

Studies of human whole blood have made many important contributions to understanding human mycobacterial immunity.^{99,132,177,219,226} The small volume requirements for the BCG-GFP-LuxFO assay of a relatively accessible minimally invasive sample type means that it is well-suited to research in paediatric populations.

The assay quantifies luminescence, which correlates with bacterial load. However, luminescence could also be contributed to by additional factors beyond the number of bacteria. As mentioned in 4.7, the location of the bacteria within the sample has not been characterised. The bacteria could be in the medium, associated with the surface of cells, or intracellular. Cells found in whole blood that are known to phagocytose bacteria include neutrophils, monocyte/macrophages, cells of dendritic cell lineage and $\gamma\delta$ T cells.^{99,237,241–244} These cell types have different life spans *in vivo* and *in vitro* with neutrophils having a twelve hour life span in circulation in a non-activated condition, much shorter *in vitro*.²⁴⁵ At any given point in time, the physicochemical properties of the compartment within which the bacteria are localised, activation of the host immune response, and the bacterial metabolic state could influence the luciferase operon activity and therefore detectable luminescence.²³² However, all samples in this series of experiments were subject to the same conditions. Although whole blood in culture represents a complex biological matrix, bioluminescence by recombinant mycobacteria is detectable *in vivo* in mice and also correlates with bacterial load.^{230,231}

In the context of the clinical and research facilities available at the study site, ethical considerations for research in children, combined with the challenges of obtaining appropriate samples of sufficient volumes, the BCG-GFP-LuxFO assay represents a novel and robust methodology with which to study the kinetics of mycobacterial-paediatric interactions *in vitro*.

CHAPTER 5: PARTICIPANT CHARACTERISTICS

5.1 Recruitment to the study

Children were enrolled in the study as described in Chapter 3. All compounds that were enrolled in the household contact platform at MRC Unit The Gambia between 1st January 2015 and 31st December 2015 were evaluated for inclusion in this study. The study recruitment profile is shown in Figure 16.

5.2 Demographic and epidemiological characteristics of the study participants

A total of 70 children were recruited to the study, from 29 compounds. Demographic features of the study participants together with descriptive statistics are detailed in Table 6. Gender was evenly distributed across the HEI, HEU, and LEU groups and did not significantly differ between groups. The proportion of children whose parent was the index case did not significantly differ between groups. HEI children were significantly older than HEU children. There was no significant difference in age between LEU children and HEI or HEU children. 54 (93%) of the highly exposed children (HEU+HEI) were sleeping in the same house as the adult index case, with 4 (7%) in the same room as the adult index case.

5.3 Tuberculin skin test results in study participants

At the initial screening performed by the contact tracing platform, all of the HEU and LEU children had a tuberculin skin test result of 0mm. HEI children had a median TST result of 18mm with a range of 13mm to 25 mm (interquartile range 16 to 20mm). Repeat TST in HEU and LEU children between 3 and 12 months after the initial TST was 0mm in all 41 children. Two-way ANCOVA using classification as HEI/HEU/LEU and age as a continuous variable and with an interaction term

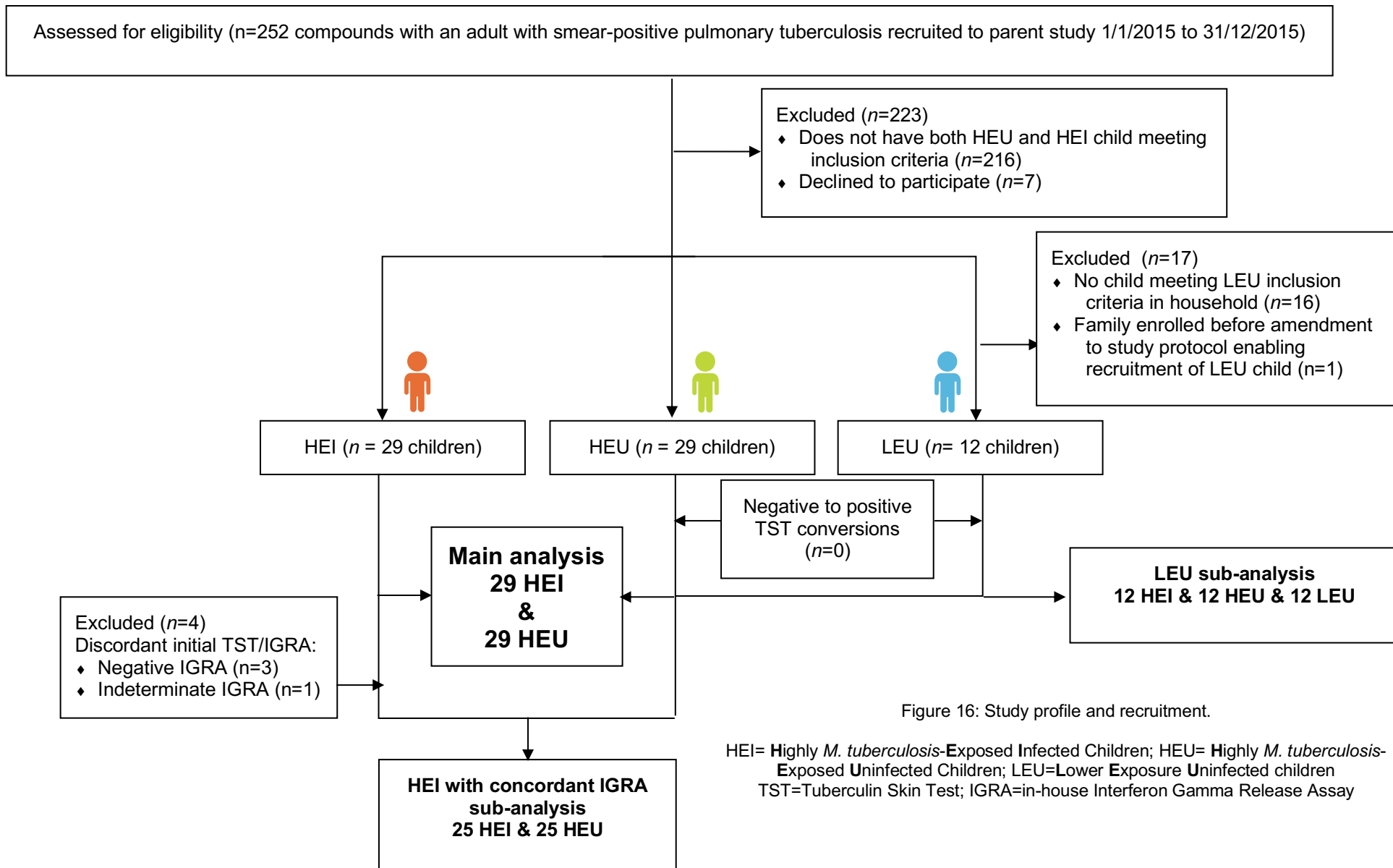


Figure 16: Study profile and recruitment.

HEI= Highly *M. tuberculosis*-Exposed Infected Children; HEU= Highly *M. tuberculosis*-Exposed Uninfected Children; LEU=Lower Exposure Uninfected children
TST=Tuberculin Skin Test; IGRA=in-house Interferon Gamma Release Assay

Characteristic		HEU children	HEI children	p vs HEU	LEU children	p vs HEU	p vs HEI
n		29	29		12		
Sleeping Proximity	Same Room	2	2		0		
	Same House	27	27		0		
	Different House	0	0		12		
Median age in years (IQR)		7.89 (7.01-10.86)	10.37 (9.43-12.36)	0.027*	9.99 (8.94-12.53)	0.93*	0.43*
% Males		14 (48)	16 (55)	0.62‡	6 (50)	1.0‡	1.0‡
HIV infection in child (n=29)		-	0	-	-	-	-
HIV infection in adult index case (adult consented to test n=28)		0					
Relationship of child to adult index case (%)	Child	4 (13.8)	7 (24.1)	0.45‡	1 (8.3)	1.0‡	0.62‡
	Cousin	6 (20.7)	2 (6.9)		3 (25.0)		
	Distant Relation	1 (3.4)	1 (3.4)		1 (8.3)		
	Grandchild	1 (3.4)	2 (6.9)		0 (0.0)		
	Nephew or Niece	9 (31.0)	6 (20.7)		3 (25.0)		
	Sibling	3 (10.3)	10 (34.5)		0 (0.0)		
	Unrelated	5 (17.2)	1 (3.4)		4 (33.3)		

Characteristic		HEU children	HEI children	p vs HEU	LEU children	p vs HEU	p vs HEI
Relationship of child to HEI (%)	Cousin	12 (41.4)	-	-	8 (66.7)		
	Distant Relation	0 (0.0)	-	-	1 (8.3)		
	Nephew or Niece	2 (6.9)	-	-	0 (0.0)		
	Sibling	10 (34.5)	-	-	0 (0.0)		
	Uncle or Aunt	1 (3.4)	-	-	0 (0.0)		
	Unrelated	4 (13.8)	-	-	3 (25.0)		
Median initial TST result in mm (IQR)		0 (0)	18 (16-20)	0.000*	0 (0)	1.0*	0.002*
Median Repeat TST result in mm (IQR)		0 (0)	-	-	0 (0)	-	
Initial Interferon Gamma Release Assay	Positive	-	25 (86.2%)	-	-		
	Negative	-	3 (10.3%)	-	-		
	Indeterminate	-	1 (0.3%)	-	-		
Median absolute White blood cell count k/ μ L (IQR)		6.38 (5.58-7.18)	6.02 (5.6-6.75)	0.563*	5.79 (4.85-6.75)	0.424*	0.374*
Mean absolute monocyte count k/ μ L (95% CI)		0.467 (0.381-0.552)	0.443 (0.352-0.534)	0.657 [†]	0.439 (0.324-0.553)	0.618 [†]	0.841 [†]
Median absolute neutrophil count k/ μ L (IQR)		2.44 (1.88-2.83)	2.61 (2.17-3.19)	0.492*	2.22 (1.82-2.59)	0.722*	0.061*
Mean absolute lymphocyte count k/ μ L (95% CI)		2.90 (0.99-4.80)	2.67 (0.91-4.44)	0.391 [†]	2.83 (1.49-4.17)	0.905 [†]	0.539 [†]
Mean Monocyte:Lymphocyte ratio (95% CI)		0.174 (0.141-0.206)	0.171 (0.138-0.204)	0.905 [†]	0.152 (0.127-0.178)	0.318 [†]	0.224 [†]

Table 6: Summary of participant characteristics.

* denotes Wilcoxon matched pairs signed rank test; ‡ McNemar's χ^2 test; † denotes paired t-test; Haematological statistics based upon results available from CellDyn machine (HEI n=25, HEU n=25, LEU n=11)

between the two variables showed only HEI/HEU/LEU as a significant factor to initial TST result (age: $p=0.549$; group: $p=0.000$; interaction between group and age: $p=0.587$).

5.4 IGRA status

Three of the 29 HEI TST positive children had a negative IGRA on the sample taken at the initial MRC Childhood TB clinic visit. One of the 29 HEI children had an indeterminate IGRA sample.

5.5 HIV infection status and risk in study participants

None of the 29 HEI children who had an HIV test as part of their initial evaluation at the MRC Childhood TB clinic were infected with HIV. 28 of the 29 adult index cases consented to an HIV test as part of their routine clinical care through the National Leprosy and Tuberculosis Program and had negative test results. The index case that declined an HIV test was not biologically related to the children recruited to this study who lived in the same compound, and the HEI child from this compound was negative for HIV infection.

5.6 Haematology parameters in study participants

Full blood count results from the Celldyn machine (that provides monocyte counts) were available for 25 HEI children, 25 HEU children and 11 LEU children. There were no significant differences between groups for white cell count, lymphocyte count, neutrophil count, monocyte count or monocyte: lymphocyte ratio (Table 6).

5.3 Discussion

During the course of this study, a total of 70 children (29 HEI, 29 HEU, and 12 LEU) were recruited in matched groups from 29 compounds. The demographic and epidemiological characteristics were well-balanced with equal gender distribution. Matched non-parametric testing on a pair-wise basis suggested that HEI children were significantly older than HEU children. *A priori*, one might have predicted HEI children to be older as in general TST positivity increases with age.^{34,40,41} However, two-way ANCOVA analysis showed that age was not a significant contributory factor to initial TST result.

Subsequent subgroup analyses by IGRA status of HEI children will compare 25 HEI children with concordant IGRA to four with discordant results. We do not know whether these four children would have converted to positive IGRAs after an interval. The proportions of TST+/IGRA- (10.3%) and TST+/IGRA indeterminate (0.3%) children are broadly comparable with the literature.^{10,17,18}

All 41 of the HEU and LEU children had an initial TST result of 0mm (≤ 5 mm was the inclusion criterion) and after an interval of three to twelve months, TST results remained 0mm. Persistently negative TST results in non-HIV infected asymptomatic children represent exactly the intended study population of interest for this study who may represent a subpopulation with an effective immune response against *M tuberculosis*.

Every consenting eligible compound during a twelve-month period was recruited into this study and the sample size after exclusions approached the predicted number for

80% power to detect a significant difference at the 5% level based upon the pilot data. This sample size was smaller than the planned 40 matched groups. However, the sample size calculation was based upon historical luminescence growth ratio pilot data, and as described in Chapter 4, a novel auto-luminescent whole blood assay was used in this study, with serial measurements of the same tubes during the experiment, enabling linear mixed effect modelling to take into account the luminescence dynamics over time. Therefore, the data was reviewed in consultation with the statistical team following recruitment at this stage, and it was felt that further increase in the sample size would not be likely to yield the hypothesised differences in luminescence between HEU children and HEI children at the early time points of 4 and 24 hours. Therefore, recruitment to the study was closed in April 2016.

CHAPTER 6 : LUMINESCENCE IN BCG-GFP-LUXFO WHOLE BLOOD ASSAY

6.1 Introduction

In this chapter I report the results of the experiments conducted in whole blood using the BCG GFP Lux FO assay, the methodological development of which is described in Chapter 4. The application of this methodology to HEI/HEU/LEU children has generated dynamic experimental data to assess functional differences in the ability to control mycobacteria metabolism/growth that might correspond to the phenotypic case definitions. The data reported here relate to Hypothesis 1:

HEU children are better able to restrict mycobacterial growth in an *in vitro* whole blood assay than their matched HEI peers.

6.2 Consideration of data adjustment requirements

6.2.1 Reproducibility of starting inoculum across experiments

The assays on the 29 groups of children were carried out over the course of 20 sets of experiments. The target RLU/ml/s for the inoculum was 3.3×10^5 RLU/ml/s. The actual inoculum had a mean of 3.29×10^5 RLU/ml/s (95% CI: 3.06 - 3.52×10^5 RLU/ml/s) (Figure 17). This represented an acceptable degree of consistency in the inoculum across the study and therefore no adjustment for starting inoculum has been made.

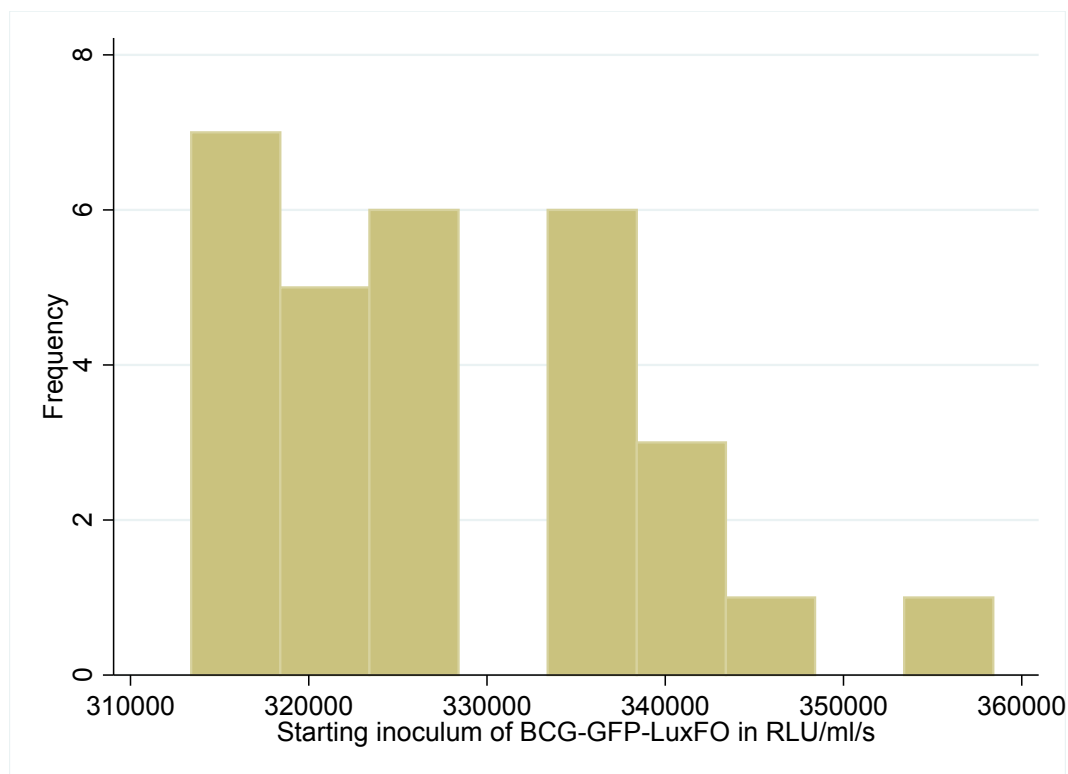


Figure 17: Distribution of starting inoculum of BCG-GFP-LuxFO.

6.3 Comparing all HEI and HEU children

6.3.1 Size and distribution of the dataset

There were 29 compounds where matched pairs of HEI and HEU children were recruited. Therefore data for this comparison is available from 58 children each of whom had sequential luminescence measurements of each of the triplicate incubations after 1, 4, 24, 48, 72, and 96 hours following the addition of BCG GFP LuxFO to the samples. This dataset consists of 1,044 luminescence measurements. Histograms and quantile-normal plots by transformation showed a log transformation of the data approximated a normal distribution and this was used for subsequent mixed effects modelling.

6.3.2 Non-parametric testing of luminescence by group for each time point

Figure 18 (graph) and Figure 19 (column scatterplots) compare the luminescence of the 58 HEI and HEU children at each time point. HEI children have significantly lower luminescence at baseline (HEI: median 402.4 RLU/s, IQR: 327.6→416.7 RLU/s; HEU 439.7 RLU/s, IQR: 356.4→554; $p=0.0069$) and at 96 hours (HEI: median 11,511 RLU/s, IQR: 8,210→15,272 RLU/s; HEU 15,895, IQR 9,655 →19,585; $p=0.0455$).

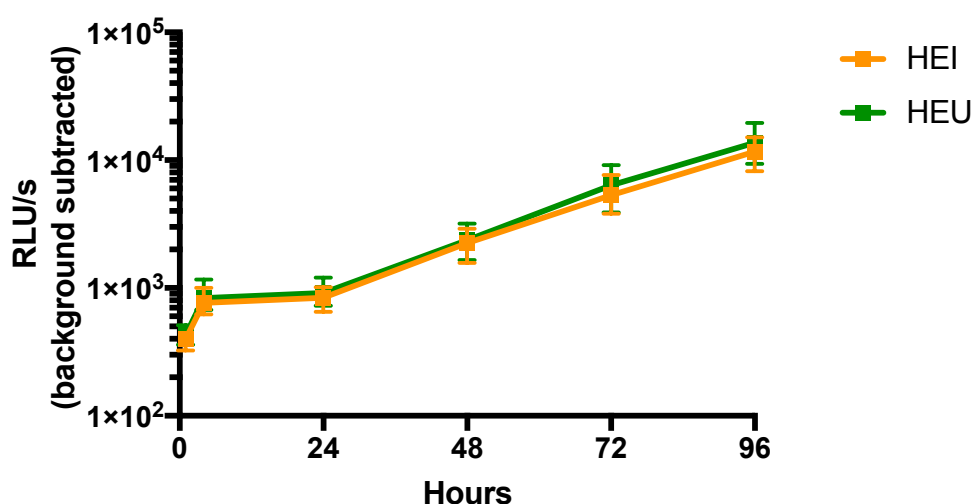


Figure 18: Kinetics of luminescence for the 29 HEI and 29 HEU children.

Median values and interquartile ranges are shown.

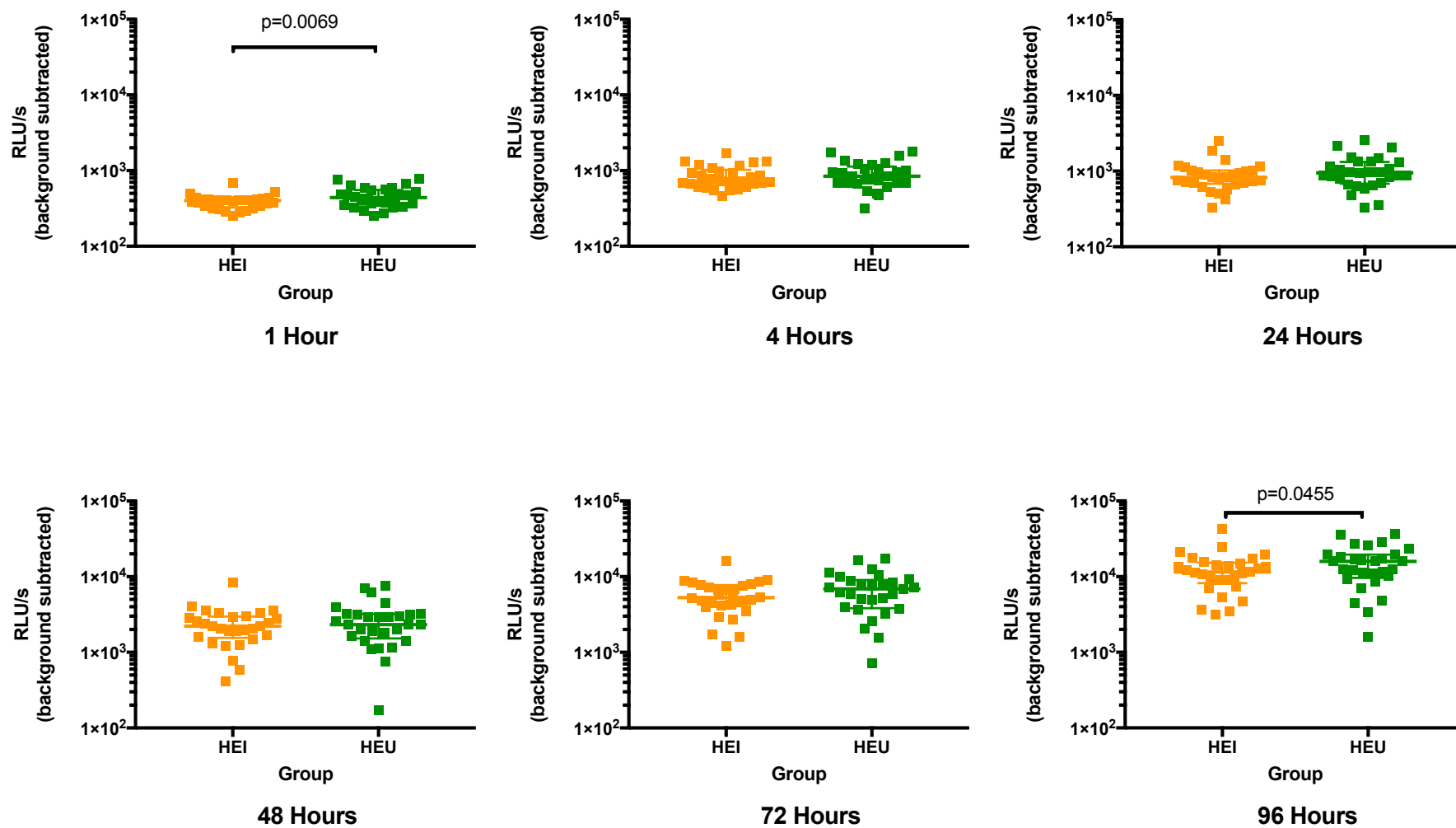


Figure 19: Column scatterplots and non-parametric analysis of luminescence of the 29 HEI and 29 HEU children.

p values from Wilcoxon matched pairs signed rank test. Bars represent median and IQR.

6.3.3 Mixed effects modelling of luminescence for all HEI vs HEU

The output of the model is shown in Appendix A.4.1. The estimated marginal means with the 95% confidence interval for each group from this model treating age as balanced are shown in Figure 20.

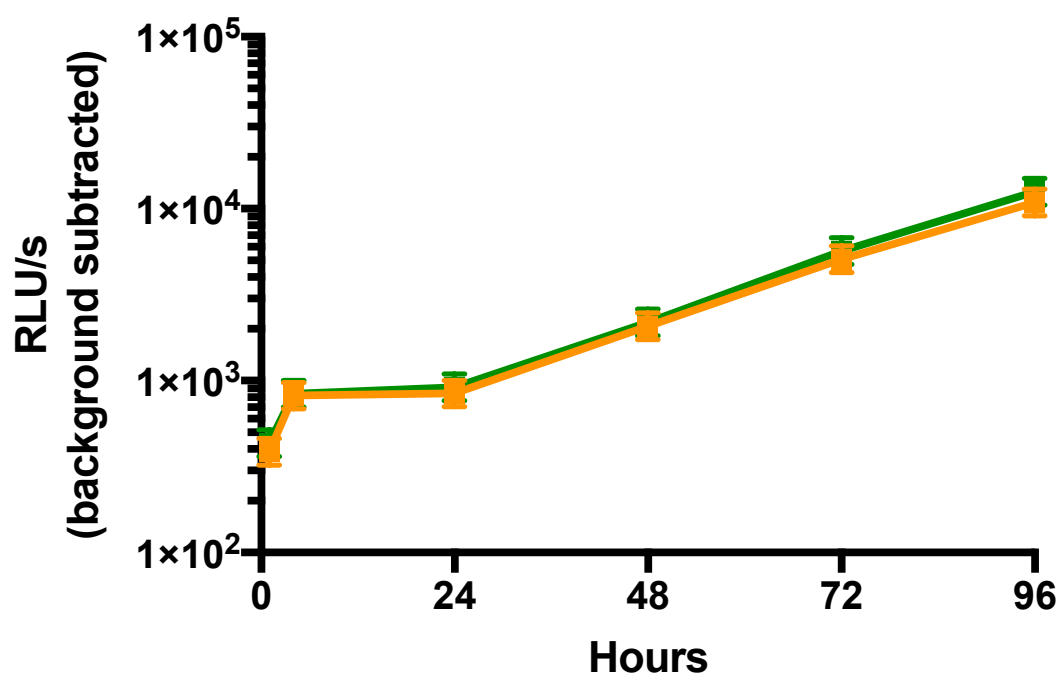


Figure 20: Modelled estimated marginal means for luminescence of 29 HEI and 29 HEU children

Error bars represent 95% confidence interval of estimated marginal means.

At baseline, HEI children again had lower modelled luminescence than HEU children, although this was not statistically significant (HEI: Mean: 386.6 RLU/s, 95% CI: 323.6-→ 461.8; HEU: Mean: 431.6, 95%CI 361.0→ 515.9; p value=0.155). HEI children had lower modelled luminescence than HEU children at 96 hours, which approached the threshold of statistical significance (HEI estimated marginal mean: 10,862 RLU/s, 95% CI: 9,092→12,976; HEU estimated marginal mean: 12,574

RLU/s, 95% CI: 10,519→15,031, pairwise comparison $p=0.059$). Infection status was not a significant variable independent of specific time points ($p=0.161$).

6.4 Subgroup analysis by IGRA status of HEI children

6.4.1 Graphical representation of data from HEI children by IGRA status

To evaluate whether data from HEI children (all of whom had positive TST>10mm) with negative or indeterminate IGRA status differed from those with positive IGRA at screening, the data for the 29 HEI children were plotted by IGRA status (Figure 21).

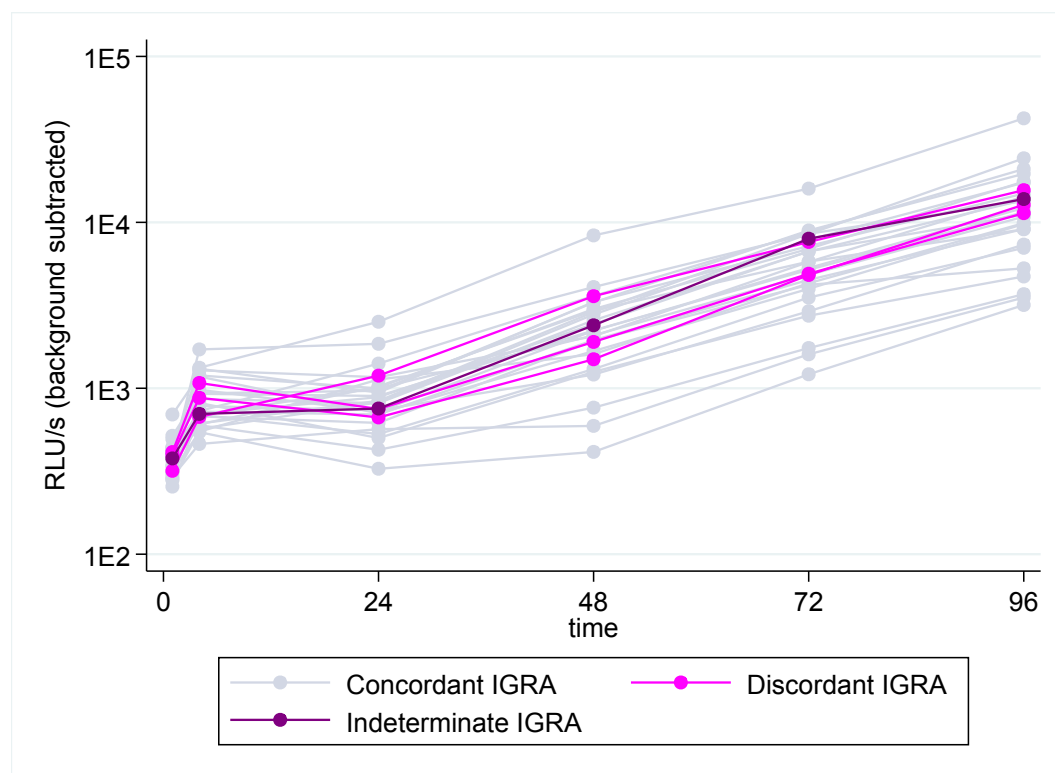


Figure 21: Comparison of luminescence kinetics for HEI children by IGRA status

Concordant IGRA $n=25$, Discordant IGRA $n=3$, Indeterminate IGRA $n=1$. $1E5=1 \times 10^5$ etc.

Luminescence responses of HEI children with negative IGRAs or indeterminate IGRAs fall well within the range of responses of HEI children with positive IGRAs.

6.4.2 Non-parametric testing and mixed effects modelling of data following exclusion of compounds where HEI children had initial discordant IGRA

The subgroup analysis dataset consists of 900 luminescence observations from 50 children. Log transformation of the data again approximated a normal distribution.

Data are summarised in Figure 22 (graph) and Figure 23 (column scatterplot). Non-parametric statistical testing of data from the 25 compounds where the HEI child had a positive IGRA at screening again showed that HEI children had significantly lower luminescence at baseline (HEI: median 403 RLU/s, IQR 325→424.8; HEU 427.6 RLU/s, IQR 339.6→579.7; $p=0.0483$). The difference at 96 hours was no longer significantly different ($p=0.141$).

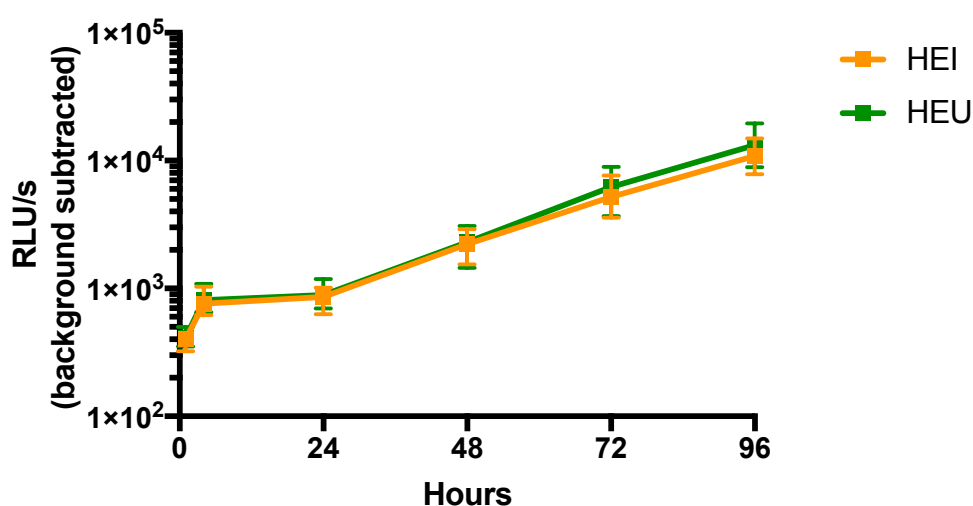


Figure 22: Kinetics of luminescence of the 25 HEI children with concordant IGRA and the 25 matched HEU children.

Error bars represent medians and interquartile ranges.

The same mixed effects model in 6.3.2 when run with log-transformed data only from the subgroup analysis generated similar estimated marginal means and confidence intervals (appendix A.4.2). Pairwise comparisons at each time point showed that HEI children had lower luminescence at baseline and 96 hours but this did not reach statistical significance (baseline: $p=0.224$, 96 hours: $p=0.098$). Infection status was not a significant variable independent of specific time points ($p=0.256$).

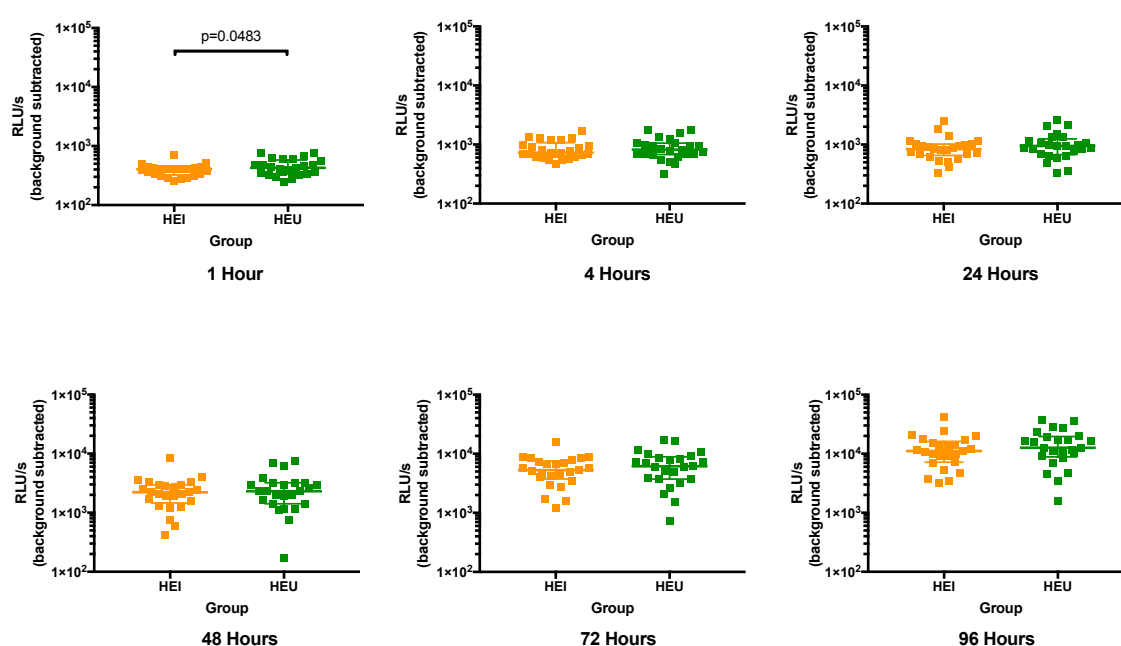


Figure 23: Column scatterplots and non-parametric analysis of luminescence of the 25 HEI children with concordant IGRA and their matched 25 HEU children.

P values are derived from Wilcoxon matched pairs signed rank test. Bars represent median and IQR.

6.5 Subgroup analysis by compounds where LEU children also recruited

6.5.1 Size and distribution of the dataset

There were 12 compounds where LEU children were also recruited as well as matched pairs of HEI and HEU children. Therefore, data for this comparison is

available from 36 children with a total of 648 luminescence observations. A log transformation of the data was used for subsequent mixed effects modelling.

6.5.2 Non-parametric testing of luminescence by group for each time point

The data for each group at each time point is shown in Figure 24 (graph) and Figure 25 (column scatterplots) with non-parametric statistical test results displayed where $p < 0.05$.

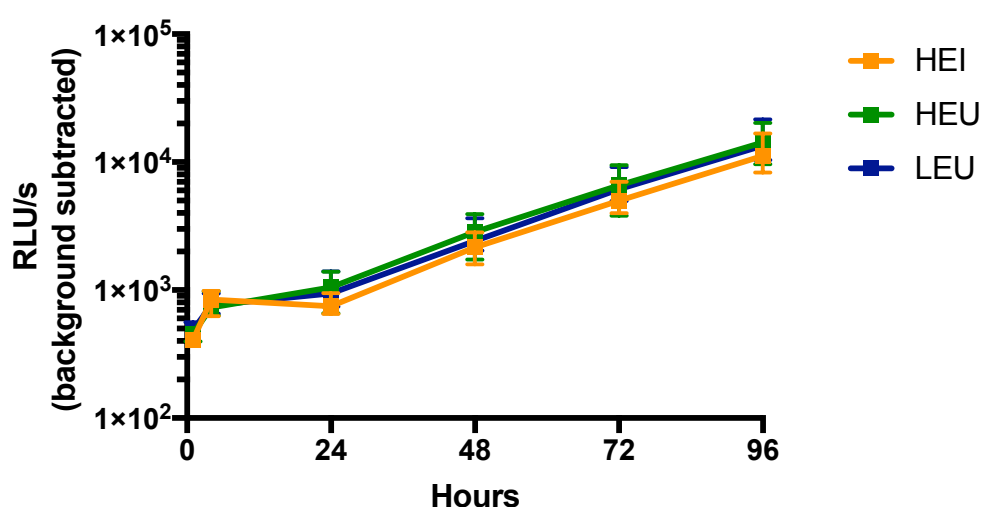


Figure 24: Kinetics of luminescence of the 36 children from compounds where a LEU child was recruited

Error bars represent medians and interquartile ranges.

These data show that LEU children have significantly higher levels of luminescence than HEI children at baseline (HEI: 410.5 RLU/s, IQR 388.5→436.6; LEU 479.7 RLU/s, IQR 436→578.1, $p=0.0125$), 24hours (HEI: 743.3, IQR 673.8→990.2; LEU 920.4 RLU/s, IQR 731.7→1388; $p=0.0122$) and 72 hours (HEI 4,861 RLU/s, IQR 4,275→7,619; LEU 6,239 RLU/s, IQR 5,127→8,749; $p=0.0425$). No statistically significant differences between LEU and HEU were noted.

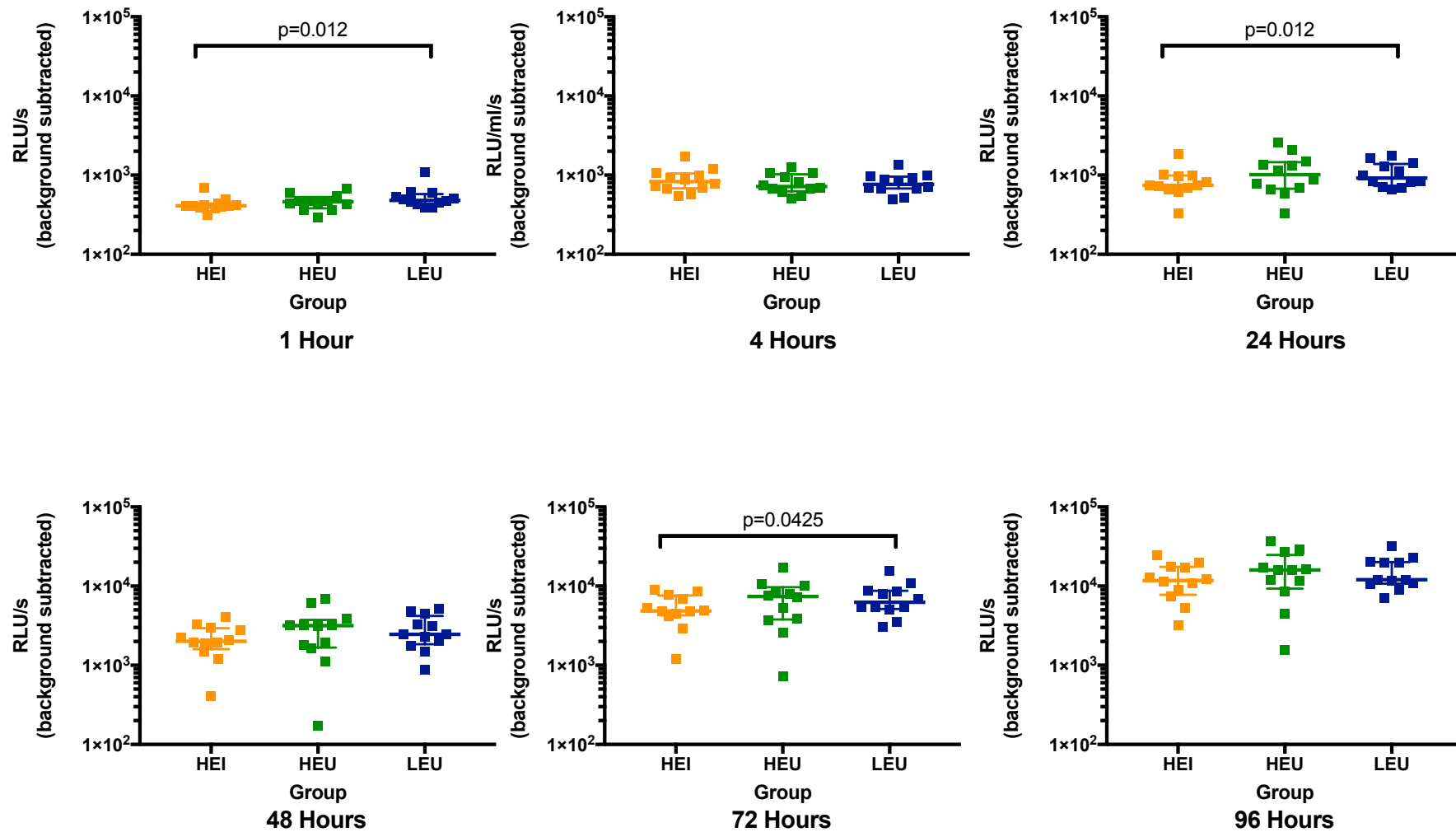


Figure 25: Scatterplots and non-parametric analysis of luminescence of the 36 children from the 12 compounds where a LEU child was recruited.

P values are derived from Wilcoxon matched pairs signed rank test. Bars represent median and IQR.

6.5.3 Mixed effects modelling

The output of the model is shown in Appendix A.4.3. The estimated marginal means with the 95% confidence interval for each group from this model treating age as balanced are shown in Figure 26 together with p values for LEU vs HEI. The model predicts that LEU children have higher luminescence than HEI children from 24 hours onwards. LEU children had higher luminescence than HEI children independent of specific time points (LEU vs HEI: $p=0.038$). There were no statistically significant differences between LEU and HEU children.

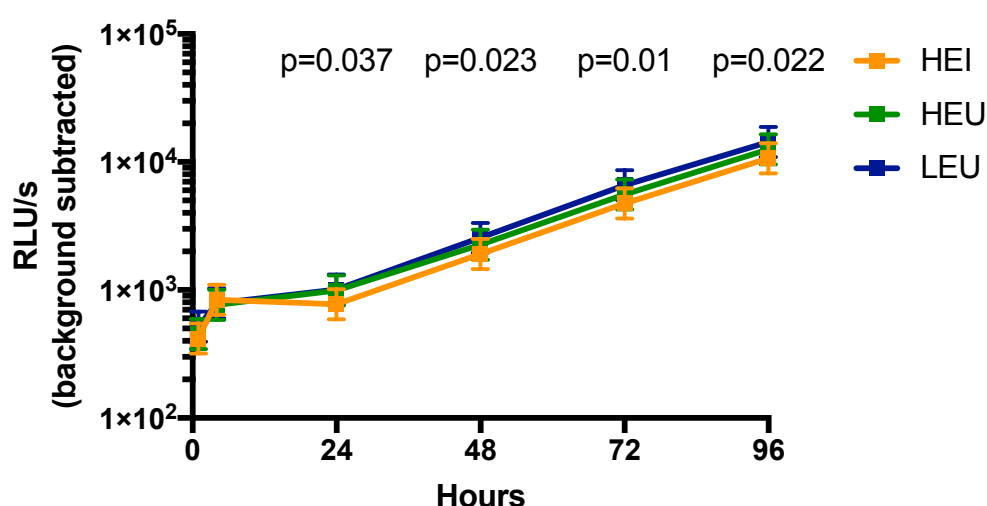


Figure 26: Modelled estimated marginal means for data from 36 children from 12 compounds where a LEU child was recruited.

Estimated marginal means with 95% confidence intervals. P values shown are pairwise comparisons between predicted values for LEU vs HEI at that time point. Differences were significant from 24hours onwards.

6.6 Discussion

Here I present real-time measurements of the kinetics of paediatric-mycobacterial interactions with greater temporal resolution than has been possible with previous methodologies. The most striking findings are that with non-parametric statistical testing, HEI children have significantly lower levels of luminescence than HEU

children at baseline ($p=0.0069$) and at 96 hours ($p=0.0455$). However, although the trends were the same, the baseline effect was not statistically significant in mixed effects modelling ($p=0.155$) and the effect at 96 hours was on the threshold of statistical significance ($p=0.059$). The rationale for using mixed effects models in this study is to account for the nature of both the study design and the data (Section 3.7.2). In addition to the matching, the mixed effects model also considers the longitudinal nature of the data, age of the children (which was significantly different between the groups), and variance in the data due to variability between individual children. Therefore, where there is a meaningful discrepancy in the results of two such statistical methods, the mixed effects model carries greater weight for interpretation. Accordingly, the apparent difference at baseline is likely to be attributable to factors that are adjusted for in the model; namely, the combination of age and variability between individual children at this first time point.

This raises the possibility that age, or another factor associated with it, could account for the apparent difference in non-parametric testing. HEI children were significantly older than HEU children (Table 6). Potential explanations could include immune differences between pre- and post-adolescent, and/or pre- and post-pubertal children. Adolescence is known to be associated with an increased risk of pulmonary tuberculosis, following the relative protection of five to ten year old children.^{6,22–24} Adolescence is associated with a number of sociocultural and behavioural changes, and also physical and hormonal changes associated with puberty. Differences in immunity between males and females with onset in puberty are well recognised.²⁴⁶ These include: an increase in CD4/CD8 ratios, absolute CD4 T cell numbers, immunoglobulins and B cells in females; and an increase in CD8 T cells and T_{reg}

cells in males.²⁴⁶ In one study, pubertal children had larger TST responses than pre-pubertal children.²⁴⁷ Although pubertal stage is confounded by age, it did also seem to be associated with differences in TST responses.²⁴⁷ However, the absolute difference in age in our study was small (HEU median age: 7.89 years, IQR 7-10.8; HEI median age: 10.37 years, IQR 9.4-12.4). The majority of Gambian children within these age ranges would still be pre-pubertal.^{248,249} Gender did not differ between groups in matched statistical analysis (Table 6).

In addition to the statistical and epidemiological explanations for the observed differences, it is also worth considering whether there are any plausible biological explanations for a detectable effect within an hour of addition of the bacteria. As discussed in section 4.11, luminescence correlates with bacterial load, but may also be contributed to by other factors including precise localisation within the sample or a cell, and metabolic state of the bacteria. Therefore, given the slow growth of BCG, any differences at baseline are more likely to reflect such changes in metabolic activity or be associated with phagocytosis rather than reflect differences in bacterial replication. HEI children were selected on the basis of a cell-mediated recognition of mycobacterial antigens but a recall adaptive response would not be compatible with a difference at baseline. It is possible that, contrary to our hypothesis, alongside the adaptive immune response, HEI children have also developed a trained innate immune response. HEI circulating monocytes and NK cells may therefore be epigenetically primed to rapidly respond to mycobacteria. As discussed in 9.4 and shown in Figure 69, RNA sequencing of stored samples would enable testing for differences in microRNA and long non-coding RNA that could reflect epigenetic mechanisms underlying differences in trained immunity. Neonatal BCG vaccination

rates are estimated to be 98% in Gambia and therefore BCG-mediated trained immunity is unlikely to differ between HEI and HEU children.¹⁸⁴ It is noteworthy that there were no statistically significant differences at 4 hours with either statistical technique.

Finally, another possibility to consider is whether the apparent difference at baseline could be artefactual and due to an unidentified technical experimental factor. All experiments were carried out following the same protocol described in Section 4.10. All tubes were inoculated simultaneously and transferred to the incubator at the same time. Given the number of patients, conditions and tubes for each experiment and the need to identify each tube in the rocking incubators and rapidly measure luminescence before returning it to the incubator, it was not feasible to randomise the order of the tubes and therefore HEI tubes were measured before HEU tubes in keeping with the tube labelling system. The time differences between an HEI tube being read at baseline compared to an HEU tube was 60 minutes post inoculation/45 minutes post incubation compared to approximately 70 minutes post inoculation/55 minutes post incubation. However, if there was such a systematic experimental reason for the difference, the difference would be present within every single matched pair and therefore both the matched non-parametric and the mixed effects modelling would be expected to arrive at similar levels of statistical significance. The matched analysis captures both children's matched exposure to the same index case, and that their samples were handled the same way on the same day in the same experiment.

My findings contradict the pilot data (section 3.2.4) upon which this hypothesis was based. The failure to reproduce the pilot data could reflect several differences in design and methodology between this study and the pilot data. The original hypothesis was derived from unpublished pilot data from a household study in Cape Town using the BCG-*lux* assay. As discussed in Chapter 4, the equivalent MOI for the BCG-GFP-LuxFO assay is relatively high compared to the BCG-*lux* assay. The statistical analysis of the pilot data was not matched by exposure to the index case, nor did it adjust for age. The pilot study also had a much smaller sample size, mismatch in the number of children per group, and different setting. Studies using the BCG*lux* assay in the UK showed a similar pattern to these Gambian children, with TST-positive subjects having lower growth ratios than TST-negative adults.^{220,224} A study published after this thesis was submitted to the examiners showed no difference with either BCG or *M. tuberculosis* versions of the whole blood Mycobacterial Growth Inhibition assay between healthy *M. tuberculosis* IGRA+ and IGRA- 8 year-old children.^{72,212}

As absolute luminescence levels are low at early time points, it may be that with this sample size, the sensitivity of this assay was insufficient to detect biologically relevant differences within the first 24 hours. However, the tendency for HEI children to have greater mycobacterial control was already evident with this sample size and therefore a substantial increase in sample size well beyond that of our original calculation would have been necessary. The data presented here shows that luminescence increased with time in the BCG-GFP-LuxFO assay. Therefore, in future iterations of the assay, the starting inoculum could be decreased further as the dynamic range at the lower end of the sensitivity of the luminometer was not utilised.

Taking all of the above into account, the overall picture is of similar luminescence profiles between HEU and HEI children, with HEI children having lower luminescence at 96 hours on the threshold of statistical significance. Therefore, the proposed hypothesis that HEU children would be better able to control mycobacterial luminescence in this whole blood assay is rejected.

The other statistically significant finding was that HEI children had lower luminescence levels than LEU children over the assay in general, and at all time points from 24 hours onwards. There were no statistically significant differences between LEU and HEU children. The pattern suggests that LEU responses did *not* encompass HEI responses, and therefore the observed differences likely reflect a consequence of infection in the HEI children. The fact that this effect was observed from 24 hours onwards would be consistent with the timeframe to recall an existing adaptive immune response. Therefore, the limited luminescence data available from LEU children support the null hypothesis that observed differences between HEU and HEI children are a consequence of infection.

CHAPTER 7: CYTOKINE RESPONSES OF TB-EXPOSED CHILDREN

7.1 Introduction

In this chapter, I report the evaluation of cytokine responses of HEI, HEU, and LEU children in the Gambia to BCG. The results specifically relate to Hypothesis 2:

The cytokine response to mycobacterial infection of whole blood *in vitro* differs between HEU and HEI children. HEU children are predicted to have an early pro-inflammatory profile (with high levels of $\text{TNF}\alpha$, IL-12, IL-1 α , and IL-1 β , and low levels of IL-10, IFN- α , and IFN- β) whilst the adaptive response of HEI children will lead to late elevated levels of IFN- γ .

7.2 Interferon alpha and beta and IL-12 quantification

Results from pilot quantification of IFN- α 2, IFN- β , IFN- γ , IL-10, IL-12 (p40) with samples from 8 children from three compounds showed that IFN- α 2 and IFN- β were not detectable in any sample, whilst levels of IL12p40 were extremely low (Figure 27).

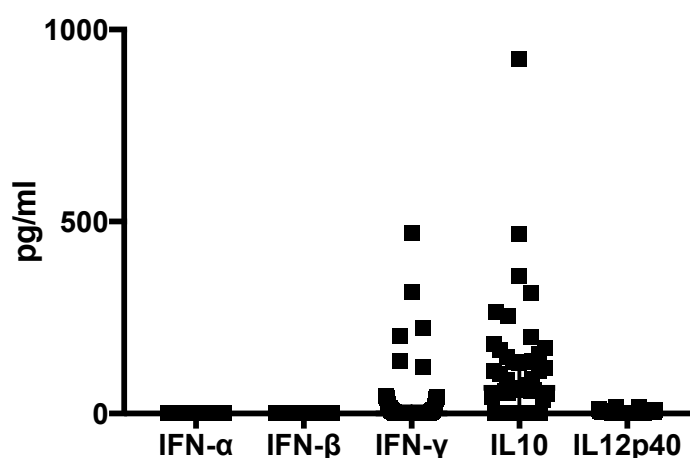


Figure 27: Pilot quantification of IFN α , IFN β and IL12p40 in supernatants.

Median and interquartile ranges.

Although IFN- α 2, IFN- β and IL-12 are important cytokines in mycobacterial immunology,^{105,132–135,250} low or undetectable levels of IFN- α 2 and IL-12 have been reported elsewhere in serum or human whole blood stimulation assays.^{251,252} Although there is significant variation in experimental methodology, other studies have shown relatively low levels of these cytokines. IFN- α levels in PBMCs following stimulation with Herpes Simplex Virus were <600pg/ml in healthy controls, and 10pg/ml in patients with TB.²⁵³ IFN- β was detectable at 24 hours from unstimulated primary human monocytes at approximately 50pg/ml with levels of up to 200pg/ml in response to mycobacterial stimulation.¹³⁴ Levels of IL12p40 following 7 day stimulation of whole blood with mycobacterial antigens in one Gambian study were predominantly <500pg/ml.⁷⁶

In light of the undetectable or very low levels of IFN- α 2, IFN- β and IL-12 in this pilot, we switched the multiplex assay to one containing the following cytokines:

- IFN- γ ,
- IL-1 α ,
- IL-1 β ,
- IL-10
- Tumour Necrosis Factor α .

7.3 Consideration of need for data adjustment

7.3.1 Variability across plates

In order to evaluate for plate-by-plate variability, the “early unstim” and “late stim” quality control samples, aliquots of which were run on each plate, were plotted for each of the five cytokines (Figure 28).

Plates 6 and 7 had higher values than the other 5 plates for all analytes except IL1- β , although this did not reach the threshold for statistical significance ($p=0.095$). Plate 6 and 7 were done on the same day in parallel and the QC samples appear to be consistent between plates 6 and 7.

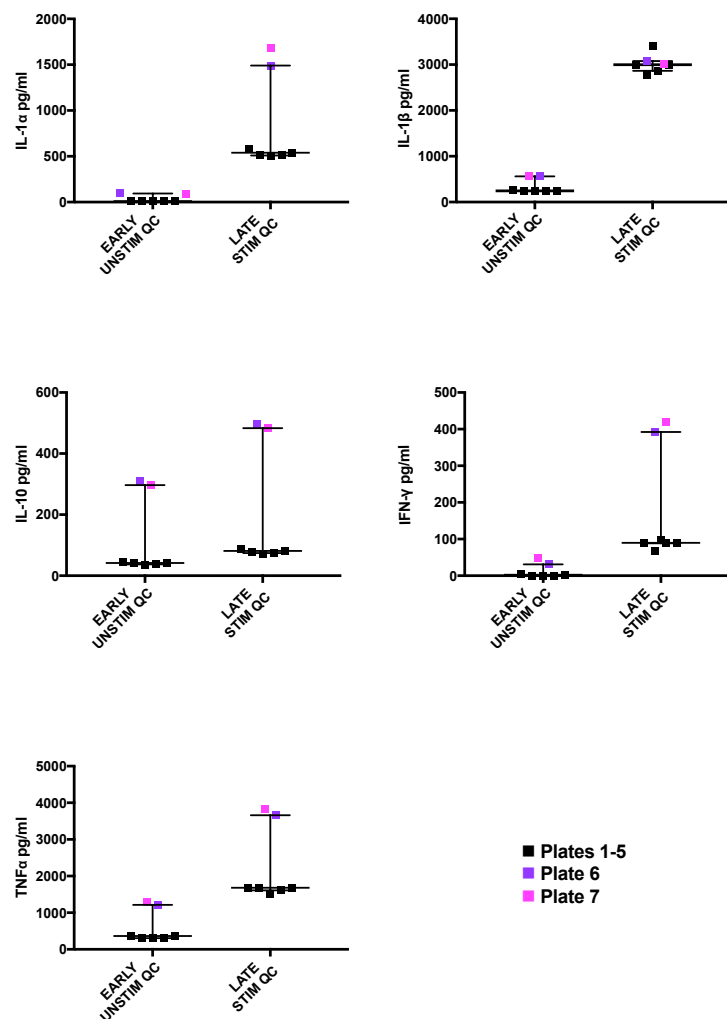


Figure 28: Comparison of quality control values between plates for cytokine multiplex.

Error bars show median and interquartile ranges.

To verify this difference, data plots for the BCG-stimulated experimental samples by plate for each of the five analytes are presented here in Figure 29.

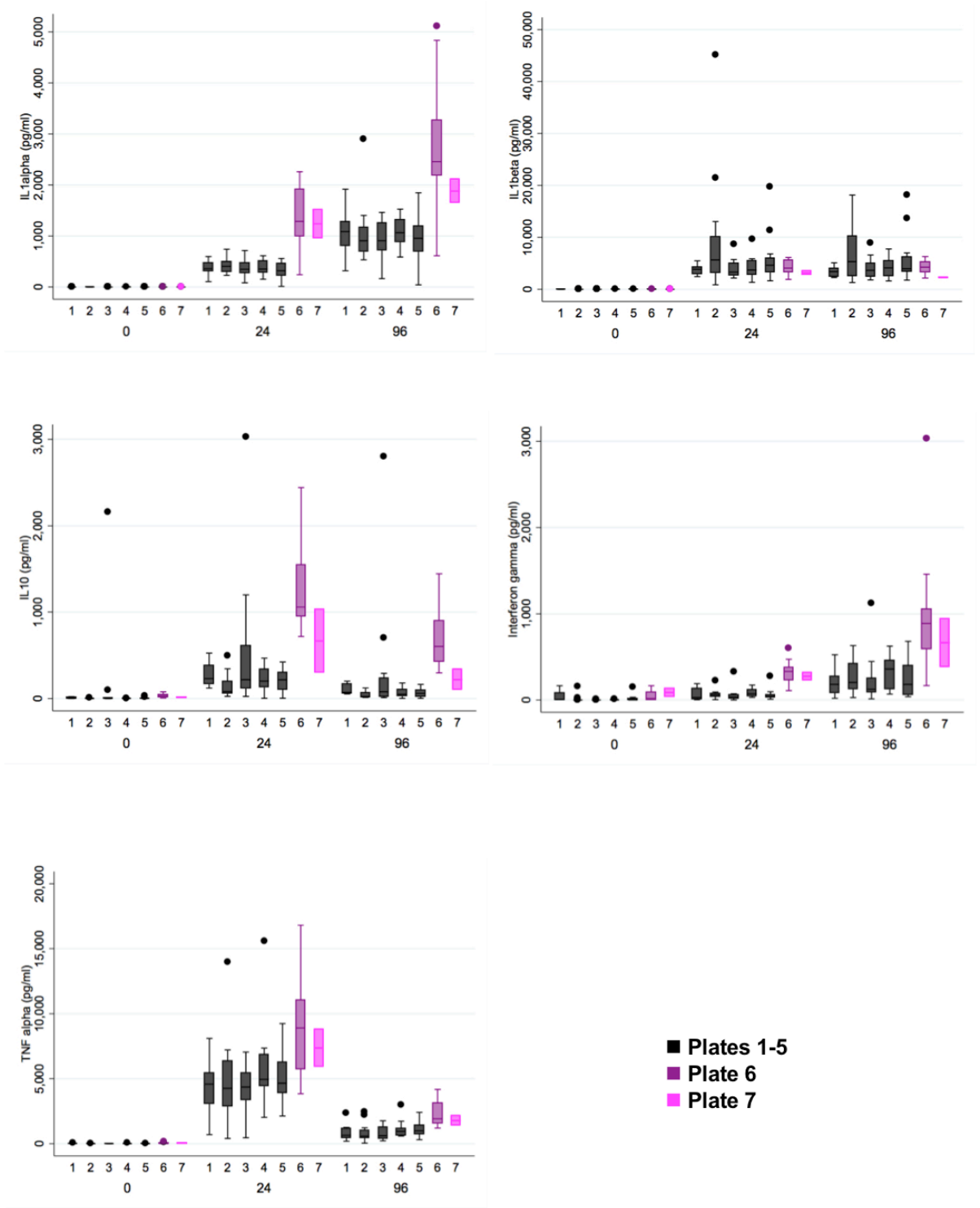


Figure 29: Cytokine values for BCG-stimulated samples over time plotted by plate.

Central line represents the median and the box the interquartile range. The whiskers represent next adjacent value and dots represent outside values.

Again, experimental sample data for plates 6 and 7 show markedly higher values for all analytes except IL-1 β .

Given that plates 6 and 7 were outliers for four of the five cytokines, were run on the same day, and had comparable values for the pooled quality control samples, an adjustment for IL-1 α , IL-10, IFN- γ , and TNF- α was applied to data for plates 6 and 7. The multiplication factor was calculated in order to adjust values such that the mean of the highest QC value for each cytokine was consistent across the plates:

$$\text{Multiplication factor} = \left(\frac{\text{Mean LATESTIMQC Plates 1 – 5}}{\text{Mean LATESTIMQC Plates 6\&7}} \right)$$

Adjusted values falling below the quantification level of the assay were again substituted with values $\frac{1}{4}$ of the lowest measured level.

7.3.2 Size of the dataset

For each child at each time point, pooled sterile-filtered supernatants from triplicate tubes for each of the two conditions (with BCG-GFP-LuxFO and with medium only) were analysed, with the exception of:

- For one child there were duplicate tubes only for the medium only condition at 96 hours. This was due to loss of supernatant from the third tube at the time of collection.
- For one child, there were singlicate tubes only at baseline and no samples at 24 hours due to insufficient blood volumes at phlebotomy.

Therefore for each of the five cytokines there are 209 datapoints for with BCG-GFP-LuxFO added and 209 datapoints for medium only control.

7.3.3 BCG-specific cytokine responses

To generate BCG-specific cytokine responses, the cytokine levels from the medium only condition for the same child at the same time point were subtracted from those where BCG had been added, to generate BCG-specific cytokine responses.

7.3.4 Data distributions of cytokine responses

The data for each of the cytokines and each of the experimental conditions were not normally distributed and there were no transformations that approximated a normal distribution. Therefore, only non-parametric testing is presented in this chapter.

7.4 Interleukin-1 α

7.4.1 Non-parametric testing for all HEU vs HEI

IL1 α levels with medium only, in the presence of BCG, and BCG-specific responses at each time point for all 29 compounds are shown in Figure 30. There were no statistically significant differences between the groups.

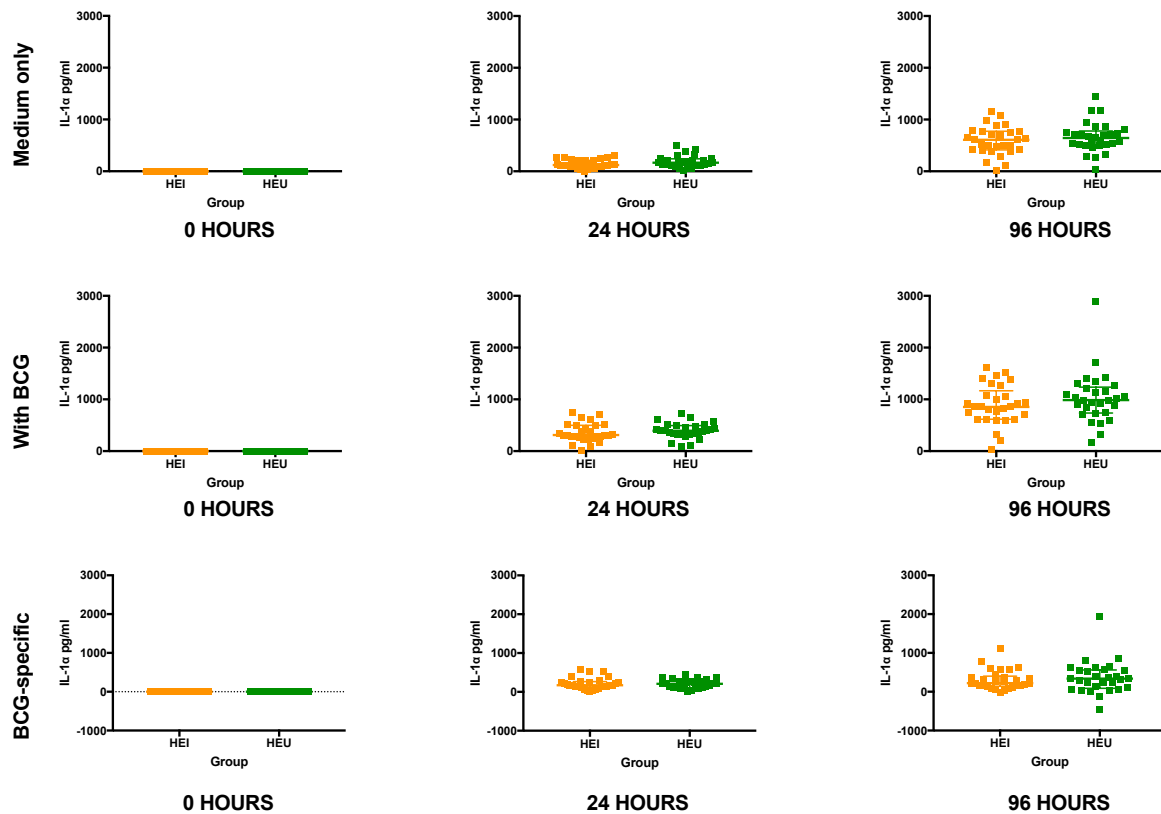


Figure 30: IL-1 α levels for the 29 HEI and 29 HEU children

Median and interquartile ranges

7.4.2 Subgroup analysis by HEI with discordant IGRA

Non-parametric testing of the HEI and HEU children from the 25 compounds where the HEI child had a positive IGRA also showed no difference between the groups at any of the time points or experimental conditions.

7.4.3 Subgroup analysis of compounds where LEU were recruited

Data from the 12 compounds where LEU children were also recruited are shown in Figure 31.

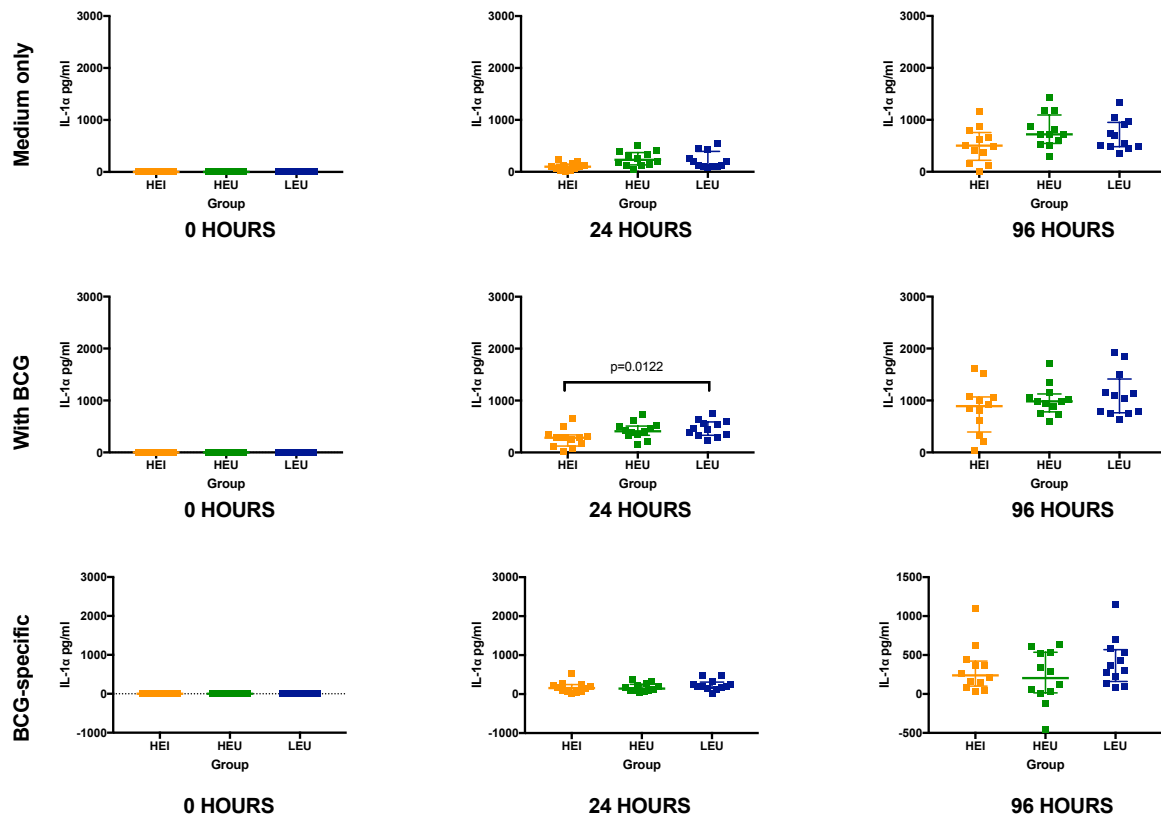


Figure 31: IL-1 α levels for the 36 children from compounds where a LEU child was recruited.

Error bars represent median and interquartile ranges.

At 24 hours in the presence of BCG, LEU children had higher levels than HEI children (HEI 284.1pg/ml, IQR 123.2→337.6; LEU 453.4, IQR 332.1→588.6, $p=0.0122$).

7.5 Interleukin-1 β

7.5.1 Non-parametric testing for all HEU vs HEI

IL1 β levels with medium only, in the presence of BCG, and BCG-specific responses at each time point for all 29 compounds are shown in Figure 32. There were no statistically significant differences between the groups.

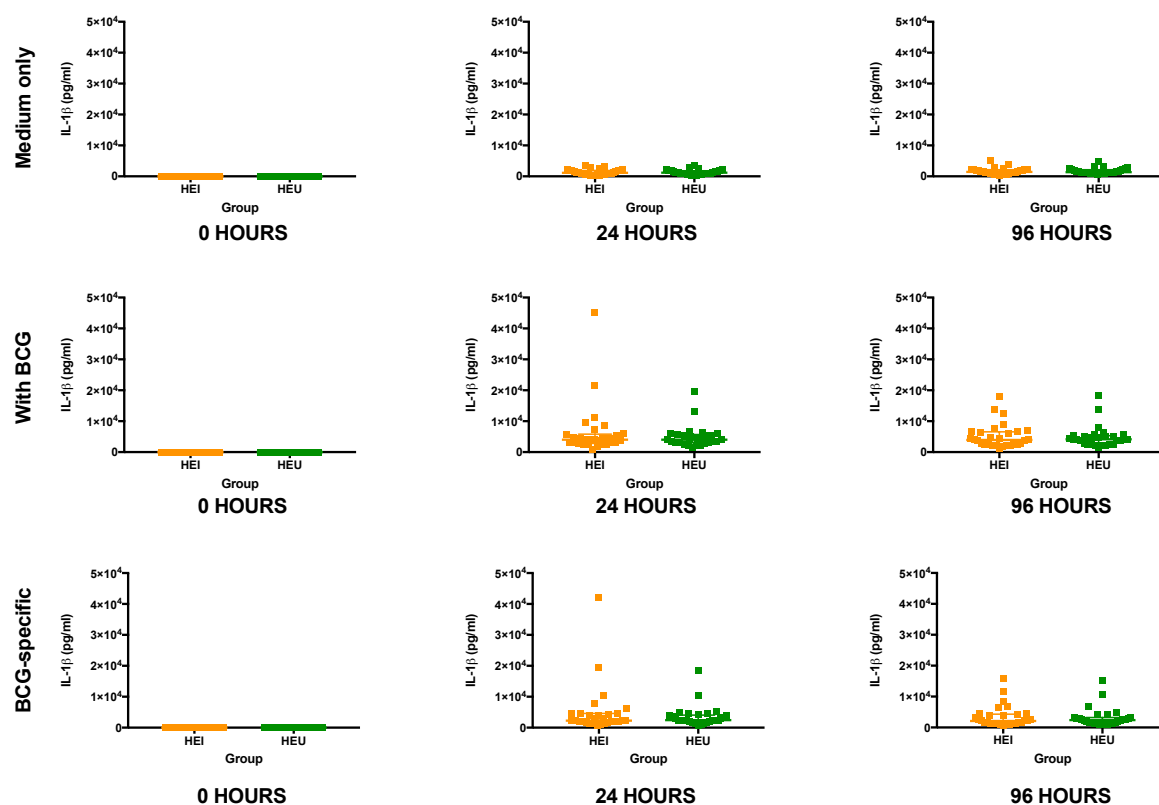


Figure 32: IL-1 β levels for the 29 HEI and 29 HEU children

Median and interquartile ranges

7.5.2 Subgroup analysis by HEI with discordant IGRA

Non-parametric testing of the HEI and HEU children from the 25 compounds where the HEI child had a positive IGRA showed no difference between the groups at any of the time points or experimental conditions.

7.5.3 Subgroup analysis of compounds where LEU were recruited

No statistically significant differences between LEU and HEU or HEI children were noted when analysing only the 12 compounds where all three children were recruited (Figure 33).

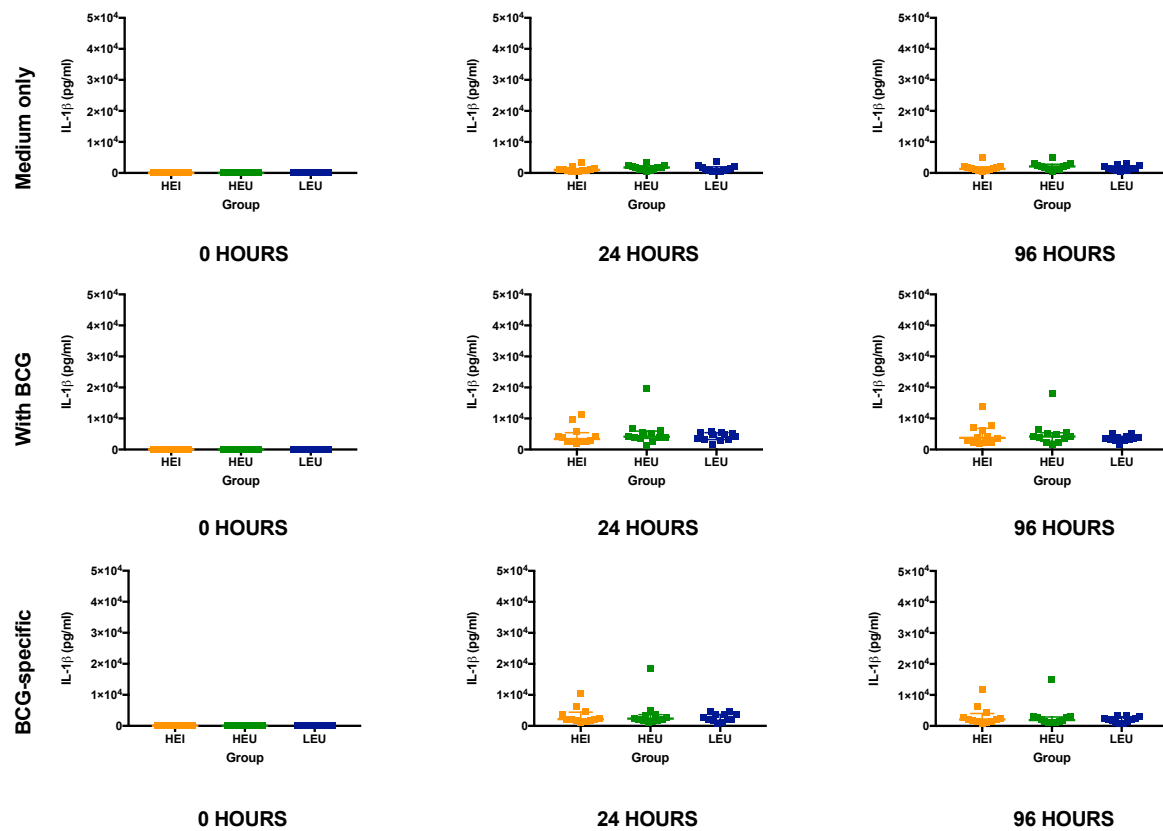


Figure 33:IL-1 β levels for the 36 children from compounds where a LEU child was recruited.

Median and interquartile range

7.6 Interleukin-10

7.6.1 Non-parametric testing for all HEU vs HEI

IL10 levels with medium only, in the presence of BCG, and BCG-specific responses at each time point for all 29 compounds are shown in Figure 34. There were no statistically significant differences between the groups.

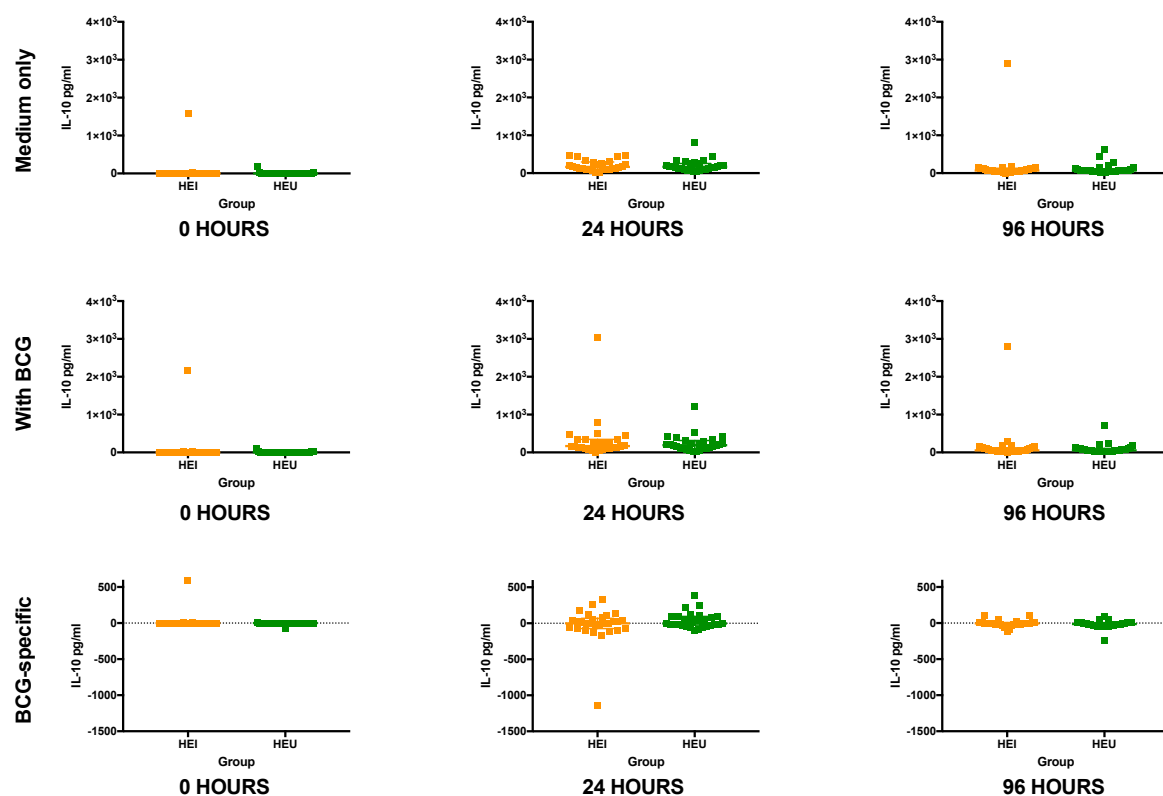


Figure 34: IL-10 levels for the 29 HEI and 29 HEU children.

Median and interquartile ranges.

7.6.2 Subgroup analysis by HEI with discordant IGRA

Non-parametric testing of the HEI and HEU children from the 25 compounds where the HEI child had a positive IGRA showed no difference between the groups at any of the time points or experimental conditions.

7.6.3 Subgroup analysis of compounds where LEU were recruited

No statistically significant differences were found between any groups in the 12 compounds where all three groups of children were recruited (Figure 35)

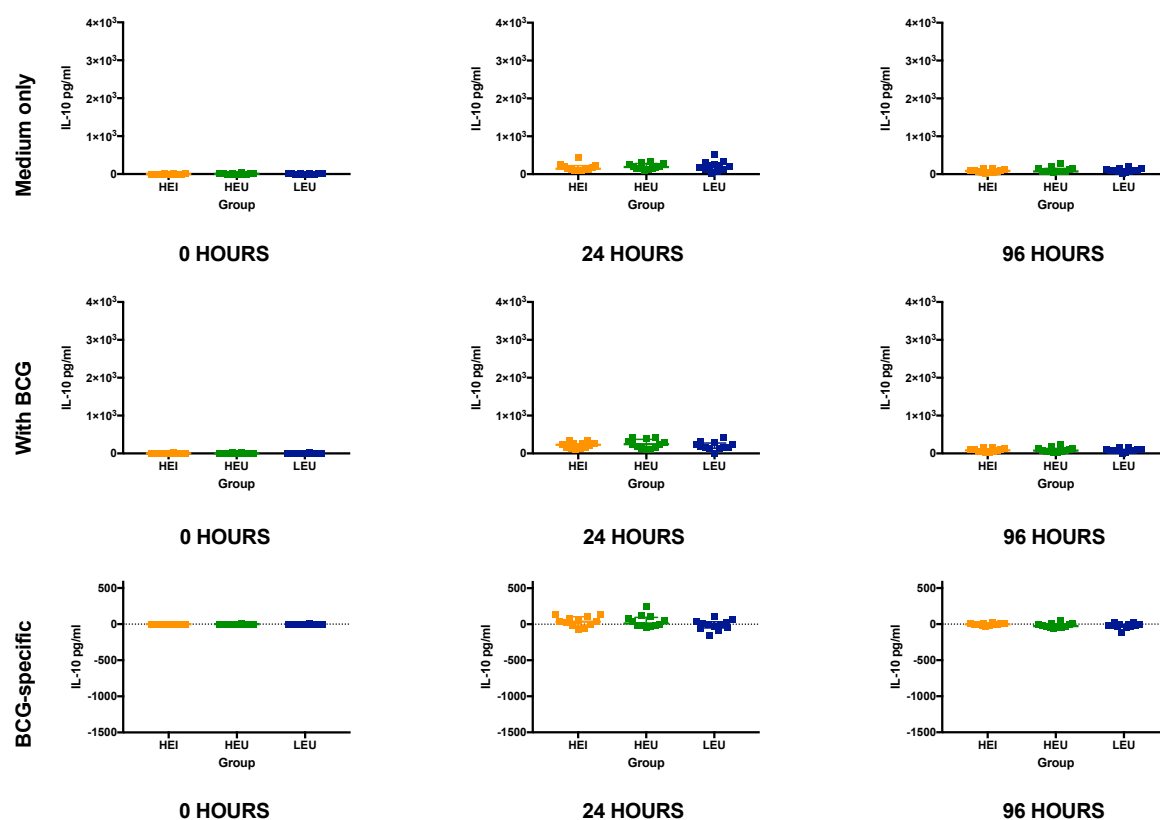


Figure 35: IL-10 levels from the 36 children from compounds where a LEU child was recruited

Medians and interquartile ranges.

7.7 Interferon- γ

7.7.1 Non-parametric testing for all HEU vs HEI

Interferon- γ levels with medium only, in the presence of BCG, and BCG-specific responses at each time point for all 29 compounds are shown in Figure 36.

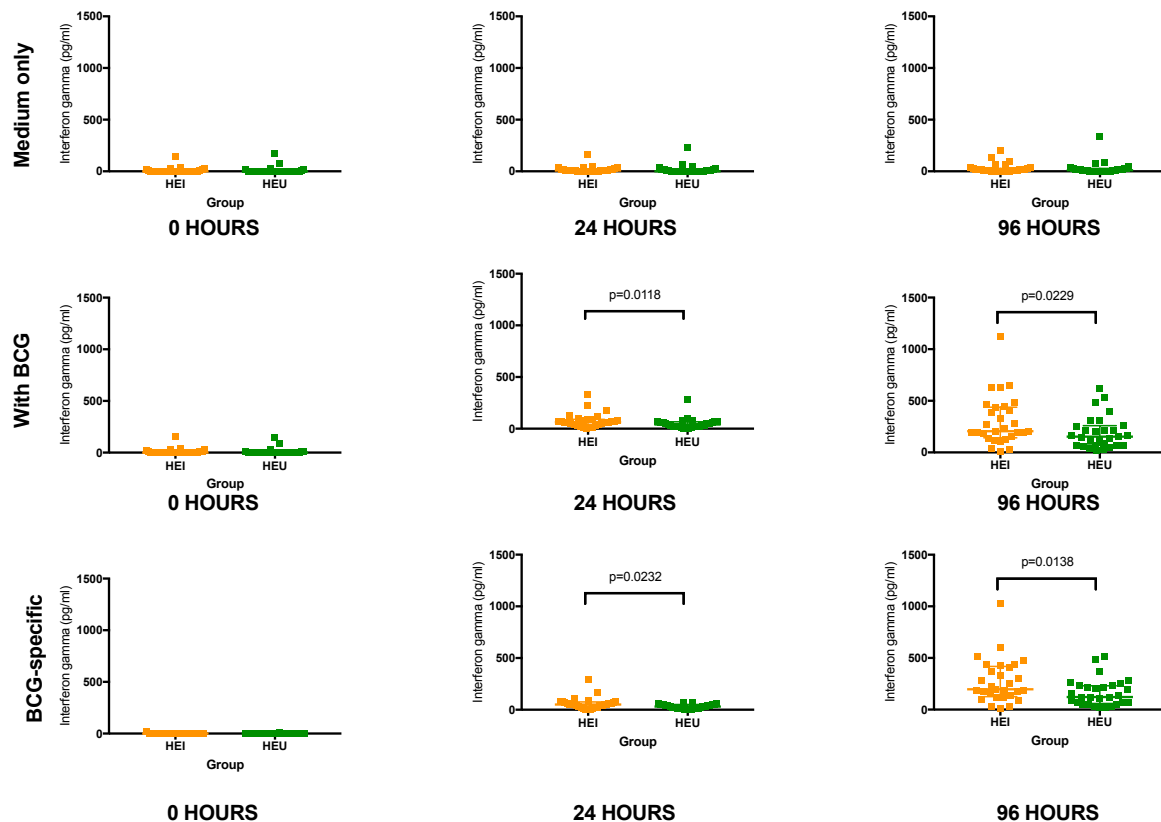


Figure 36: Interferon- γ levels for the 29 HEI and 29 HEU children.

Median and interquartile ranges. P values from Wilcoxon matched pairs signed rank test.

HEI children produced significantly more interferon- γ in the presence of BCG than HEU children at 24 hours (HEI median 63.67pg/ml, IQR 36.38→90.9; HEU 40.06, IQR 23.63→67.24; $p=0.0118$) and at 96 hours (HEI median 205.5 pg/ml, IQR 142.1→438.7; HEU 153.3 pg/ml, IQR 71.26→259; $p=0.0229$). BCG-specific responses were also higher in HEI children at 24 hours (HEI median 50.44, IQR

23.15→75.36; HEU 30.06, IQR 14.64→50.86, $p=0.0232$) and at 96 hours (HEI median 197.2pg/ml, IQR 125.4→418.3; HEU 121.4, IQR 59.53→231; $p=0.0138$)

7.7.2 Subgroup analysis by HEI with discordant IGRA

Interferon γ production by the HEI children with discordant IGRA results compared to HEI children with positive IGRA results for the three experimental conditions is shown in Figure 37. Differences were not statistically significant with the Mann-Whitney U Test.

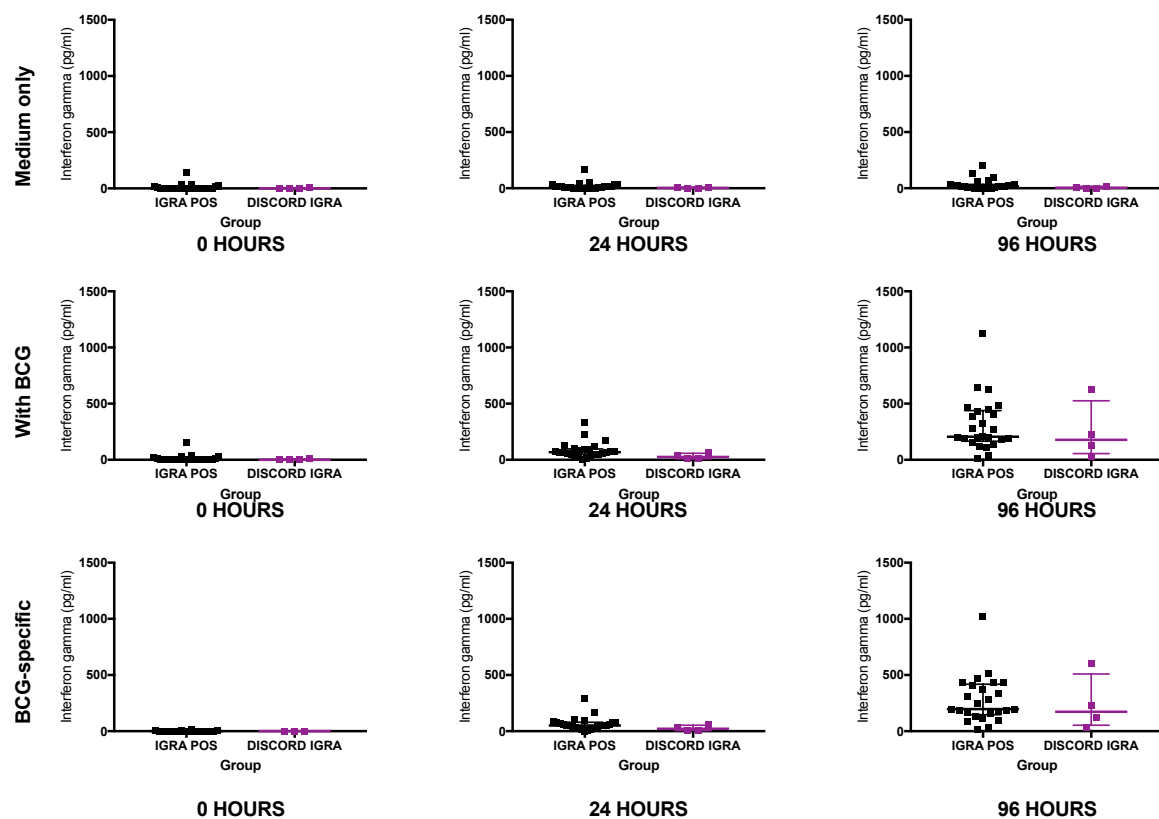


Figure 37: Interferon- γ levels of the 29 HEI children by IGRA status

Median and interquartile range

Non-parametric statistics for only the 25 compounds where HEI children had a positive IGRA are shown in Figure 38. HEI children had a higher interferon- γ response to BCG at 24 hours (HEI median 68.19pg/ml, IQR 46.45→96.34; HEU 40.06 pg/ml, IQR 24.15→67.24; $p=0.0053$) and at 96 hours (HEI median 205.5pg/ml, IQR 166.2→438.7; HEU 149.2, IQR 71.26→239.3; $p=0.0275$). BCG-specific responses were also increased at 24 hours (HEI median 51.04pg/ml, IQR 28.14→78.9; HEU 30.06 pg/ml, IQR 14.64→54.42 ; $p=0.0179$) and at 96 hours (HEI median 197.2 pg/ml, IQR 146.4→418.3; HEU 114, IQR 59.53→222; $p=0.0219$).

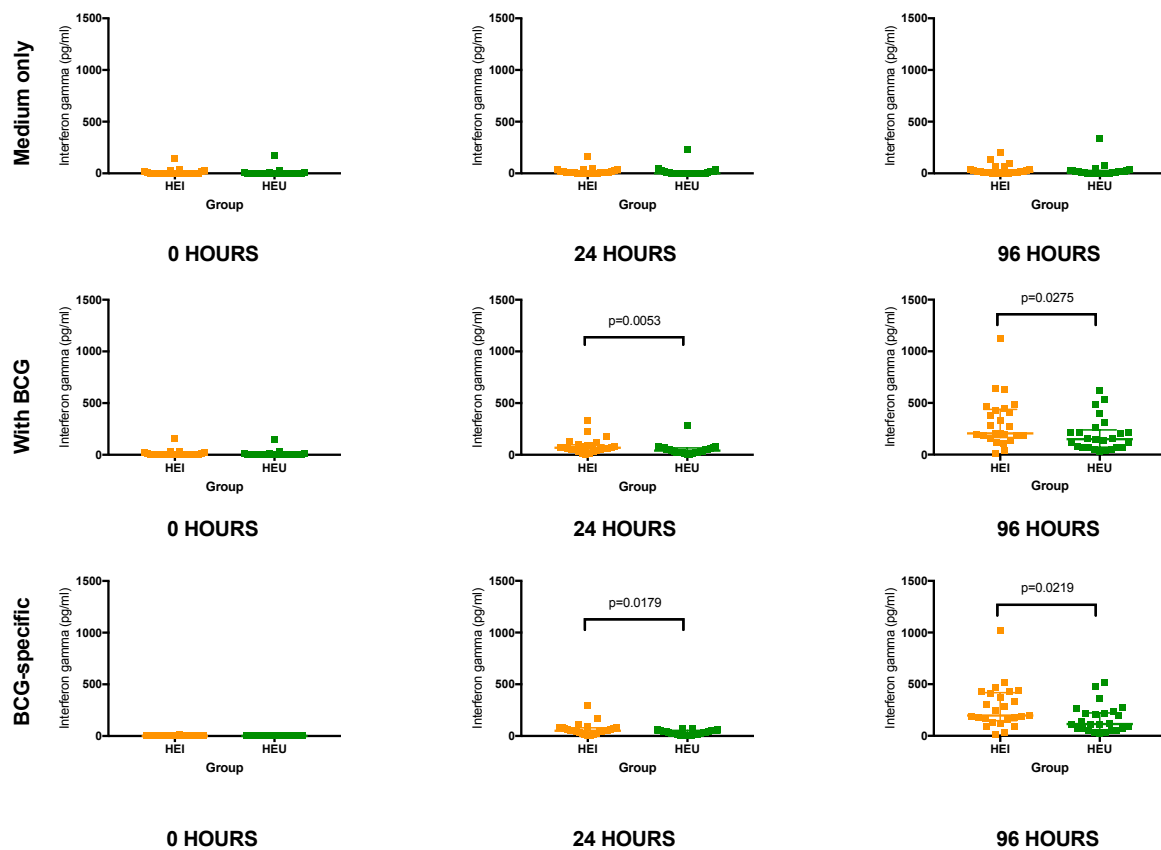


Figure 38: Interferon- γ levels for the 25 HEI children with concordant IGRA and the matched 25 HEU children.

Medians and interquartile ranges.

7.7.3 Subgroup analysis of compounds where LEU were recruited

There were no differences in interferon- γ between LEU children and HEI or HEU children. (Figure 39)

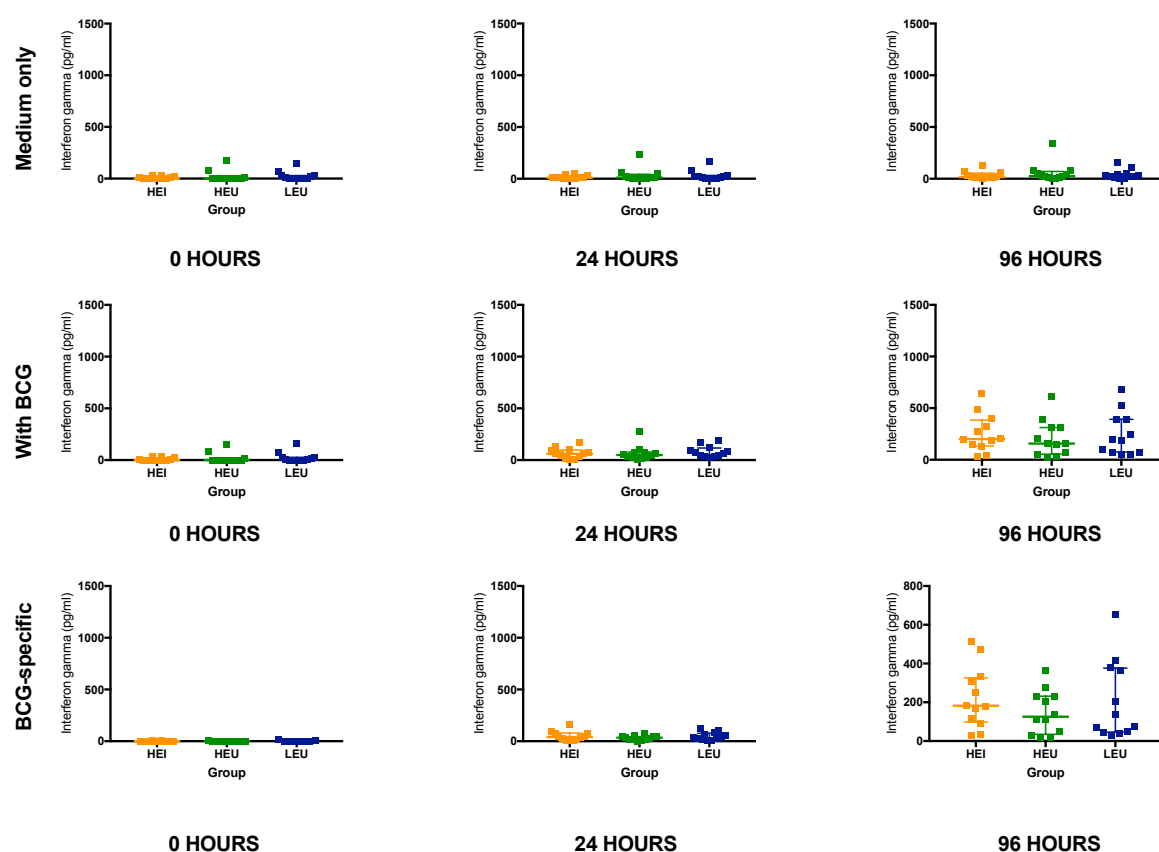


Figure 39: Interferon- γ levels from the 36 children from compounds where a LEU child was recruited.

Medians and interquartile ranges

7.8 Tumour Necrosis Factor α

7.8.1 Non-parametric testing for all HEU vs HEI

TNF α levels with medium only, in the presence of BCG, and BCG-specific responses at each time point for all 29 compounds are shown in Figure 40. There were no statistically significant differences between the groups.

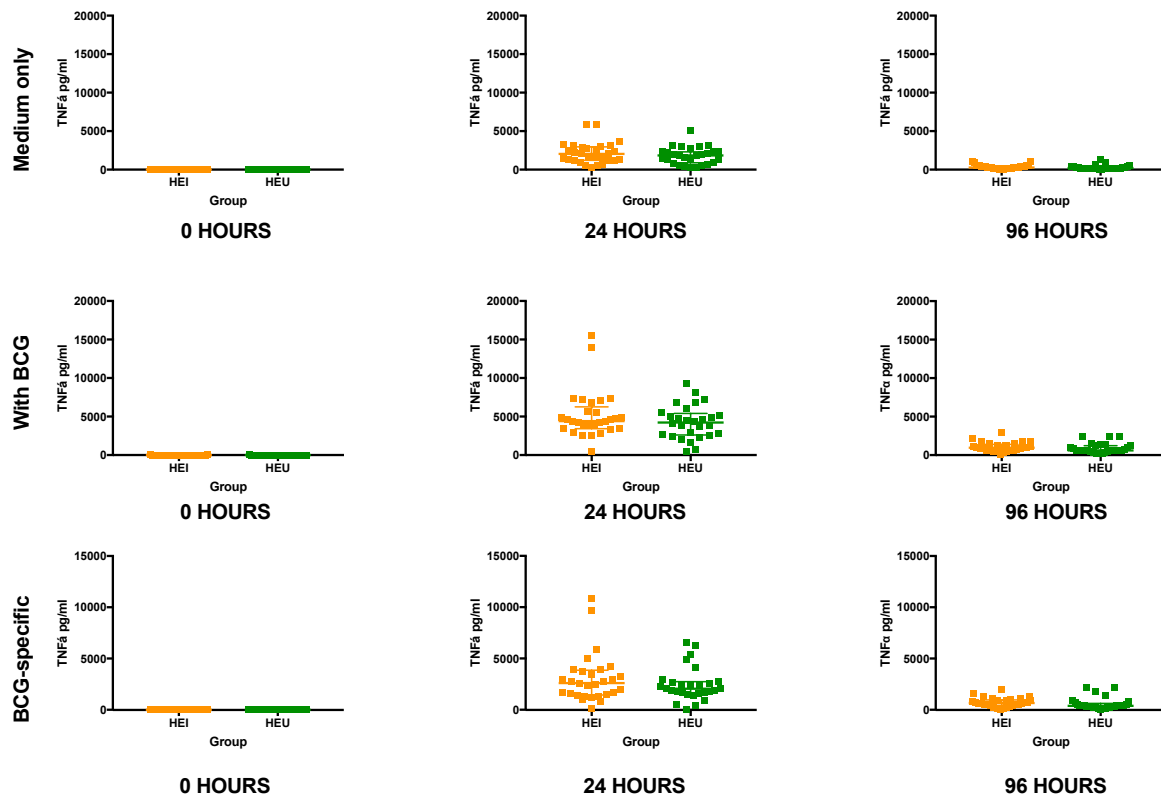


Figure 40: TNF α levels for the 29 HEI and 29 HEU children

Median and interquartile ranges.

7.8.2 Subgroup analysis by HEI with discordant IGRA

Non-parametric testing of the HEI and HEU children from the 25 compounds where the HEI child had a positive IGRA showed no difference between the groups at any of the time points or experimental conditions.

7.8.3 Subgroup analysis of compounds where LEU were recruited

BCG-specific TNF α responses of LEU children were significantly lower than those of HEI children at 96 hours (Figure 41: HEI 816.3 pg/ml, IQR 509.3→1,280, p vs LEU=0.0269; HEU 372.4pg/ml, IQR 205.2→529.5, p vs LEU=0.176; LEU 466.4, IQR 425.9→688.2).

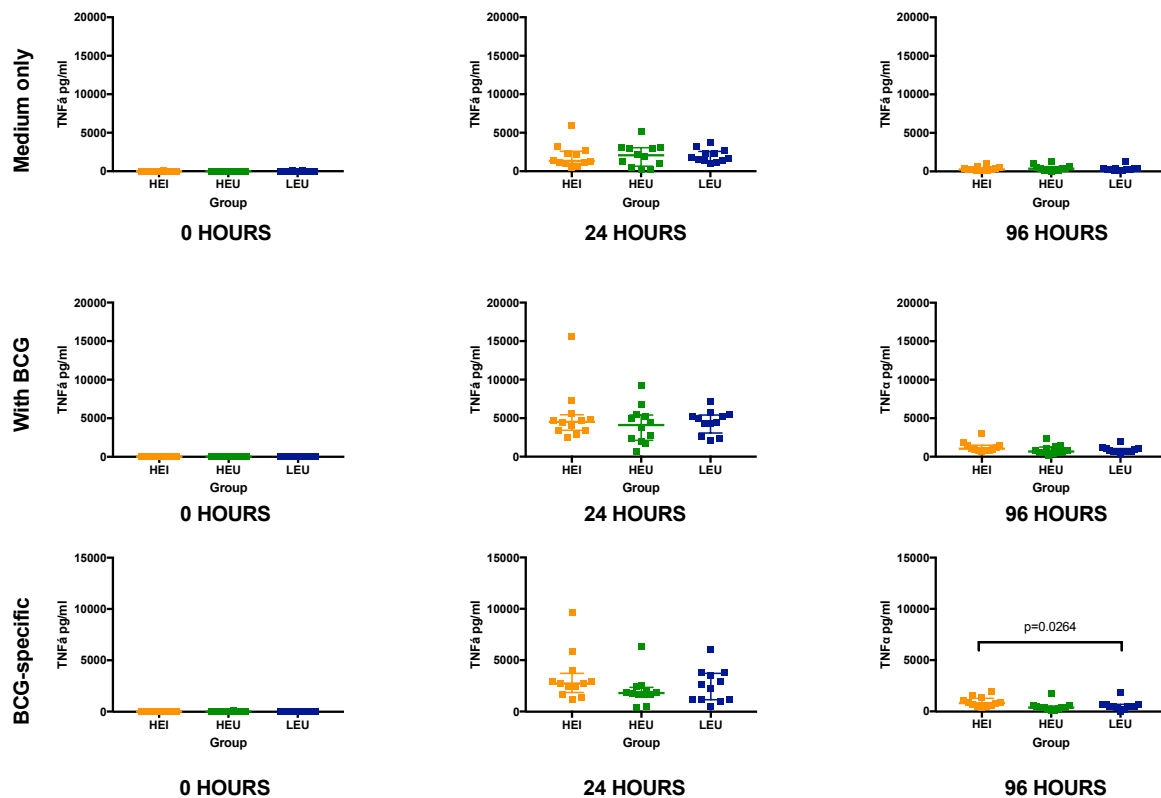


Figure 41: TNF- α levels from the 36 children from compounds where LEU children were recruited. Medians and interquartile ranges.

7.9 Conclusion

We conducted an extensive panel of cytokine measurements in supernatants from the different experimental conditions. The only significant difference in cytokine levels between HEU and HEI children detected in this study was the greater production of interferon- γ by HEI children at 24 hours and 96 hours in response to BCG. This is in keeping with the selection of HEI children based upon an adaptive immune response to mycobacteria and has also been shown in Ugandan and Pakistani cohorts.^{56,60,61} The HEI children who had a negative IGRA at screening did not have significantly different interferon- γ responses compared to the other HEI

children with positive IGRAs. Excluding compounds where HEI children had discordant IGRAs did not produce notably different results for any of the cytokines.

The heritability of TNF- α production in response to mycobacterial stimulation and the proximity of the associated genetic locus to TST1 (absence of TST reactivity), meant we analysed the TNF- α levels in our study with great interest.^{49–51} There were no difference in TNF- α levels between HEI and HEU children. Although disappointing, these results are in keeping with other experimental data where no differences were observed in household contact studies.^{53,56,60,78}

Overall, the alternative hypothesis that there is a qualitatively different innate immune response between HEU and HEI children is rejected with the cytokines studied and time points chosen. We did not look at specific cytokine-producing cell populations, although these have not shown statistically significant differences in paediatric case-control studies of children with tuberculosis disease.^{113,114}

LEU children had higher IL1- α levels in the presence of BCG at 24 hours and lower BCG-specific TNF- α levels at 96 hours compared to HEI children. Surprisingly, there was no statistical difference between IFN- γ levels between LEU and HEI children,²⁵⁴ which may reflect the small sample size. The null hypothesis that differences between HEU and HEI children are a consequence of infection is maintained.

Vitamin D levels have not been measured in the study participants reported in this thesis although this would be a valuable follow-on study with stored serum samples.

Vitamin D is an immune mediator with IFN- γ -dependent and IFN- γ and TNF- α -independent mechanisms to control *M. tuberculosis* including through the antimicrobial peptide cathelicidin.^{98,99,134,255–259} There are conflicting results from randomized controlled trials of vitamin D supplementation during tuberculosis treatment and whether it shortens time to sputum conversion overall, or only in a genotype-dependent manner.^{257,260,261} Low levels of vitamin D were associated with extrapulmonary tuberculosis in a UK study, and with TST conversion in contacts of patients with TB in a Spanish cohort.^{82,83,258} In contrast, a Gambian adult study showed higher Vitamin D levels were associated with progression to pulmonary tuberculosis in household contacts.⁸⁰ Mean vitamin D levels in Gambian children have been estimated between 50 and 95nmol/L, which are considered sufficient by international definitions.^{262–264} In a pilot paediatric randomized controlled trial in Mongolia, there was a borderline significant reduction in TST conversion rate from negative to positive in the vitamin D supplementation group compared to placebo group.⁸¹ Quantifying Vitamin D levels in this cohort and investigating their associations with cytokine production is proposed to build on the results reported in this thesis (Section 9.4, Figure 69).

CHAPTER 8 : EICOSANOID RESPONSES OF TB-EXPOSED CHILDREN

8.1 Introduction

Here I report the evaluation of eicosanoid responses of HEI, HEU, and LEU children in the Gambia in the BCG-GFP-LuxFO whole blood assay. This relates to:

Hypothesis 3: The eicosanoid response to mycobacterial infection of whole blood *in vitro* differs between HEU and HEI children, with HEU children having an early pro-inflammatory profile (low levels of LXA4 and high levels of PGE2 and LTB4).

8.2 Consideration of data adjustment requirements

8.2.1 Variability across plates

No batch effect was detectable in Principal Component Analysis (Figure 41), nor Partial Least Squares-Discriminant Analysis models generated by my collaborator, Dr. Arnaud Wolfer.

8.2.2 BCG-specific eicosanoid responses

To generate BCG-specific eicosanoid responses, the eicosanoid levels from the medium only condition for the same child at the same time point were subtracted from the BCG-GFP-LuxFO condition to generate BCG-specific eicosanoid responses.

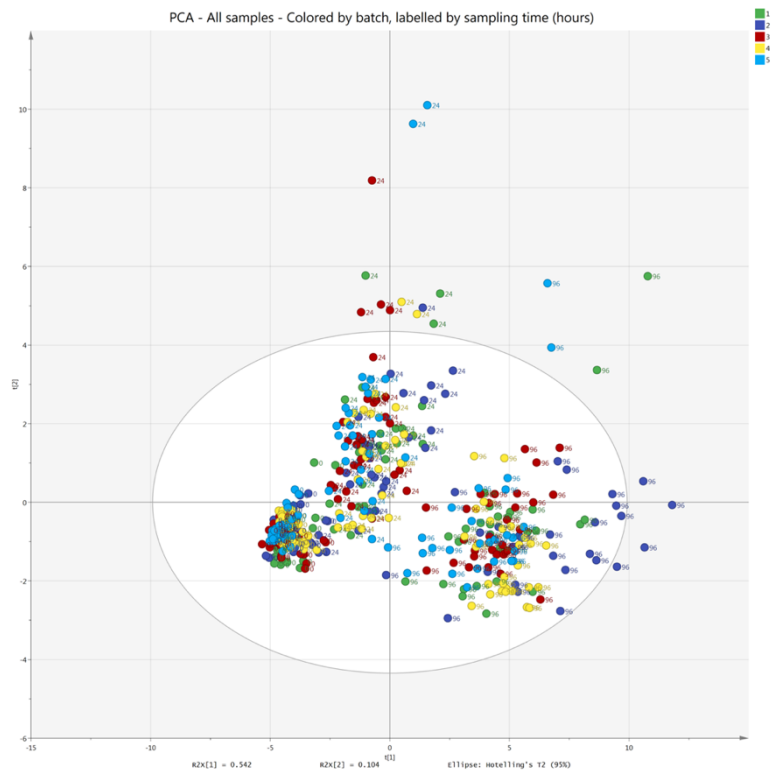


Figure 42: Principal component analysis of mass spectrometry samples.

Colour represents plate (legend top right). Adjacent to each sample is the time point. Courtesy of Dr. A. Wolfer. Analysis carried out on SIMCA, Sartorius Stedim Biotech, Aubagne, France

8.3 Overview of the data

8.3.1 Size of the dataset

For each child at each time point, pooled 0.22µm sterile-filtered supernatants from triplicate tubes for each of the two conditions (with BCG-GFP-LuxFO and with medium only) were analysed, with the exception of:

- For one child there were duplicate tubes only for the medium only condition at 96 hours. This was due to loss of supernatant from the third tube at the time of collection.
- For one child, there were singlicate tubes only at baseline and no samples at 24 hours due to insufficient blood volumes at phlebotomy.

Therefore for each of the eicosanoids there are 418 datapoints, of which 416 represent pooled triplicates, one represents pooled duplicate, and one is a singlicate reading.

8.3.2 Summary of the eicosanoids present at detectable levels

Of the eicosanoids assayed with the targeted mass spectrometry for this study, LTB₄, lipoxin A₄, lipoxin B₄ and 16(R)-Hydroxyeicosatetranoic Acid were not detectable in our samples with this method. Mixed effects models were generated for all detected eicosanoids. Henceforth, data is only displayed for those eicosanoids which were detectable and hypothesized to be of interest (i.e. PGE₂ and 12oxoLTB₄, a metabolite of LTB₄²⁶⁵) and those that were tested as the hypothesis-generating component that demonstrated significant differences between groups.

8.4: 12-oxo-LTB₄

8.4.1 Non-parametric testing for all HEI vs HEU

No statistically significant differences were found with non-parametric testing for each experimental condition (Figure 43 graphs, Figure 44 Column Scatterplots).

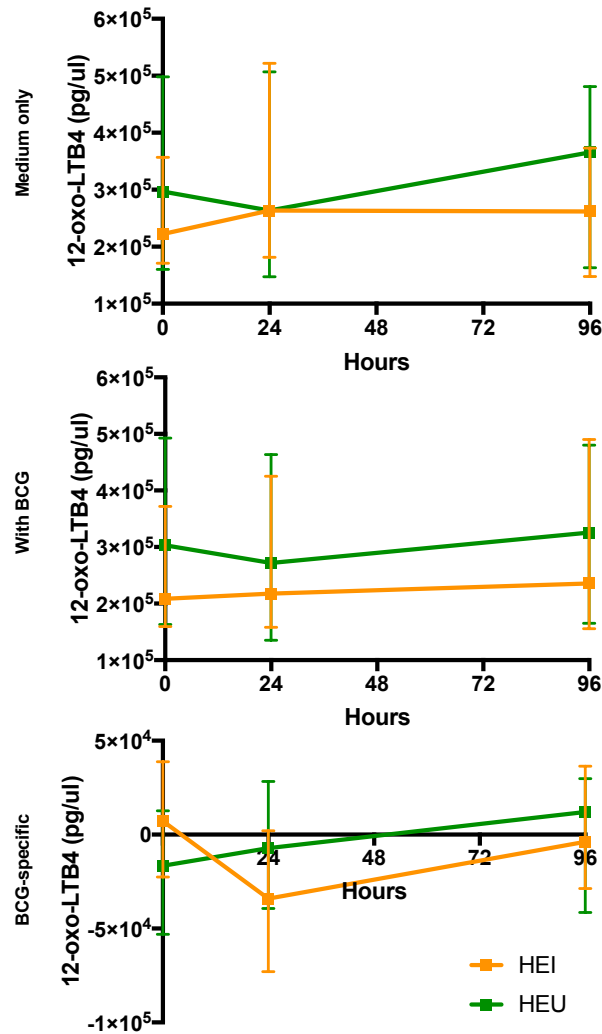


Figure 43: Kinetics of 12oxoLTB4 by experimental condition for 29 HEI vs HEU

Top row: medium only condition; middle row: with BCG condition; bottom row: BCG-specific values (medium only values subtracted from with BCG condition). Medians and interquartile ranges.

8.4.2 Mixed effects modelling for all HEI vs HEU – medium only condition

Log-transformed data was used for mixed effects modelling for this condition.

The model is shown in appendix A.4.4. Pairwise comparisons showed no differences between the groups.

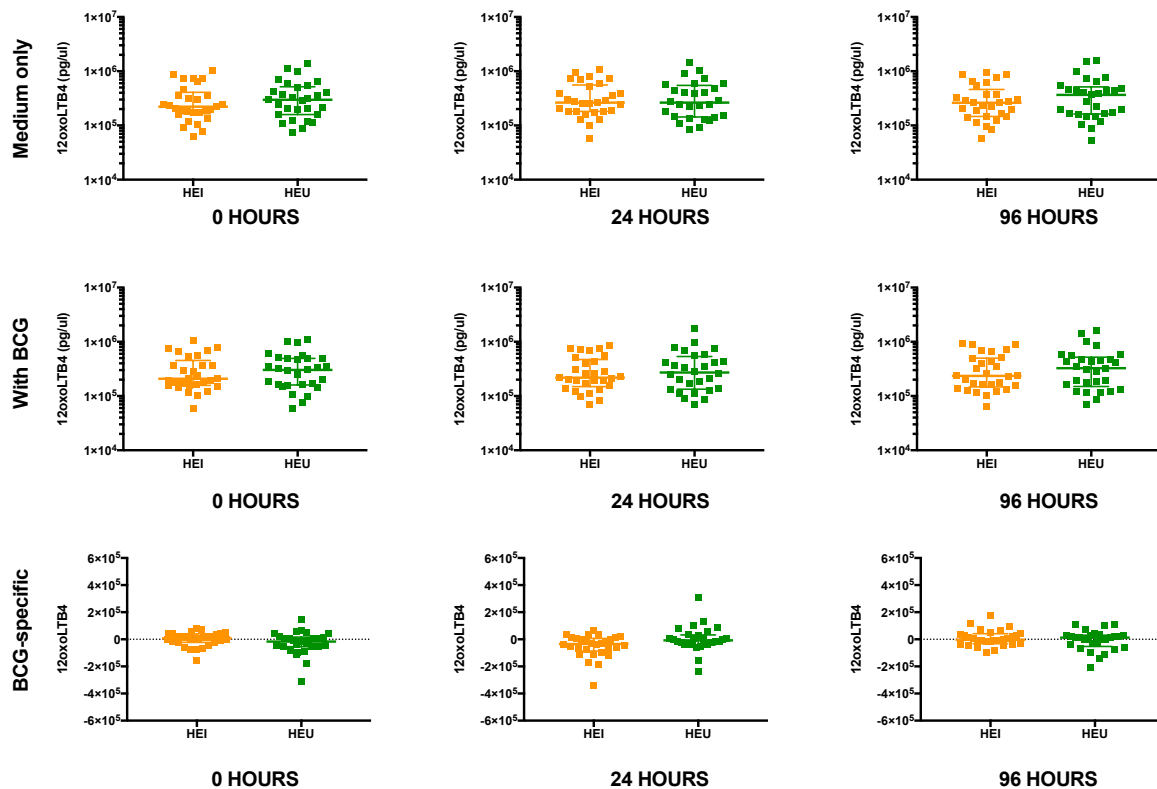


Figure 44: Scatterplots of 12-oxo-LTB4 levels by experimental condition and time for 29 HEI vs 29 HEU

Top row: medium only condition; middle row: with BCG condition; bottom row: BCG-specific values (medium only values subtracted from with BCG condition). First column: Baseline; Second column: 24 hours; Third column: 96 hours. Median and interquartile ranges.

8.4.3 Mixed effects modelling for all HEI vs HEU – with BCG

Log-transformed data was used for mixed effects modelling.

The model is shown in appendix A.4.5. Pairwise comparisons revealed no statistically significant differences between groups.

8.4.4 Mixed effects modelling for all HEI vs HEU – BCG-specific levels

Untransformed values were used for mixed effects modelling.

The model is shown in appendix A.4.6. Predictions from the model are shown in Figure 45. At 24 hours, the model predicts that HEI children have suppressed levels

of 12oxoLTB4 in the presence of BCG ($-53,970\text{pg}/\mu\text{l}$, 95%CI: $-81,698 \rightarrow -26,241$) in contrast to HEU children ($5,881$, 95%CI $-22,355 \rightarrow 34,118$, $p=0.003$).

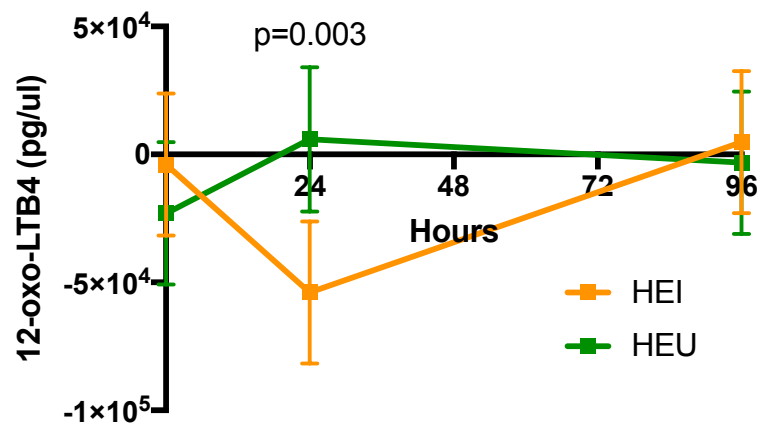


Figure 45: BCG-specific modelled estimated marginal means with data from 29 HEI and 29 HEU

Estimated marginal means and 95% confidence intervals. P values shown are from pairwise comparisons between predicted values at each time point.

8.4.5 Subgroup analysis by HEI with discordant IGRA

Values from HEI children with discordant IGRAs do not significantly differ from those with positive IGRAs (Figure 46). The mixed effects model for BCG-specific 12oxoLTB4 with only compounds where the HEI child had a positive IGRA generates similar predictions with a statistically significant modelled difference at 24 hours (Figure 47: HEI $-60,710\text{pg}/\mu\text{l}$, IQR $-91,763 \rightarrow -29,657$; HEU $4,760$, IQR $-26,906 \rightarrow 36,426$, $p=0.004$).

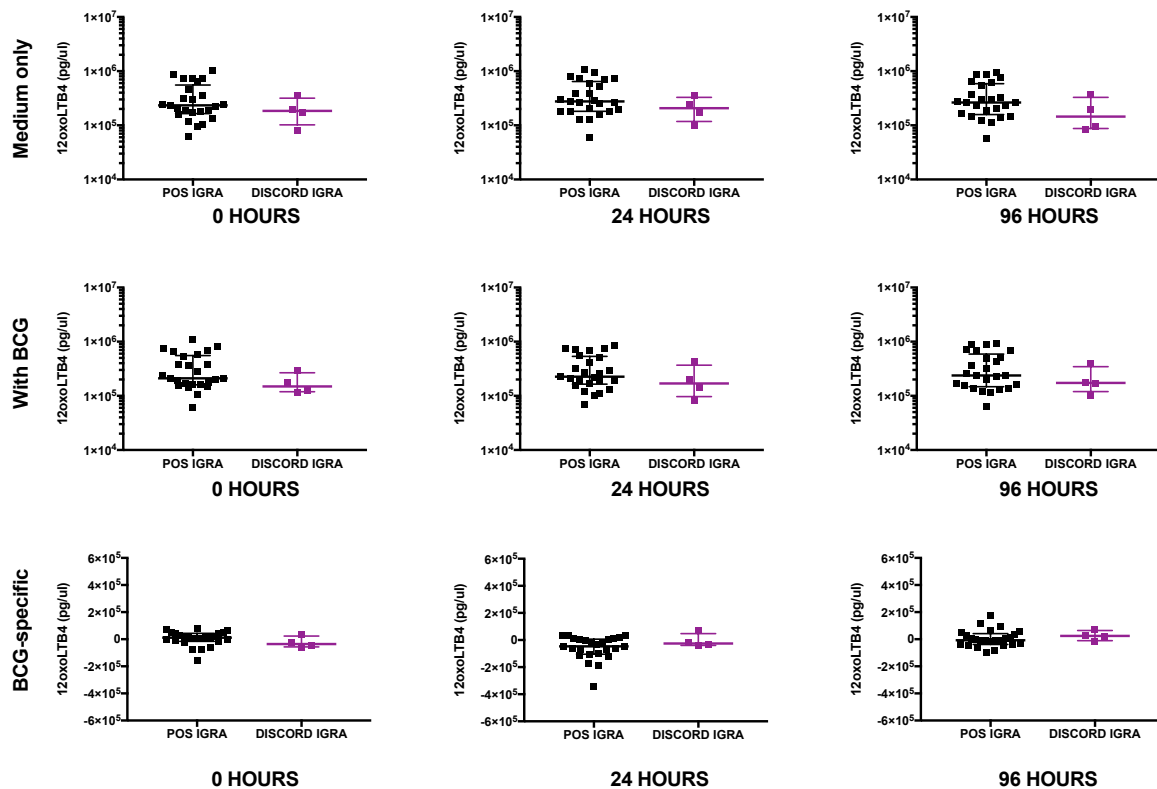


Figure 46: 12oxoLTB4 levels by IGRA status for the 29 HEI children

Top row: medium only condition; middle row: with BCG condition; bottom row: BCG-specific values (medium only values subtracted from with BCG condition). First column: Baseline; Second column: 24 hours; Third column: 96 hours. Median and interquartile ranges.

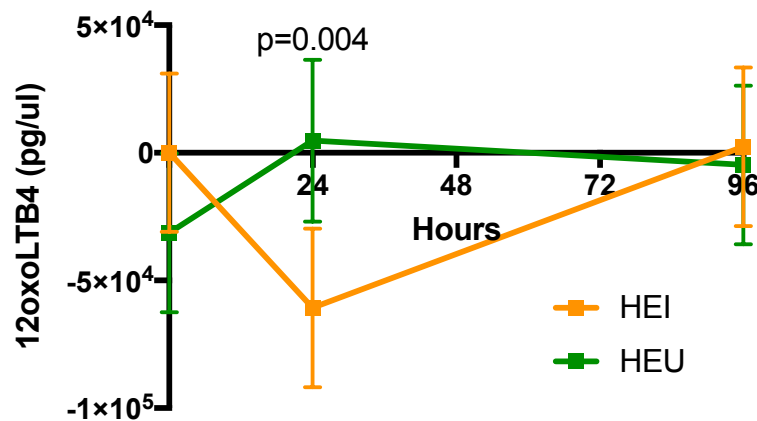


Figure 47: BCG-specific modelled estimated marginal means of 12oxoLTB4 from 25 HEI with concordant IGRA and 25 matched HEU

Estimated marginal means with 95% confidence intervals. P values shown are pairwise comparisons between predicted values at that time point.

8.4.6 Subgroup analysis of compounds where LEU were recruited

Non-parametric testing (Figure 48 graphs, Figure 49 column scatterplots) and mixed effects modelling demonstrated no significant differences between HEI, HEU, and LEU children.

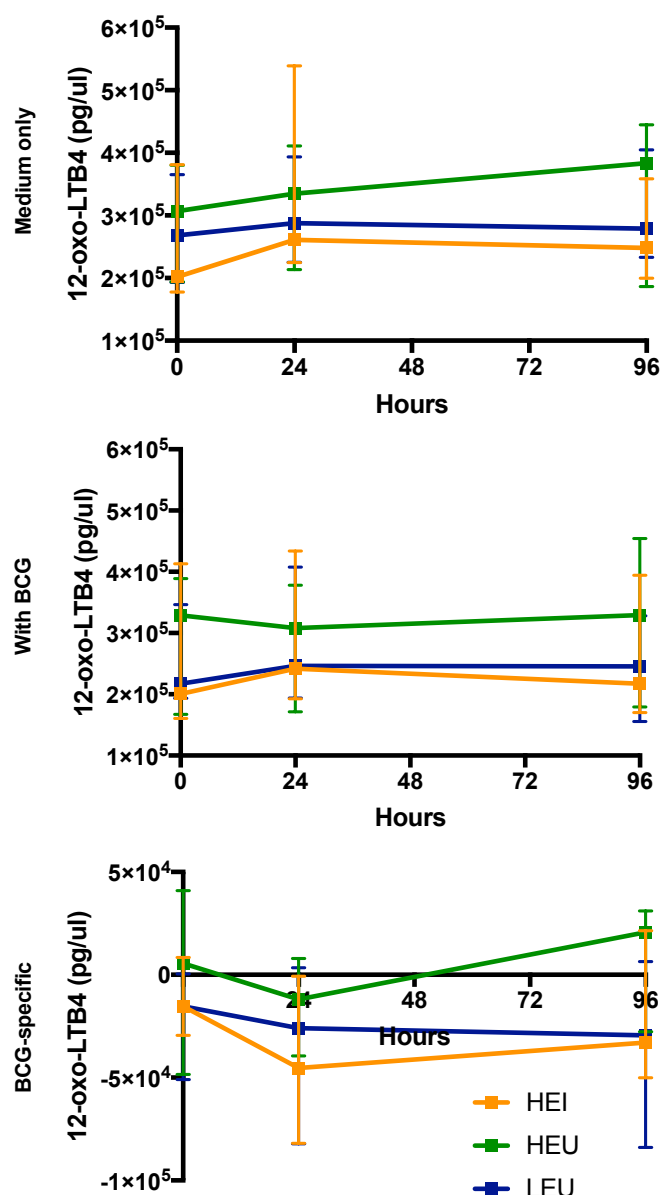


Figure 48: Kinetics of 12oxoLTB4 levels from the 36 children from compounds where a LEU child was recruited.

Top row: medium only condition; middle row: with BCG condition; bottom row: BCG-specific values (medium only values subtracted from with BCG condition). Medians and interquartile ranges.

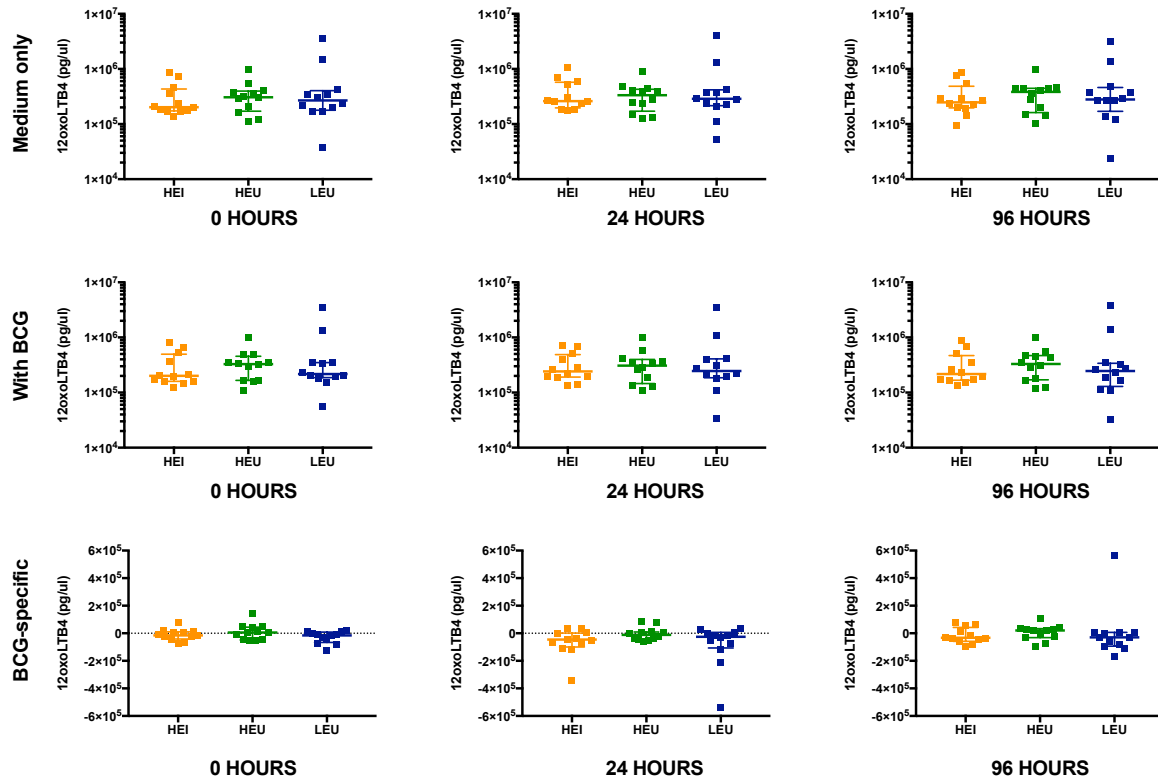


Figure 49: Scatterplots of 12-oxo-LTB₄ levels by experimental condition for 36 children from compounds where LEU was recruited.

Top row: medium only condition; middle row: with BCG condition; bottom row: BCG-specific values (medium only values subtracted from with BCG condition). First column: Baseline; Second column: 24 hours; Third column: 96 hours. Median and interquartile ranges.

8.4.7 Summary of 12oxoLTB₄ findings

Mixed effects modelling of the data adjusting for age and accounting for matching and the longitudinal nature of the data predicts that HEI children have negative values of BCG-specific 12oxoLTB₄ at 24 hours (i.e. the presence of BCG is associated with *lower* levels of 12oxoLTB₄ than when unstimulated), and that these levels are significantly lower than those of HEU children. The effect holds true when compounds where the HEI child had a discordant IGRA are excluded from the analysis. This is in accordance with hypothesis 3.

8.5 Prostaglandin E₂

8.5.1 Non-parametric testing for all HEU vs HEI

No statistically significant differences were found between HEI and HEU children with non-parametric testing (Figure 50 graphs, Figure 51, column scatterplots).

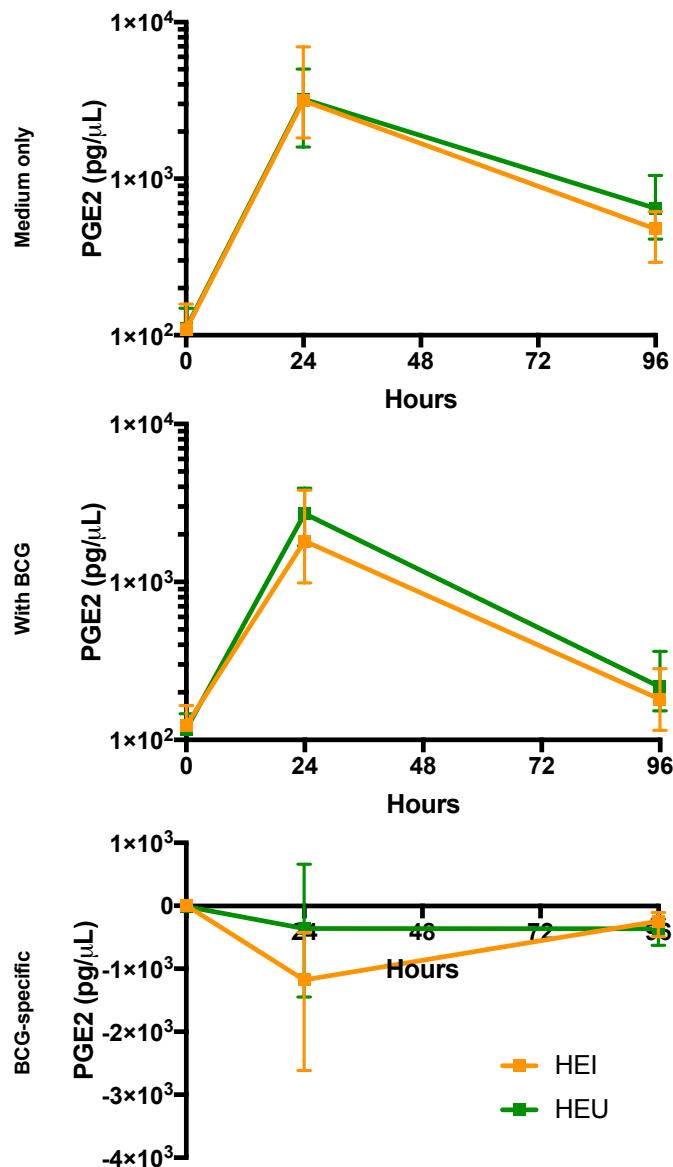


Figure 50: Kinetics of PGE₂ levels by experimental condition for 29 HEI and 29 HEU children

Top row: medium only condition; middle row: with BCG condition; bottom row: BCG-specific values (medium only values subtracted from with BCG condition). Medians and interquartile ranges.

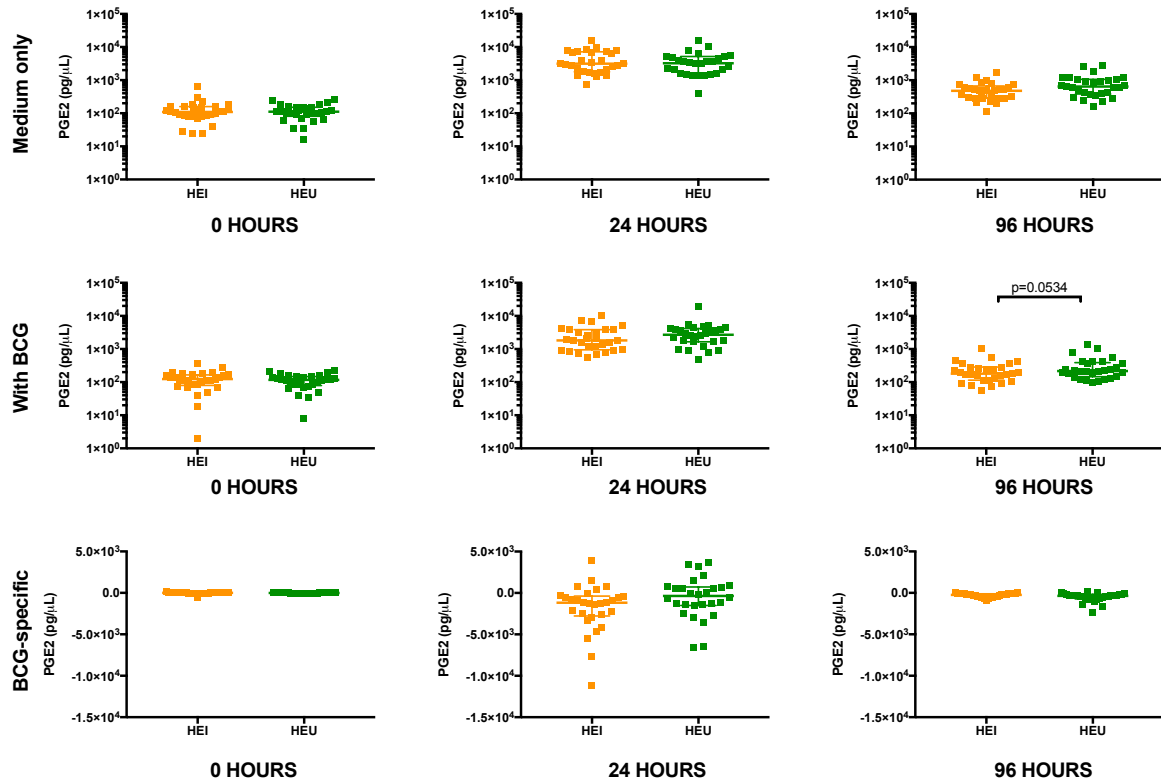


Figure 51: PGE2 levels by experimental condition and time for 29 HEI and 29 HEU children

Top row: medium only condition; middle row: with BCG condition; bottom row: BCG-specific values (medium only values subtracted from with BCG condition). First column: Baseline; Second column: 24 hours; Third column: 96 hours. Median and interquartile ranges.

8.5.2 Mixed effects modelling for all HEU vs HEI with medium only

Log-transformed data were used for mixed effects modelling.

The model is shown in appendix A.4.7. The predicted marginal means and 95% confidence intervals for the groups (medium only condition) from the model are shown in Figure 52. HEU children had significantly higher predicted PGE2 values at 96 hours (HEI estimated mean: 446.42pg/μl, 95% CI 347.3→573.8; HEU estimated mean: 668.3pg/μl, 95% CI 519.3→860.0, $p=0.028$). [Note although the lower 95% CI of HEU overlaps with the upper 95% CI of HEI, the odds that both values are at opposite extreme ends of the confidence interval are much less than the odds that

only one of them will be and therefore the difference between groups can still be significantly different]

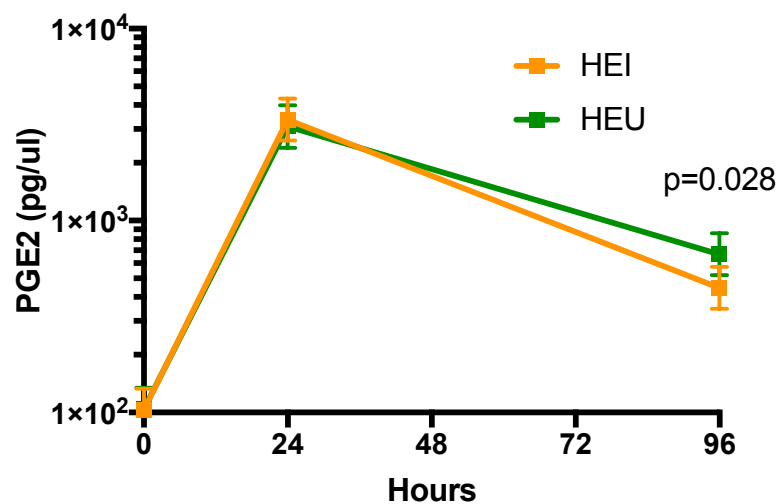


Figure 52: Modelled estimated marginal means for medium only PGE2 from 29 HEI and 29 HEU

Predicted marginal means with 95% confidence interval. P values from pairwise comparisons between groups at that time point.

8.5.3 Mixed effects modelling for all HEU vs HEI in presence of BCG

Log-transformed data were used for mixed effects modelling. Pairwise comparisons of predicted data showed no statistically significant differences.

8.5.4 Mixed effects modelling for all HEU vs HEI: BCG-specific

Data were not normally distributed and there was no transformation of the data that approximated a normal distribution therefore mixed effects modelling was not carried out for this condition.

8.5.5 Subgroup analysis by HEI with discordant IGRA

Non-parametric test comparisons of HEI children with a positive IGRA and those with a discordant IGRA showed no statistically significant differences (Figure 53)

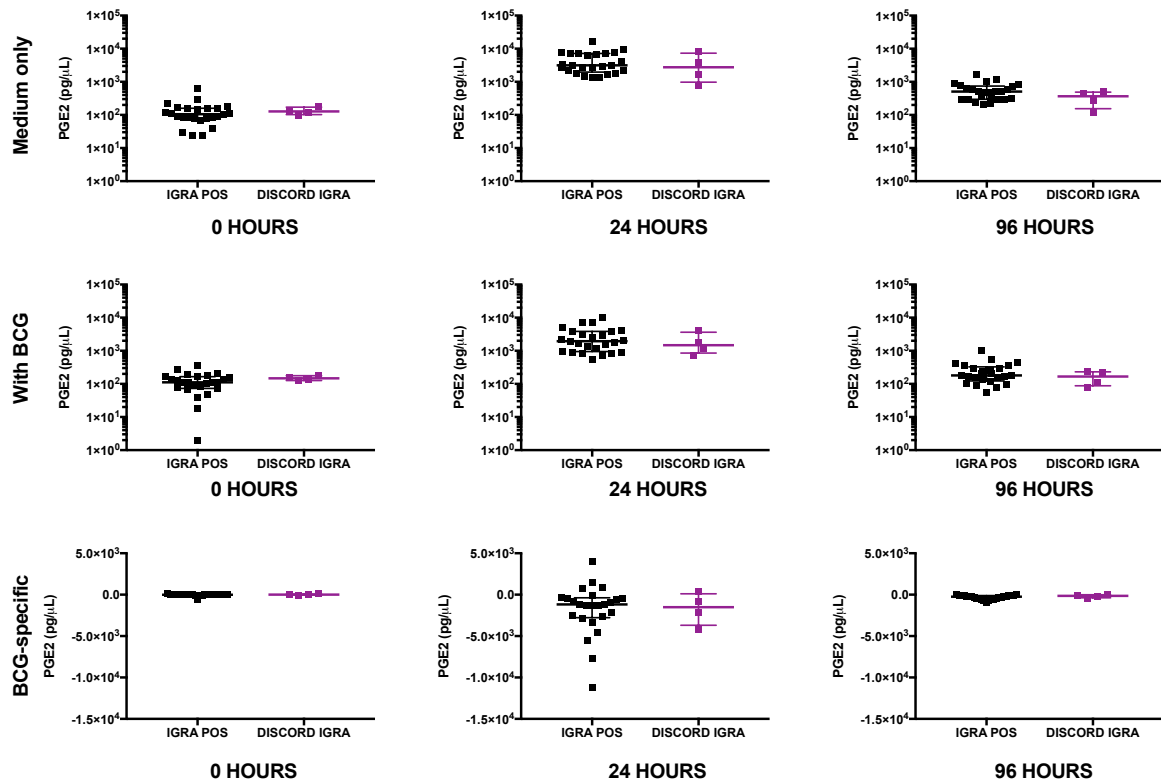


Figure 53: PGE2 levels by IGRA status of 29 HEI children

Top row: medium only condition; middle row: with BCG condition; bottom row: BCG-specific values (medium only values subtracted from with BCG condition). First column: Baseline; Second column: 24 hours; Third column: 96 hours. Median and interquartile ranges.

Repeating the non-parametric analysis shown in 8.5.1 following exclusion of data from those compounds where HEI had discordant IGRAs again showed no difference between groups by condition or time point (data not shown).

Log-transformed data approximated normal distributions with medium only and BCG present and therefore the same mixed effects models in 8.5.2 and 8.5.3 were run.

There were no statistically significant differences between groups. There were no transformations of BCG-specific levels of PGE2 that approximated a normal distribution and therefore mixed effects modelling was not performed.

8.5.6 Subgroup analysis of compounds where LEU were recruited

There were no statistically significant differences between groups with non-parametric testing (Figure 54, graphs, Figure 55, column scatterplots)

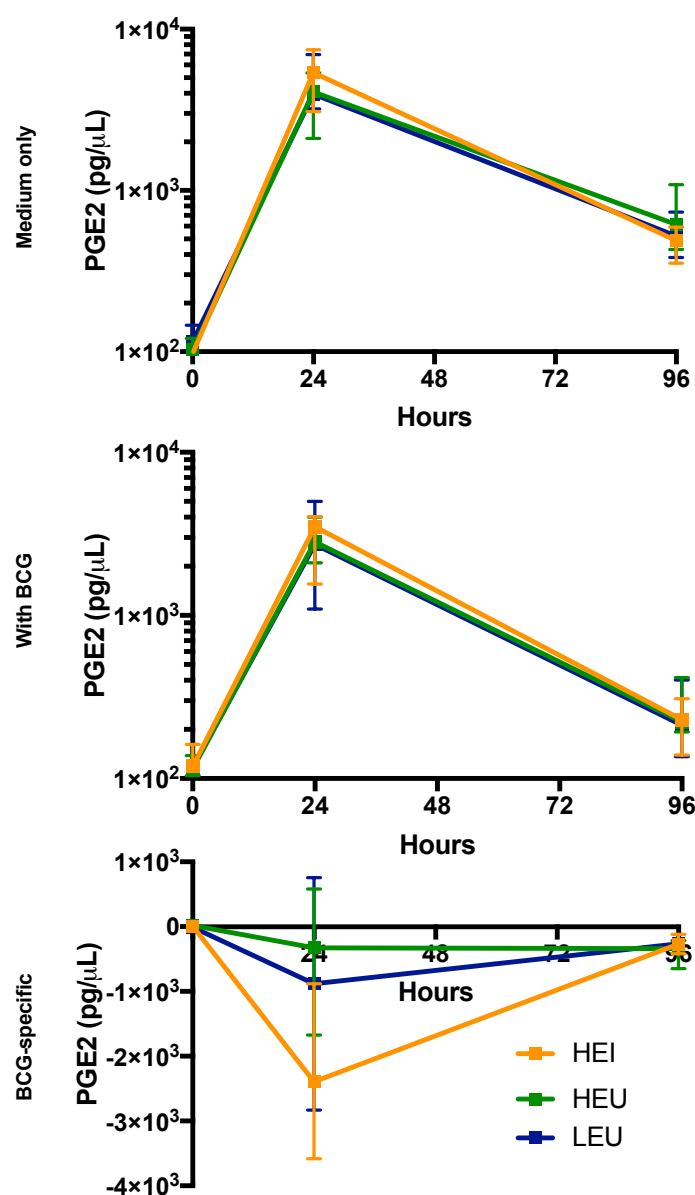


Figure 54: Kinetics of PGE2 levels by experimental condition for the 36 children from compounds where a LEU child was recruited.

Top row: medium only condition; middle row: with BCG condition; bottom row: BCG-specific values (medium only values subtracted from with BCG condition). Medians and interquartile ranges.

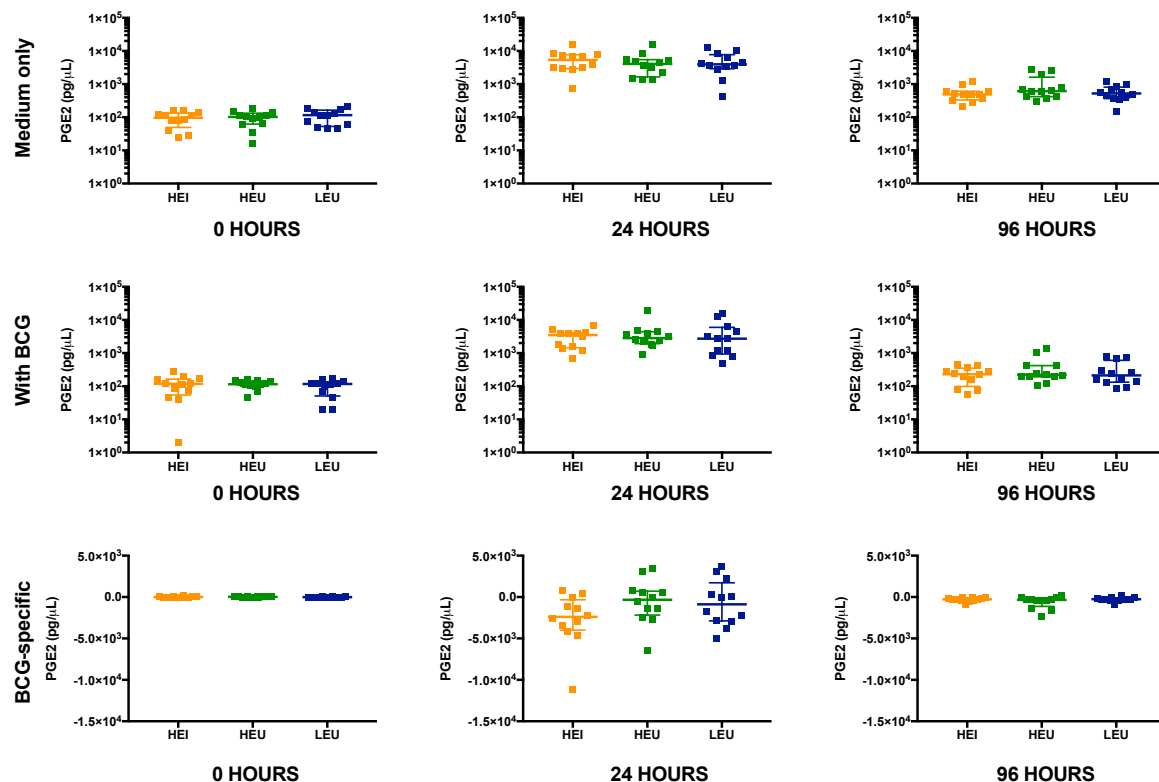


Figure 55: PGE2 levels by experimental condition for the 36 children from compounds where a LEU child was recruited.

Top row: medium only condition; middle row: with BCG condition; bottom row: BCG-specific values (medium only values subtracted from with BCG condition). First column: Baseline; Second column: 24 hours; Third column: 96 hours. Median and interquartile ranges.

Log-transformed data for the medium only and with BCG conditions approximated normal distributions therefore mixed effects modelling was performed. No statistically significant differences between groups were found (data not shown).

8.5.6 Summary of PGE2 results

There were no biologically relevant differences detected in PGE2 levels between groups.

8.6: 6ketoPGF1 α

6ketoPGF1 α is the stable hydrolysed product of prostacyclin, an eicosanoid most known for its role in vasodilatation and inhibition of platelet aggregation, and as a treatment for pulmonary arterial hypertension.^{266–269} At the time of the study conception and execution it was not predicted to be an important mediator of host-mycobacterial interactions.

8.6.1 Non-parametric testing for all HEU vs HEI

Data are displayed in Figure 56 (graphs) and Figure 57 (column scatterplots)

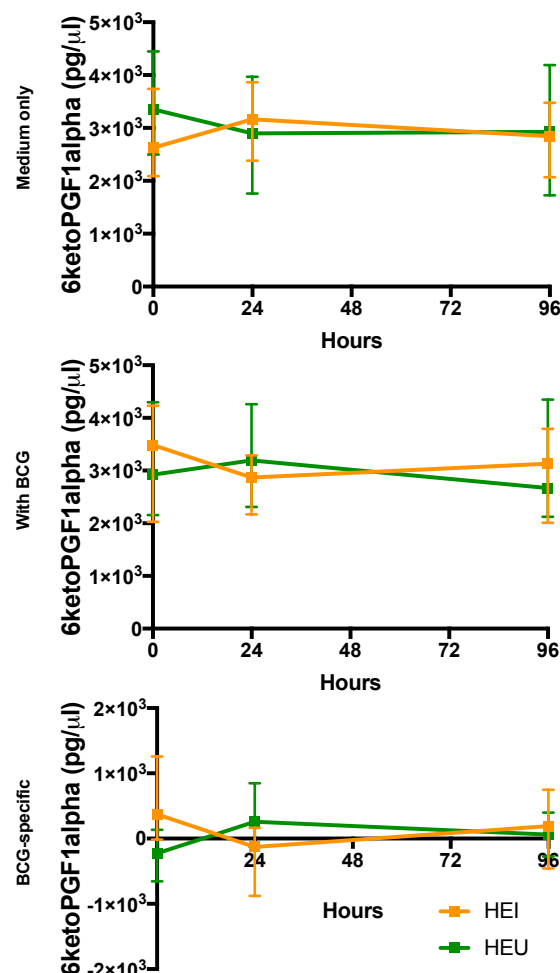


Figure 56: Kinetics of 6ketoPGF1 α levels for 29 HEI and 29 HEU children

Top row: medium only condition; middle row: with BCG condition; bottom row: BCG-specific values (medium only values subtracted from with BCG condition). Medians and interquartile ranges.

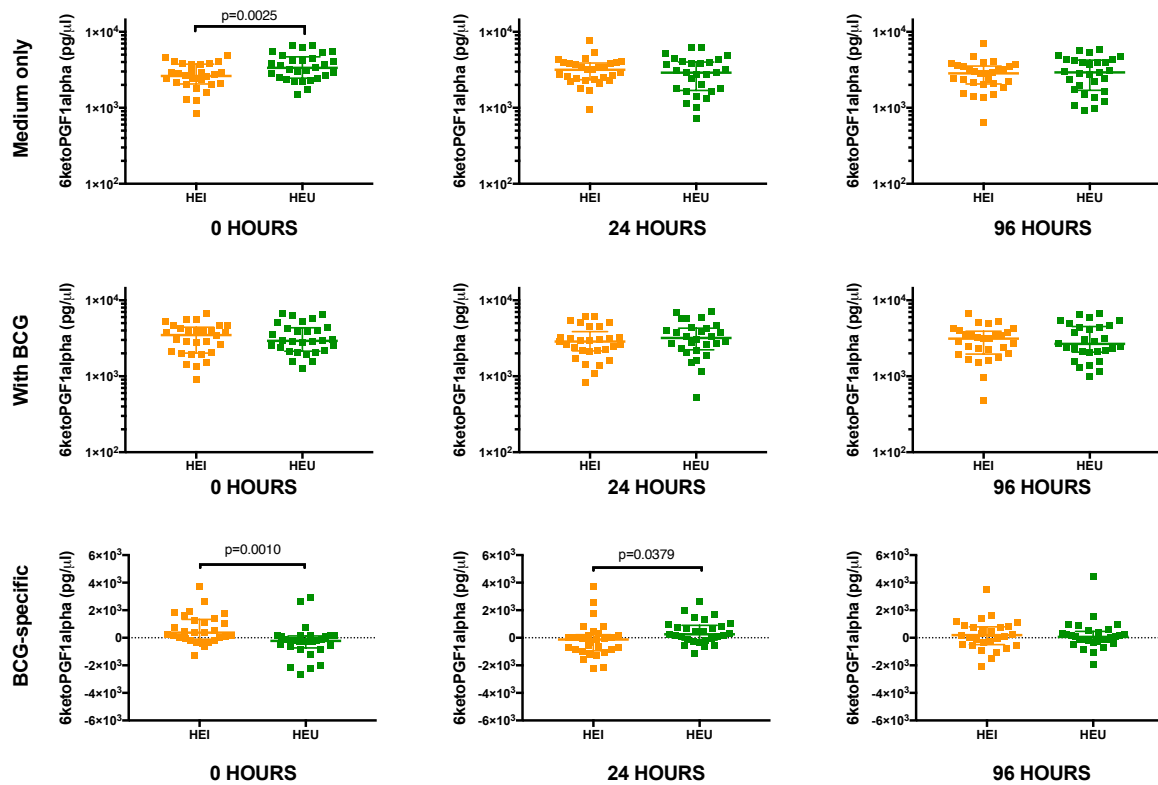


Figure 57: 6-ketoPGF1 α levels by experimental condition for the 29 HEI and 29 HEU children

Top row: medium only condition; middle row: with BCG condition; bottom row: BCG-specific values (medium only values subtracted from with BCG condition). First column: Baseline; Second column: 24 hours; Third column: 96 hours. Median and interquartile ranges.

At baseline with medium only, HEU children have higher levels of 6ketoPGF1 α (HEI median 2,627 pg/ μ l, IQR 2,056→3,751; HEU 3,352, IQR 2,451→4,694; p=0.0025).

There were time-related differences in BCG-specific levels of 6keto-PGF1alpha, with HEI children having higher levels at baseline (HEI 372.8pg/ μ l, IQR -107→1,331; HEU -224.6, IQR -735.9→137.8; p=0.001) but the pattern reversing at 24 hours with HEU children having higher BCG-specific levels (HEI median -127.2pg/ μ l, IQR -948.2→224.7; HEU 258.6 pg/ μ l, IQR -162.5→906; p=0.00379).

8.6.2 Mixed effects modelling for all HEU vs HEI – medium only

Square-root-transformed data were used for mixed effects modelling. The model is shown in appendix A.4.8. The predicted marginal means and 95% confidence intervals for the groups (medium only condition) from the model are shown in Figure 58. At baseline, HEU children were predicted to have higher levels of 6ketoPGF1 α than HEI children (predicted mean HEI: 2,668pg/ μ l; 95%CI 2,216 $\rightarrow$ 3,162; HEU 3,548pg/ μ l, 95% CI 3,020 $\rightarrow$ 4,118; p=0.007).

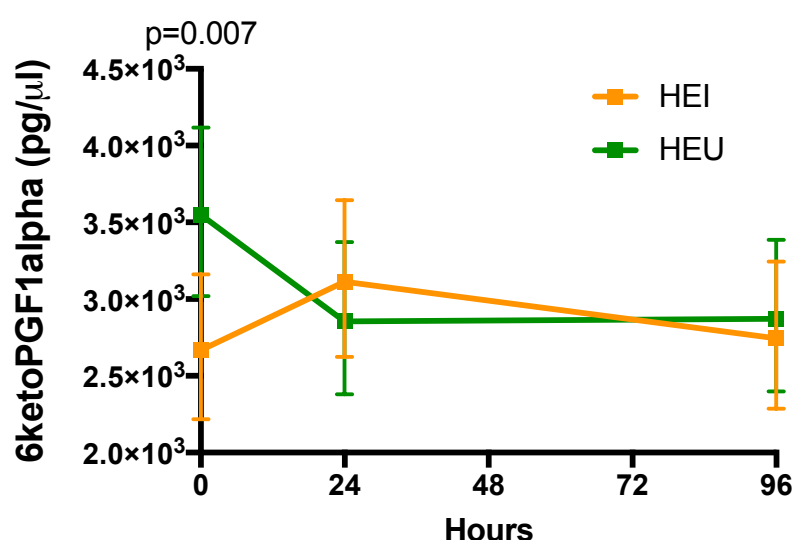


Figure 58: Modelled estimated marginal means of 6ketoPGF1 α with medium only from 29 HEI and 29 HEU

Predicted means and 95% confidence intervals shown. P values derived from pairwise comparisons between predicted data for groups at each time point.

8.6.3 Mixed effects modelling for all HEU vs HEI – with BCG

Square-root-transformed data were used for mixed effects modelling. The model is shown in Appendix A.4.9. The predicted marginal means and 95% confidence intervals for the groups (medium only condition) from the model are shown in Figure 59. There were no statistically significant differences.

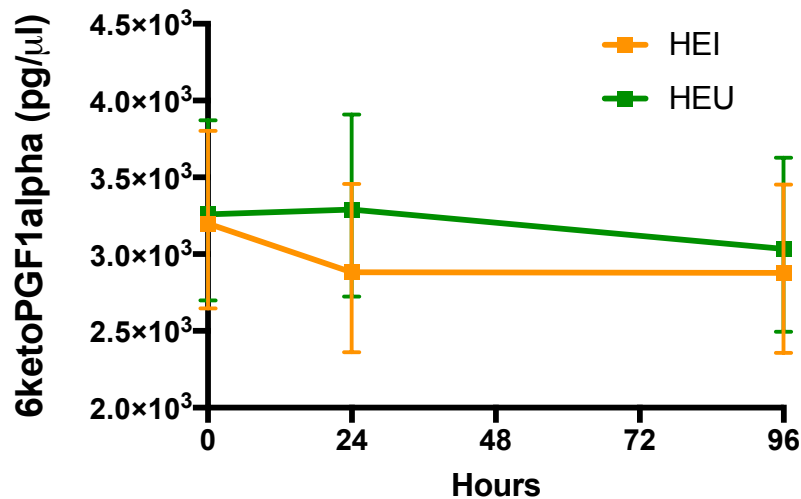


Figure 59: Modelled estimated marginal means of 6ketoPGF1 α with BCG from 29 HEI and 29 HEU

Predicted means and 95% confidence intervals shown. P values derived from pairwise comparisons between predicted data for groups at each time point.

8.6.4 Mixed effects modelling for all HEU vs HEI – BCG-specific

Untransformed data were used for mixed effects modelling. The model is shown in Appendix 4.10. The predicted marginal means and 95% confidence intervals for the groups from the model are shown in Figure 60. At baseline, HEI children were predicted to have higher BCG-specific levels than HEU children (HEI predicted mean 604.86pg/μl, 95% CI 199.31→1010.41; HEU -271.82pg/μl, 95% CI -679.04→135.40; p=0.001). The pattern was reversed at 24hours with HEU children predicted to have significantly higher levels (HEI predicted mean -170.8pg/μl, 95% CI -576.37→234.72; HEU 436pg/μl, 95% CI 22.86→849.15; p=0.028).

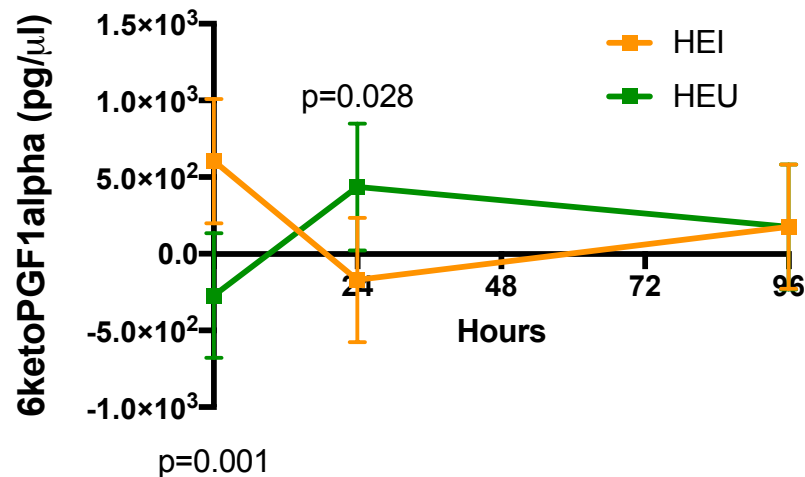


Figure 60: Modelled estimated marginal means of BCG-specific 6ketoPGF1 α from 29 HEI and 29 HEU

Predicted means and 95% confidence intervals shown. P values derived from pairwise comparisons between predicted data for groups at each time point.

8.6.5 Subgroup analysis by HEI with discordant IGRA

At 96 hours in the presence of BCG, HEI children with positive IGRAs had significantly higher levels of 6ketoPGF1 α than HEI children with discordant IGRAs (Figure 61: IGRA positive HEI median 3,223 pg/ μ L, IQR 2,345→4,126; discordant IGRA HEI 1,888 pg/ μ L, IQR 1,154→2,180; $p=0.0248$). At baseline, BCG-specific levels of 6ketoPGF1 α were also higher in IGRA positive HEI children (IGRA pos HEI median 360.8 pg/ μ L, IQR 9.0→1,496; discordant IGRA HEI -332.8 pg/ μ L, IQR -1,080→316.6; $p=0.0434$).

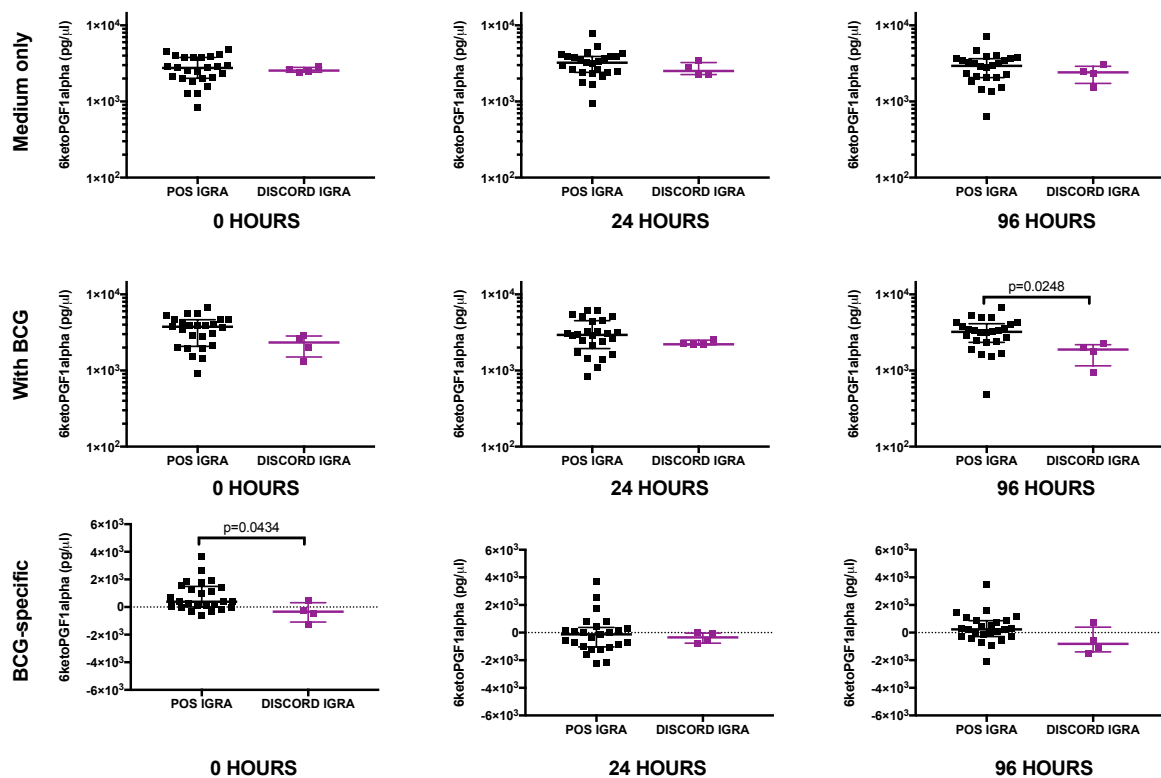


Figure 61: 6ketoPGF1 α levels by IGRA status for 29 HEI children

Top row: medium only condition; middle row: with BCG condition; bottom row: BCG-specific values (medium only values subtracted from with BCG condition). First column: Baseline; Second column: 24 hours; Third column: 96 hours. Median and interquartile ranges.

Given these significant differences between children with discordant IGRA results, I now present the subgroup analysis excluding the four compounds where the HEI child had a discordant IGRA result.

8.6.6 Non-parametric subgroup analysis - compounds where HEI had positive IGRA

Non-parametric testing showed a similar pattern as the whole dataset (Figure 62), with HEU children having higher levels at baseline with medium only (HEI median 2,763pg/ μ l, IQR 2,014 $\rightarrow$ 3,794; HEU 3,352, IQR 2,511 $\rightarrow$ 4,899, $p=0.0061$). BCG-specific responses at baseline were again higher in HEI children (median HEI 380.8pg/ μ l, IQR 8.97 $\rightarrow$ 1,496; HEU -214.7pg/ μ l, IQR -735.9 $\rightarrow$ 137.8; $p=0.0002$). The

difference in BCG-specific levels at 24 hours was now of borderline significance ($p=0.0787$).

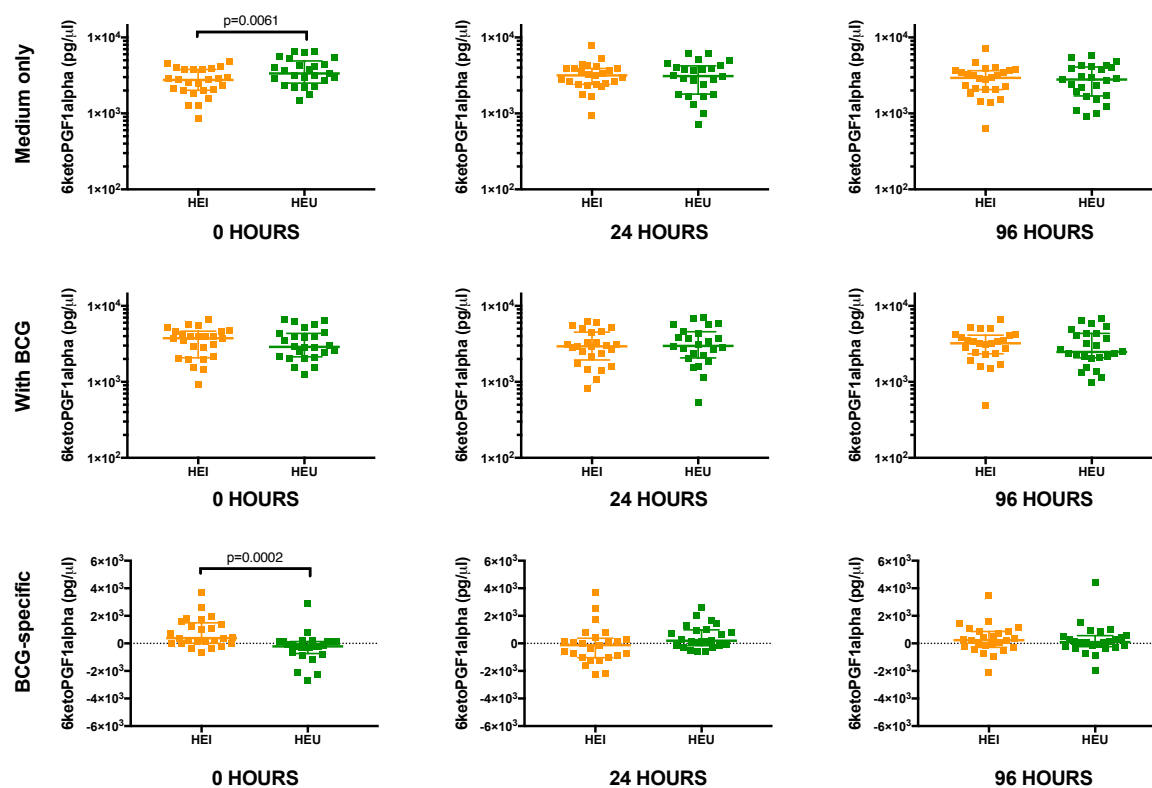


Figure 62: 6ketoPGF1 α levels for 25 HEI children with concordant IGRA and the 25 matched HEU children
Median and interquartile ranges.

8.6.7 Mixed effects modelling medium only - compounds where HEI had positive IGRA

Square-root-transformed data was used for mixed effects modelling.

The same model from 8.6.2 was run with this dataset and the output is shown in Appendix 4.11. The predicted marginal means and 95% confidence intervals for the groups (medium only condition) from the model are shown in Figure 63. At baseline, HEU children were predicted to have higher levels of 6ketoPGF1 α than HEI children (HEI predicted mean 2,678pg/ μ l, 95%CI 2184 $\rightarrow$ 3222; HEU 3594pg/ μ l, 95% CI 3016 $\rightarrow$ 4223, $p=0.009$).

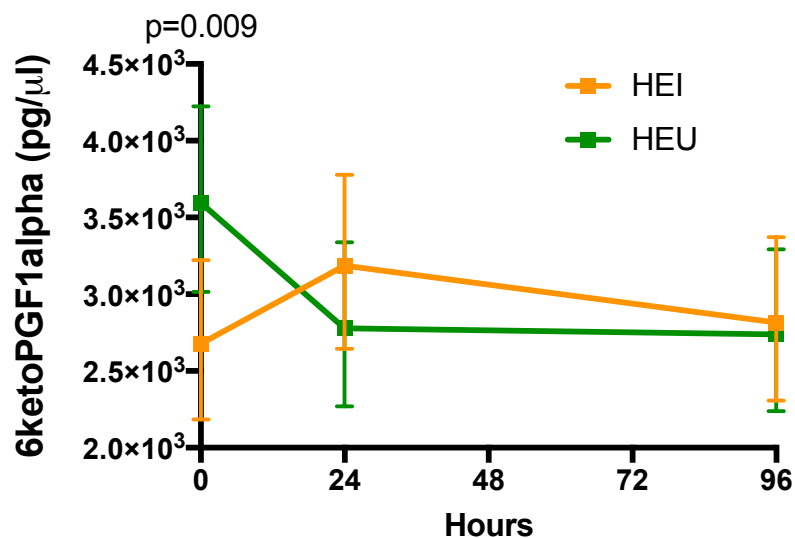


Figure 63: Modelled estimated marginal means of 6ketoPGF1 α for medium only condition from 25 HEI children with concordant IGRA and the 25 matched HEU children

Predicted means and 95% confidence intervals shown. P values derived from pairwise comparisons between predicted data for groups at each time point.

8.6.8 Mixed effects modelling with BCG - compounds where HEI had positive IGRA

Square root transformation of the data approximated a normal distribution. The same mixed effects model as shown in 8.6.3 was run and again showed no significant differences between groups.

8.6.9 Mixed effects modelling of BCG-specific levels - compounds where HEI had positive IGRA

Untransformed data was used and the mixed effects model shown in 8.6.4 was run with this dataset. The model is shown in Appendix A.4.12. The predicted marginal means and 95% confidence intervals for the groups from the model are shown in Figure 64.

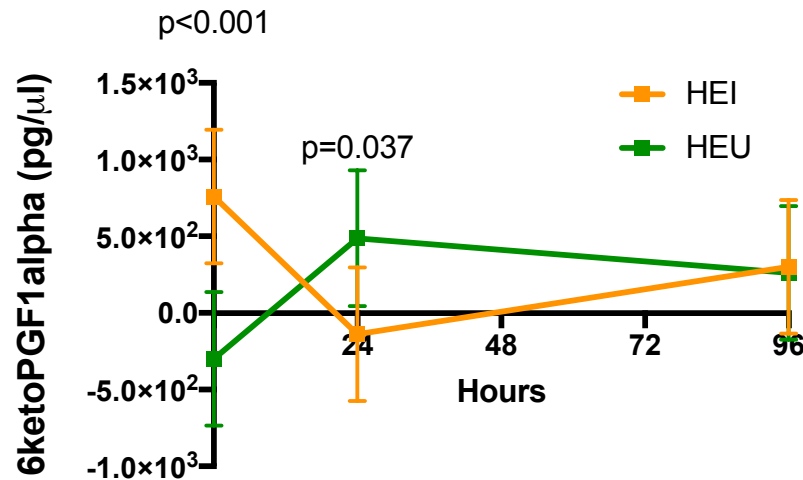


Figure 64: Modelled estimated marginal means of BCG-specific 6ketoPGF1 α from 25 HEI children with concordant IGRA and the 25 matched HEU children

Predicted means and 95% confidence intervals shown. P values derived from pairwise comparisons between predicted data for groups at each time point.

At baseline, HEI children were predicted to have higher BCG-specific levels than HEU children (HEI predicted mean 759pg/ μ l, 95% CI 324.48 $\rightarrow$ 1193; HEU -298pg/ μ l, 95% CI -733 $\rightarrow$ 137; $p < 0.001$). The pattern was again reversed at 24hours with HEU children predicted to have significantly higher levels (HEI predicted mean: -138pg/ μ l, 95% CI -573 $\rightarrow$ 296; HEU 487pg/ μ l, 95% CI 44 $\rightarrow$ 930; $p = 0.037$).

8.6.10 Subgroup analysis of compounds where LEU were recruited

With medium only (Figure 65, graphs; Figure 66 column scatterplots), LEU children have significantly higher levels of 6ketoPGF1 α at baseline than HEI children (HEI median 2,836pg/ μ l, IQR 1,724 $\rightarrow$ 3,551; LEU 4,268pg/ μ l, IQR 2,341 $\rightarrow$ 7,631; $p = 0.0269$) and at 24 hours they have significantly higher levels than both HEI and HEU children (HEI 2,549pg/ μ l, IQR 2,330 $\rightarrow$ 3,704, p vs LEU=0.0122; HEU 2,646pg/ μ l, IQR 1,457 $\rightarrow$ 3,914, p vs LEU=0.0342; LEU 4,603pg/ μ l, IQR

2,982→6,014). At baseline in the presence of BCG, LEU children have significantly higher levels than HEI children (median HEI 3,278, IQR 1,957→4,169; LEU 4,262pg/μl, IQR 2,505→6,113; p=0.021). BCG-specific levels at 24 hours were significantly lower in LEU children than HEU children (median HEU 569.7pg/μl, IQR 184.6→1,410; LEU -999.9pg/μl, IQR -1,708→-268.4; p=0.021).

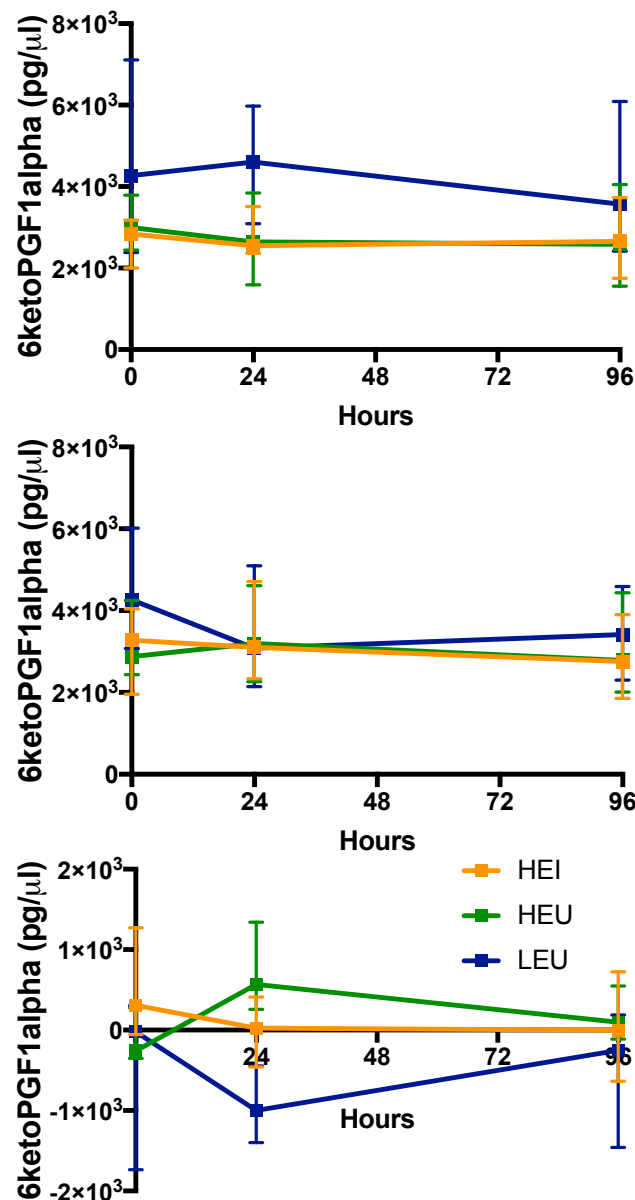


Figure 65: Kinetics of 6ketoPGF1α levels for the 36 children from compounds where a LEU child was recruited.

Top row: medium only condition; middle row: with BCG condition; bottom row: BCG-specific values (medium only values subtracted from with BCG condition). Medians and interquartile ranges.

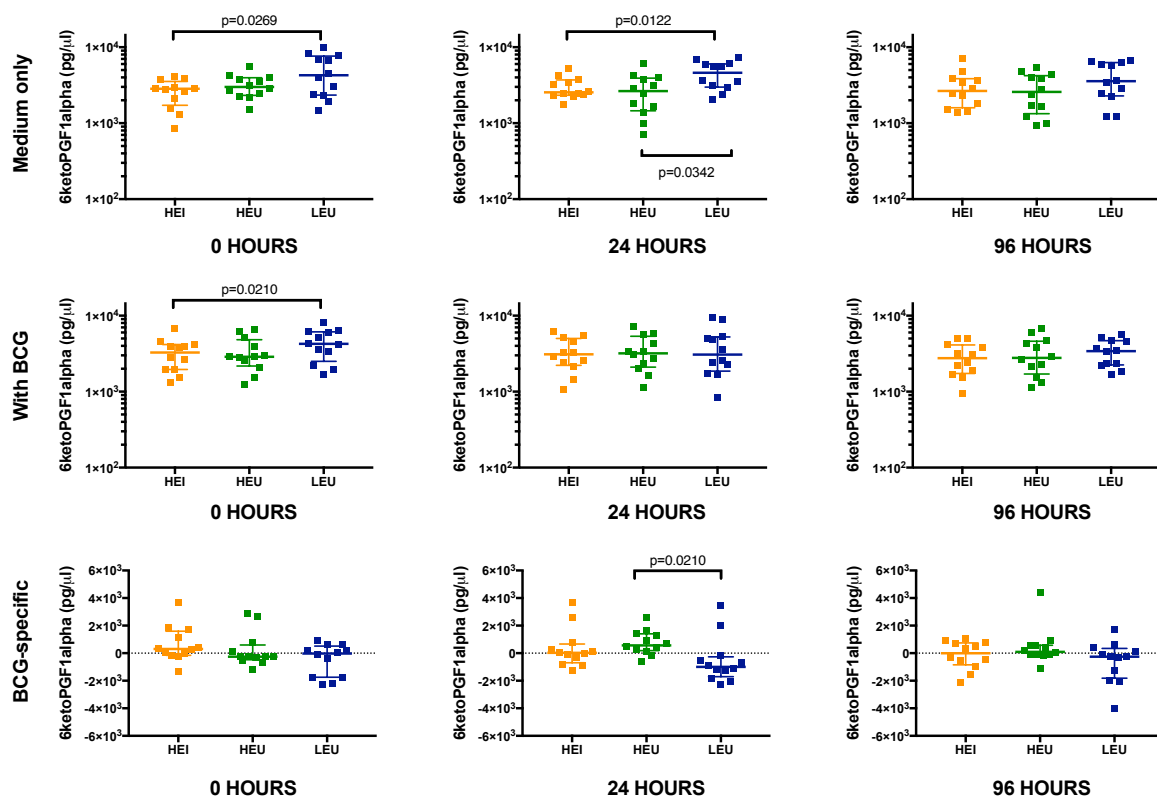


Figure 66: 6ketoPGF1alpha levels for the 36 children from compounds where a LEU child was recruited.

Top row: medium only condition; middle row: with BCG condition; bottom row: BCG-specific values (medium only values subtracted from with BCG condition). First column: Baseline; Second column: 24 hours; Third column: 96 hours. Median and interquartile ranges.

8.6.11 Mixed effects modelling of medium only condition where LEU were recruited

Square-root transformed data was used in the same model as shown in 8.6.2 with this dataset. The model is shown in Appendix 4.13. The predicted marginal means and 95% confidence intervals for the groups (medium only condition) from the model are shown in Figure 67.

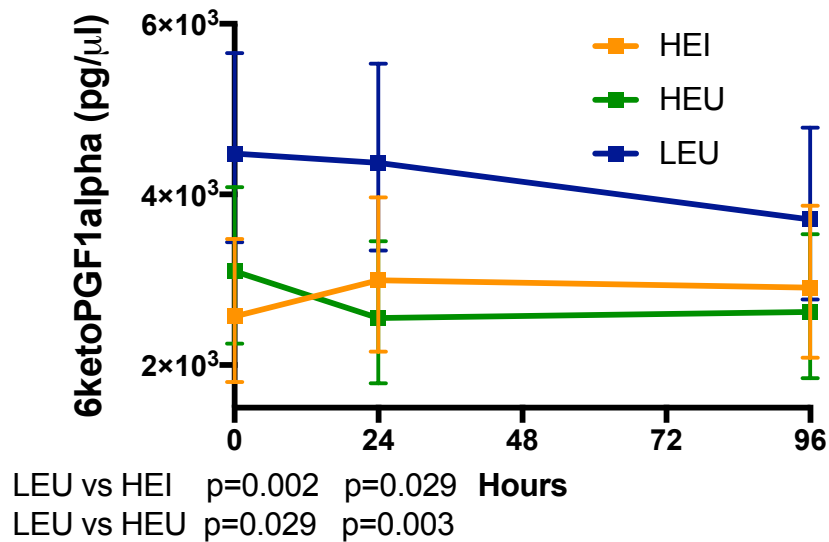


Figure 67: Modelled estimated marginal means of 6ketoPGF1 α for medium only condition from 36 children from compounds where a LEU child was recruited.

Predicted means and 95% confidence intervals shown. P values derived from pairwise comparisons between predicted data for groups at each time point.

LEU had significantly higher predicted 6ketoPGF1 α levels than either HEI or HEU children at baseline (HEI predicted mean 2571pg/ μ l, 95% CI 1801→3479, p vs LEU=0.002; HEU predicted mean 3100, 95% CI 2251→4084, p vs LEU=0.029; LEU predicted mean 4481pg/ μ l, 95% CI 3442→5657) and 24 hours (HEI predicted mean 2993 pg/ μ l, 95% CI 2156→3966, p vs LEU=0.029; HEU predicted mean 2552, 95% CI 1788→3451, p vs LEU=0.003; LEU predicted mean 4370pg/ μ l, 95% CI 3344→5532).

8.6.12 Mixed effects modelling with BCG where LEU were recruited

Square root-transformed data was suitable for modelling. The same model as shown in 8.6.3 showed no differences between groups with this dataset.

8.6.13 BCG-specific mixed effects modelling where LEU were recruited

Untransformed data was suitable for modelling. The same model as shown in 8.6.4 was run with this dataset. The model is shown in Appendix 4.14. The predicted marginal means and 95% confidence intervals for the groups from the model are shown in Figure 68.

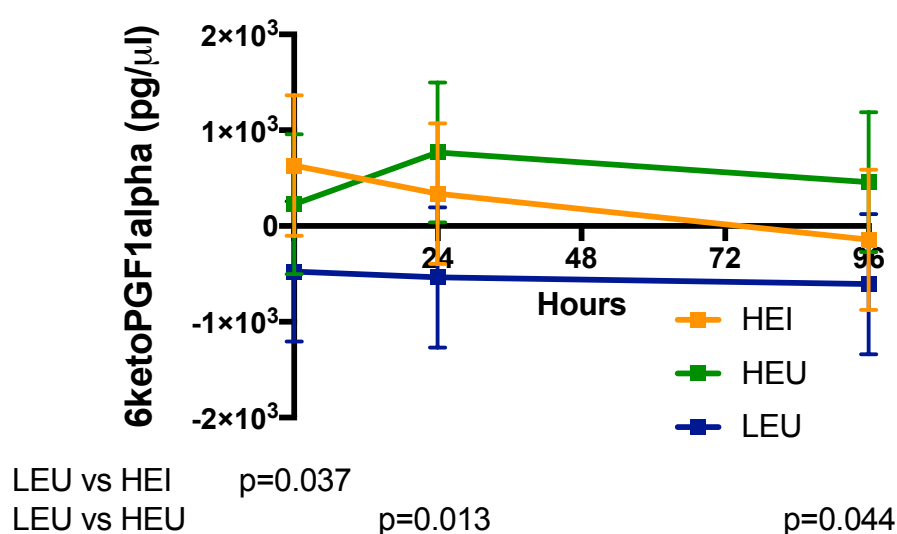


Figure 68: Modelled estimated marginal means of BCG-specific 6ketoPGF1 α from 36 children from compounds where a LEU child was recruited.

Predicted means and 95% confidence intervals shown. P values derived from pairwise comparisons between predicted data for groups at each time point.

At baseline, BCG-specific levels of 6ketoPGF1 α in LEU children were significantly lower than HEI children (HEI predicted mean 631pg/μl, 95% CI -100→ 1363, LEU predicted mean -474, 95% CI -1206→ 257, p=0.037). At 24 hours (HEU predicted mean 768, 95% CI 38→ 1498; LEU -535, 95% CI -1267→ 196, p=0.013) and 96 hours (HEU predicted mean: 459pg/μl, 95% CI -271→ 1189, LEU -605pg/μl, 95% CI -1337→ 127, p=0.044) LEU levels were predicted to be lower than those of HEU children.

8.6.14 Summary of 6ketoPGF1 α findings

Both non-parametric testing and mixed effects modelling of all pairs of HEI and HEU children showed that HEU children have higher levels at baseline in the unstimulated condition. BCG-specific levels show a dynamic difference between groups, with HEU having lower levels at baseline, and higher levels at 24 hours compared to HEI children. This metabolite is the only experimental parameter measured that did show a statistically significant difference between HEI children with positive IGRAs and those with discordant IGRAs. However, even after excluding data from these four compounds, the same pattern was broadly seen. Mixed effects modelling demonstrated that LEU children tended to have significantly higher levels of 6ketoPGF1 α when unstimulated, but lower BCG-specific levels.

8.8 Conclusion

6ketoPGF1 α showed a number of statistically significant differences of note in this study. As mentioned in 8.6, it is a stable downstream metabolite of prostacyclin, an eicosanoid with vasoactive, anti-platelet aggregation and anti-inflammatory properties.^{266–270} A role for prostacyclin was not hypothesised based on the literature at the time of study conception. The primary site of prostacyclin production has long been considered to be vascular endothelium, particularly in the lung, the site of the primary encounter with *M. tuberculosis*.²⁶⁸ This raises the question of where the prostacyclin is being produced in the whole blood assay in the absence of endothelial cells. However, macrophages and promyelocyte cell lines are known to express the enzymes required for prostacyclin biosynthesis with differential expression following activation or BCG stimulation, therefore there is a biologically plausible site for prostacyclin production within the whole blood assay.^{271–274} The role

of neutrophils in prostacyclin production is complex, with neutropaenic mice producing higher levels of prostacyclin, but neutrophils also induce prostacyclin production by endothelium.^{275,276} The enzymes required to synthesize prostacyclin are expressed in a broad range of tissues beyond vascular endothelium, including the lung and lymphoid tissue.^{277–280}

One notable finding in this study was that BCG-specific changes were detectable at baseline (in contrast to all the other eicosanoids and cytokines measured). After addition of the bacteria in a Bijou tube, 0.5ml aliquots were placed into individual 12 x 75mm tubes and then the baseline tubes were centrifuged whilst the others were placed in the incubator. Therefore 10-20 minutes would have passed before the baseline tubes were centrifuged, suggesting a surprisingly rapid effect. However, the lower BCG-specific levels in HEU children at baseline are associated with the *higher* levels of 6ketoPGF1 α in HEU children in the medium only condition rather than lower levels in the presence of BCG (see Figures 57). This suggests that the apparent BCG-specific difference at baseline is more likely to represent higher circulating levels of prostacyclin in HEU children, rather than such a rapid induction of prostacyclin production in response to BCG. In addition to these baseline differences, there were significantly higher BCG-specific levels of 6ketoPGF1 α in HEU children at 24 hours. Therefore, the combination of higher levels of 6ketoPGF1 α when unstimulated at baseline and higher BCG-specific levels at baseline and 24 hours suggest that the HEU response is broadly characterised by higher circulating levels of prostacyclin *and* increased levels in response to BCG in the first 24 hours of the assay, when compared to HEI children.

Levels of 6ketoPGF1 α also significantly differed in both of the sub-analyses carried out for this study, in contrast to the other experimental data. IGRA status of HEI children significantly affected the results – with HEI children with concordant IGRAs having higher levels in the presence of BCG at 96 hours, and higher BCG-specific levels at baseline, compared to those with discordant IGRA status. It is interesting that these 6ketoPGF1 α levels correlate better with IGRA results than IFN- γ production in the BCG-GFP-LuxFO assay and raises the possibility that 6ketoPGF1 α levels could similarly characterize subpopulations within children who have positive TST results. Of course, this hypothesis requires further validation.

LEU children had higher unstimulated levels and lower BCG-specific levels of 6ketoPGF1 α than HEU and HEI children, especially at baseline and at 24 hours. If differences in eicosanoid levels were solely a consequence of infection in the HEI children, one would expect LEU and HEU levels to be similar. In fact, LEU levels in the medium only condition were statistically significantly higher than *both* HEU and HEI children. The fact that levels differ between all three groups suggest not only that there are consequences of infection (as shown by HEI vs HEU and HEI vs LEU comparison) but there may also be consequence of exposure in the absence of infection i.e. potentially trained immunity (as shown by HEU vs LEU comparison).

Given the unexpected results, it is also important to consider whether the experimental observations could be spurious. However, levels were quantified simultaneously with the other eicosanoids using Multiple Reaction Monitoring by mass spectrometry, and none of the other analytes showed a similar pattern of results. Specifically, the 6ketoPGF1 α peak corresponds with the pure internal

standard (as does the specific fragment) and there were no neighbouring interfering peaks. The signal was clear compared to the noise, and matches the profile from the validation experiments. Given the matched study design, samples from the same compound were run on the same plate but samples were randomised within the plate. Cytokines that were measured in a different aliquot from the same original sample again did not show this profile in their results. IGRA status was determined on samples taken and analysed separately from this study, and therefore the related differences observed in 6ketoPGF1 α cannot be a consequence of the experimental procedures with the BCG-GFP-LuxFO assay.

The biological implications of these results and potential mechanisms by which prostacyclin could be involved in host mycobacterial immunity are considered further in the following chapter. Differences were also observed in levels of 12oxoLTB₄, a metabolite of LTB₄, which is implicated in host-mycobacterial interactions. HEI children had negative values of BCG-specific 12oxoLTB₄ at 24 hours (i.e. the presence of BCG is associated with *lower* levels of 12oxoLTB₄ than when unstimulated), and these levels were significantly lower than those of HEU children. The only statistically significant difference in PGE₂ levels was predicted higher levels in HEU children in the medium only condition at 96 hours. It is noteworthy that the majority of oxylipins measured did not show differences between groups, although some that were of interest such lipoxin A₄ were not detectable in our samples with this assay.

CHAPTER 9: FINAL DISCUSSION AND CONCLUSIONS

I have developed a novel whole blood autoluminescent mycobacterial assay alongside implementing a specific recruitment framework for matched groups of children exposed to tuberculosis with discordant infection status in a TB-endemic country. Mycobacterial responses of 70 children were analysed using three distinct experimental modalities – luminescence in the whole blood assay as a functional measure; cytokine production by a multiplex bead-based assay; and a novel mass spectrometry technique to quantify eicosanoid mediators.

9.1 Synthesis of results in relation to hypotheses

Hypothesis 1: HEU children are better able to restrict mycobacterial growth in an *in vitro* whole blood assay than their matched HEI peers.

In the functional luminescence assay, HEU children did not show better control of mycobacterial growth than HEI children, and therefore the alternative hypothesis was rejected.

Hypothesis 2: The cytokine response to mycobacterial infection of whole blood *in vitro* differs between HEU and HEI children. HEU children are predicted to have an early pro-inflammatory profile (with high levels of $\text{TNF}\alpha$, $\text{IL-1}\alpha$, and $\text{IL-1}\beta$, and low levels of IL-10 , $\text{IFN-}\alpha$, and $\text{IFN-}\beta$) whilst the adaptive response of HEI children will lead to late elevated levels of $\text{IFN-}\gamma$.

The only detected cytokine to show statistically significant differences between HEI and HEU children were BCG-specific interferon- γ levels at 24H and 96H, which reflects the infection status of the children. The alternative hypothesis of an early pro-inflammatory cytokine profile in HEU children was rejected.

Hypothesis 3: The eicosanoid response to mycobacterial infection of whole blood *in vitro* differs between HEU and HEI children, with HEU children having an early pro-inflammatory profile (low levels of LXA4 and high levels of PGE2 and LTB4).

At 24 hours, HEU children produced significantly higher BCG-specific levels of pro-inflammatory 12oxoLTB4 than HEI children. HEU children had higher levels of the broadly pro-inflammatory PGE2 in the medium only condition at 96 hours than HEI children but there were no differences at earlier time points or in response to BCG. LXA4 levels were not detectable in our samples with the mass spectrometry assay used. Although not hypothesized, HEU children had higher levels of a metabolite of the anti-inflammatory eicosanoid prostacyclin, than HEI children at baseline and at 24 hours. Therefore, there is conflicting evidence regarding this hypothesis and the implications of the prostacyclin results are discussed further below.

Hypothesis 4: The responses of a control group of **Lower Exposure Uninfected (LEU)** children reflect those of the general population in a high-TB-endemicity setting and encompass *both* HEU and HEI responses. This would suggest that any differences between HEU and HEI children in hypotheses

1→3 are not solely a consequence of infection in the HEI child but may represent intrinsic differences in the immune response of HEU individuals.

There were two parameters where LEU responses did not encompass both HEI and HEU responses. LEU children had significantly higher luminescence levels from 24H onwards compared to HEI children. This was in parallel to the higher luminescence levels at 96H in HEU children compared to HEI children ($p=0.059$). This would support the observed differences in luminescence being a consequence of infection in the HEI children. LEU children also had higher unstimulated levels and lower BCG-specific levels of 6ketoPGF1 α than HEU and HEI children, especially at baseline and 24 hours. The fact that all three groups HEI, HEU, and LEU had different responses suggests that not only were there differences associated with infection status, but also degree of exposure to *M. tuberculosis*. Hypothesis 4 has proven difficult to test in a definitive manner, in part complicated by the small number of compounds in which there were eligible LEU children.

9.2 Relevance of findings to the field

This study is the first to compare functional, cytokine, and eicosanoid responses to mycobacteria of children with discordant infection status but matched exposure to the same smear-positive adult tuberculosis patient in a TB-endemic country. In particular, the literature on eicosanoids and human tuberculosis has primarily been restricted to adults, genetic associations, and tuberculosis disease – including severe presentations such as meningitis and patients not responding to MDR-TB medical treatment.^{116,133,142,146,151,281,282} Therefore, this study represents to my

knowledge the first investigation of eicosanoid biology in paediatric tuberculosis infection. The intriguing 6ketoPGF1 α results also represent a novel contribution to the field, with limited prior publications on prostacyclin and tuberculosis. A PubMed search identifies two Ukrainian papers published in Russian in the 1990s, one involving patients, and the other guinea pigs.^{283,284} Levels of thromboxane and 6ketoPGF1 α were measured in venous samples from 114 patients with tuberculosis and compared to 50 healthy controls.²⁸³ Patients with tuberculosis had higher levels of 6ketoPGF1 α (16.31 $\pm$ 0.47pg/ml) than healthy controls (13.3 $\pm$ 0.31, $p < 0.01$). Levels of 6ketoPGF1 α also appeared to be higher in patients with more severe forms of TB. The only subsequent article regarding prostacyclin and tuberculosis was published in April 2016 after recruitment to this study had completed.¹⁴⁶ In this study, human granulomas in pneumonectomy samples from patients with MDR-TB were analysed in cross-section by proteomic techniques and complemented by HPLC tandem mass spectrometry. A pro-inflammatory milieu was shown in the centre of caseous and necrotic granulomas with high levels of TNF α and the LTA4H enzyme that synthesizes LTB₄. One piece of evidence supporting a contrasting anti-inflammatory profile in the rim of granulomas, was higher levels of 6ketoPGF1 α detected in the inner and outer cellular layers compared to the caseum.¹⁴⁶

The study reported in this thesis contributes several additional points of interest to this existing literature. Firstly, identifying distinctive 6ketoPGF1 α responses with a different study design and experimental methodology, in a different tissue, from a population that differs from that of Marakalala *et al*¹⁴⁶ with regard to age, and geographical location strengthens the evidence that prostacyclin is an immune

mediator of generalised relevance to human mycobacterial immunity. Secondly, the findings in a study of protection against mycobacterial infection, with time course data suggests that not only is prostacyclin implicated in granuloma formation in uncontrolled disease, but also in early innate responses that may be involved in effective early clearance of mycobacteria. This is a biologically plausible concept as the site of the primary encounter with *M tuberculosis* is the lung, where prostacyclin is known to be produced by vascular endothelium, pulmonary epithelium, and where prostacyclin analogues are used to treat pulmonary hypertension.^{268,269,278,285}

There are several mechanisms by which prostacyclin might exert a protective effect in human immunity against mycobacteria. There is a large and consistent literature base from cardiovascular, cancer, and dentistry research showing the angiogenic properties of prostacyclin and its analogues, mediated by prostacyclin receptor, peroxisome proliferator-activated receptors, and vascular endothelial growth factor release.^{286–300} Angiogenesis has been recently implicated in the pathogenesis of tuberculosis with mycobacteria inducing angiogenesis in granulomas associated with vascular endothelial growth factor.^{301,302} On the basis of this, the pro-angiogenesis effect of prostacyclin would appear to favour mycobacteria, although the kinetics of the interaction may be crucial in determining effective early clearance versus containment within the granuloma. Prostacyclin appears to have tumour-preventive properties related to its angiogenic properties.^{303–306} Its relevance in a paediatric population has been shown with samples from children with pulmonary hypertension treated with a prostacyclin analogue demonstrating increased angiogenesis *in vitro* through increased numbers of endothelial colony forming cells.³⁰⁷

A further potential mechanism relates to modulatory effects of prostacyclin upon cell death. Prostacyclin inhibits cigarette-smoke-induced endothelial apoptosis, decreases pro-apoptotic Bax/Bcl-2, and is also expressed in peripheral lymphoid tissue where it inhibits T-cell proliferation and delays spontaneous apoptosis.^{280,308,309} In parallel, manipulation of cell death modality by pathogenic mycobacteria is thought to be an immune evasion mechanism, although there are conflicting reports in the literature.^{148,236,310–313} Macrophage apoptosis following MTB infection is associated with better mycobacterial control than necrosis.^{137,139,314} Apoptosis of MTB-infected macrophages is required for the early initiation of T cell immunity.³¹⁴ In comparison to this wealth of *in vitro* and animal model data, patient studies have varied in methodology and results.^{314–318} Related to the prostacyclin effect upon cigarette-smoke-induced endothelial apoptosis, smokers are known to be at higher risk of TB, and unengulfed apoptotic MTB-infected macrophages can undergo secondary necrosis with increased mycobacterial growth.³¹⁹

Thirdly, it could exert a protective effect through its direct anti-inflammatory properties. Prostacyclin agonists decrease expression of IL-1 β , macrophage inflammatory protein-1 α , and increased IL-10 expression in an animal model of heart failure, together with decreased neutrophil and macrophage infiltration.³⁰⁹ A recent Nature Medicine paper demonstrated that prostacyclin promotes axonal remodelling and motor recovery in a mouse model of central nervous system inflammation.²⁹⁴

Finally, prostacyclin can modulate function of group 2 innate lymphoid cells, T-cells, and B-cells, and therefore its influence may be as part of regulation of the innate and adaptive immune systems.^{279,285,320}

Regarding the functional arm of this study, the luminescence results did not replicate the pilot data as discussed in section 6.6. Therefore, the overall pattern was consistent with the literature of either no detectable difference between groups or greater mycobacterial control by latently infected individuals.^{72,224,321} Similarly, although there seems to be a strong genetic association between $\text{TNF}\alpha$ production and TST reactivity, the cytokine data reported here are consistent with other functional studies where no differences has been shown between latently infected and uninfected participants.^{49–51,53,56,60,78} No differences were observed with $\text{IL}1\alpha$, $\text{IL}-1\beta$ or $\text{IL}-10$ and differences in $\text{IFN}\gamma$ levels at 24H and 96H reflected infection status. Therefore, the luminescence and cytokine multiplex arms of this study add to the body of literature highlighting that a functional correlate of protection against infection remains elusive.

A summary schematic diagram of key factors in paediatric immunity to mycobacterial infection is shown in Figure 69. In bold are mediators evaluated in this study, including prostacyclin and its potential mechanisms. Although hypothesized to be relevant, $\text{LXA}4$ was not detectable in our samples with this mass spectrometry assay. The figure also includes factors known to be important such as genetic loci associated with TST reactivity and the role of CD4 T-cells as shown by the impact of HIV infection. What constitutes an effective early response from cellular players such as antigen-presenting cells, NK cells, neutrophils, $\gamma\delta$ T-cells and macrophages remains unclear.⁷² It is possible that an effective immune response is mediated by a different or additional component of the adaptive immune system than simply T-cell mediated cellular immunity, and the role of B cells and antibody has seen renewed interest.^{322,323} Epigenetic factors and the role of trained innate immunity remains as

yet unexplored in paediatric immunity to *M tuberculosis* infection. The role of vitamin D in LTBI is not consistent across settings and there is limited paediatric data.^{80–83,258} The TNF α data reported here are consistent with other functional studies where no differences has been shown between latently infected and uninfected participants, despite the genetic associations.^{49–51,53,56,60,78}

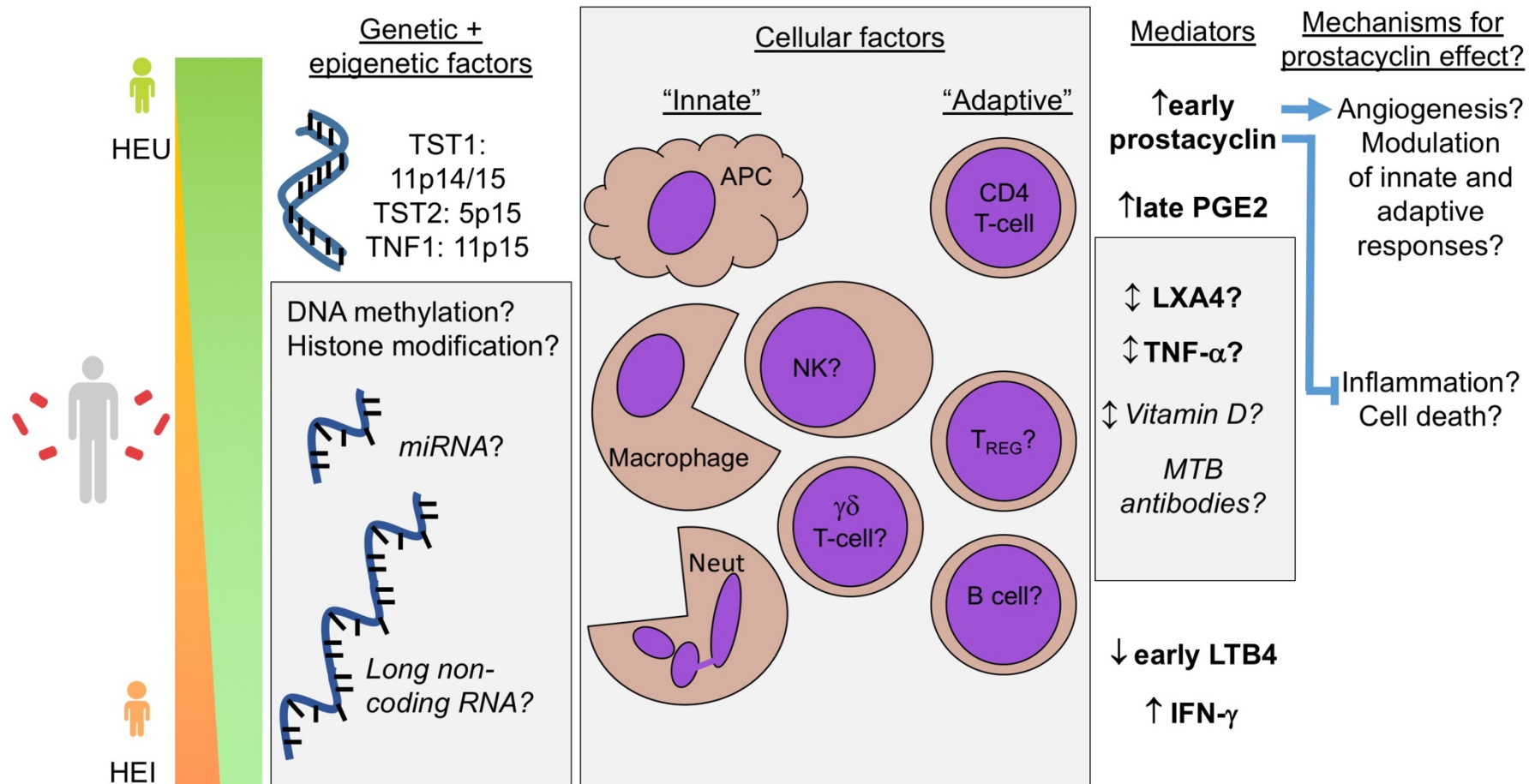


Figure 69: Schematic diagram of current understanding of immunological factors underlying paediatric *M tuberculosis* infection.

The bar on the left represents risk of going onto develop tuberculosis disease. Factors in **bold** represent mediators evaluated in this thesis. Factors in *italics* represent hypotheses that could be tested with stored samples from this thesis. Factors marked with "?" represent unknown contributions. Where factors are grouped together within a grey box, their contribution along the spectrum from HEU to HEI is not known. Factors without "?" in normal font are known to be involved from the published literature. Unstim=unstimulated/medium only condition. BCG-spec=BCG-specific, APC=Antigen-Presenting Cell

9.3 Strengths of the study

The main strength of the study is the application of novel methodologies in a study population of carefully matched children with discordant infection status. Although, the household contact study is a well-established platform for experimental studies, most have not prospectively recruited participants matched on key epidemiological factors such as exposure to tuberculosis.

A substantial methodological contribution was made through development of the BCG-GFP-LuxFO assay. This enabled characterisation of kinetics of human-mycobacterial interactions with minimal blood volumes in a manner that was not previously possible. There is potential for the starting inoculum to be reduced together with automated luminescence reading facilitating future applications in vaccine or drug trials.

Study recruitment was effective and efficient with children from all consenting eligible compounds during the fieldwork period taking part in the study. This entailed considerable logistical and laboratory coordination to ensure that mycobacteria were in logarithmic growth phase on the day of phlebotomy for 96 hour assays using fresh whole blood.

The application of mixed effects modelling given the matched study design and longitudinal data enabled more sophisticated interrogation of the data in an age-adjusted manner than is possible with conventional non-parametric statistical testing.

9.4 Limitations

Although recruitment was effective during the study period, nevertheless we did not recruit the sample size to have 90% power to detect a difference in luminescence between HEI and HEU children. However, given that the pilot data on which the sample size calculation was based used a different laboratory method and a different study design in a different context, recruiting to the sample size target is unlikely to have dramatically changed the results. Specifically, there was a lower than expected number of compounds where there was an eligible LEU child which constrained how much could be interpreted from that study group. This study had a defined study population of children between the age of 5 and 15 years old. This was appropriate for the research question, but the exclusion of children under 5 due to isoniazid preventive therapy precluded comparison of responses between distinct age groups. As discussed in section 1.2.6, the immunophenotype of TST response on which children were recruited to this study could encompass a range of host-pathogen outcomes, and although there is an increased relative risk of disease with a positive TST, most children with *M tuberculosis* infection remain asymptomatic. There is therefore the possibility that many in the HEI group also represent effective control of the pathogen, rendering biologically relevant differences difficult to identify. Adjustment of statistical significance thresholds for multiple testing has not been applied. However, the only mediator which was not hypothesized *a priori* to be of interest was prostacyclin, which exhibited the strongest statistical significance.

The hypotheses regarding luminescence and cytokines were not confirmed, and similar patterns were observed to other functional studies. The fact the same samples yielded the novel 6ketoPGF1 α results lends scientific plausibility that the

differences observed here are of true biological relevance, and that this study is not simply an outlier.^{53,56,61,124} Nevertheless, this study was conducted in a single setting in a West African context, and therefore its applicability to other populations is unknown without further validation.

In terms of experimental methodology, the assay was used based upon the vaccine strain BCG, and not pathogenic *M tuberculosis*. This would have been ideal, although it is noteworthy that other human whole blood assays have not demonstrated a difference between pathogenic and non-pathogenic strains of mycobacteria.^{72,212,237} The limitations of the BCG-GFP-LuxFO assay have been discussed in sections 4.11 and 6.6. The sample type used is whole blood and is therefore not representative of the lung, where the initial encounter with *M tuberculosis* occurs. The inoculum used was relatively high and although luminescence correlates with CFU, other factors can influence the metabolic read-out of luminescence beyond the number of bacteria alone such as precise location of the bacteria and nutrient availability. The inclusion of GFP means the assay has the potential to incorporate cellular applications to delineate contributions and death modalities of individual cell populations within whole blood. This was not feasible in study reported here, although similar approaches with GFP-labelled mycobacteria in whole blood have shown that neutrophils and macrophages are the predominant infected cell type.^{72,237} The lack of IGRA characterisation of the HEU and LEU children is another limitation.

DNA sampling and analysis was not included in the ethics approval and therefore it is not possible to validate the genetic findings from the South African, Ugandan, and

French cohorts related to TST negativity.^{47–50,53–55} Furthermore, the sample size is likely to be too small for such an approach.

9.5 Next steps

This study reports an association between 6ketoPGF1 α levels and infection status in a BCG whole blood assay in *M tuberculosis*-exposed children. There are a number of approaches to further investigate the underlying biology and potential mechanisms of action of prostacyclin in human mycobacterial immunity. Validation of the findings in a second distinct study population would add greater confidence of its relevance. Given the differences were observed at baseline in the medium only condition, targeted interrogation of published gene expression datasets comparing latently infected and uninfected patients could provide supporting evidence.¹⁷⁷ Similarly, analysis of gene expression from cells infected with mycobacteria *in vitro* could provide further corroboration.^{58,324} Experimental manipulation to confirm functional relevance of the identified association would then be necessary. This could be recapitulated *in vitro* with whole blood and addition of prostacyclin analogues and antagonists. Prostacyclin is known to act via specific prostacyclin receptors, as well as peroxisome proliferator-activated receptors, and serotonin receptors so the site of action could also be explored.^{286,297,303,325} In an attempt to more finely delineate the source and target of prostacyclin, primary or immortalised cell lines could be infected with mycobacteria, again with pharmacological manipulation, genetic knockouts, or use of small interfering RNAs. Evaluation of any differences between infection with pathogenic and avirulent mycobacteria may elucidate bacterial mechanisms to evade the host inflammatory response. *In vivo* experiments in a genetically tractable model of mycobacterial disease such as zebrafish or mice would add strong evidence of functional relevance at whole organism level. Use of cell death modality

approaches including flow cytometry and advanced microscopy and quantification of mediators such as vascular endothelial growth factor would be needed to elucidate the precise mechanism of action of prostacyclin of those proposed in section 9.2.

As highlighted in Figure 69, there are additional research questions that can be addressed using stored samples from this study. Whole blood RNA samples are available and the Royal Society of Tropical Medicine & Hygiene has already awarded me a small grant (GR000763) towards RNA sequencing based on data presented in this thesis. In addition to testing whether mRNA expression in whole blood mirrors the differences seen with prostacyclin, this also offers the opportunity to look for potential epigenetic differences mediated by microRNAs and long non-coding RNA that may reflect trained innate immunity.⁹⁴ Furthermore, such an untargeted and comprehensive analytical approach may be able to generate novel hypotheses related to paediatric control of mycobacterial infection.³²⁶ Stored serum samples are also available which could be used to investigate the association between vitamin D and TST status in this matched Gambian paediatric study.^{80–83,258} The serum could also be used to investigate differences in functional and structural properties of antibodies in tuberculosis, which have had renewed interest recently.^{322,323}

If prostacyclin is indeed a relevant host mediator in control of mycobacteria, the question becomes how clinically or immunologically tractable it is. It is currently used therapeutically for pulmonary hypertension but primarily as a parenteral drug with accompanying complications.^{268,269} Given the need for prolonged treatment with multiple anti-mycobacterial drugs, there is great interest in the use of adjunctive anti-

inflammatory agents.^{116,133,327–330} It is already standard practice to treat clinical tuberculous meningitis with steroids, and indeed those who derive the survival benefit from this seem to be those with the most hyperinflammatory state.¹¹⁶

Pharmacological inhibition of 5-lipoxygenase, which catalyses lipoxinA4 and leukotrieneB4, prevents acute mortality of Mtb-infected mice,¹³³ as can ibuprofen.³³¹

The role of eicosanoids in inducing durable protective immune responses is as yet relatively unexplored in infectious diseases, although it is a focus of interest in cancer immunotherapy.^{332,333}

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- 331 Vilaplana C, Marzo E, Tapia G, Diaz J, Garcia V, Cardona P-J. Ibuprofen therapy resulted in significantly decreased tissue bacillary loads and increased survival in a new murine experimental model of active tuberculosis. *J Infect Dis* 2013; **208**: 199–202.
- 332 Hou W, Sampath P, Rojas JJ, Thorne SH. Oncolytic Virus-Mediated Targeting of PGE 2 in the Tumor Alters the Immune Status and Sensitizes Established and Resistant Tumors to Immunotherapy. *Cancer Cell* 2016; **30**: 108–19.
- 333 Pollara JJ, Spesock AH, Pickup DJ, Laster SM, Petty ITD. Production of prostaglandin E2 in response to infection with modified vaccinia Ankara virus. *Virology* 2012; **428**: 146–55.

APPENDIX 1 – STUDY APPROVALS

Scientific Coordinating Committee

MRC Unit: The Gambia, Fajara
PO Box 273 Banjul, The Gambia
West Africa

Switchboard (+220) 4495442/6 Ext 2308
Fax (+220) 4495919/4496513

E-mail: scc@mrc.gm

Intranet: <http://mrcportal/Committees/SCC/SitePages/Home.aspx>

Webpage: <https://mrcportal.mrc.gm/Committees/SCC/SitePages/Home.aspx>



13 November 2014

Professor Beate Kampmann
Theme Leader, Vaccinology
MRC Unit, The Gambia
Fajara

Dear Professor Kampmann

SCC 1405v2, Protection against mycobacterium tuberculosis infection: Learning from exposed but uninfected children

Thank you for submitting your revised proposal and response letter both dated 12 November 2014 addressing the issues raised by the SCC at its meeting held on 4 November 2014.

I am happy to approve your proposal which will be forwarded to the Ethics Committee for further consideration at its meeting on 28 November 2014.

With best wishes

Yours sincerely

A handwritten signature in blue ink, appearing to read 'A Roca', written over a light blue rectangular background.

Dr Anna Roca
Acting Chair, Scientific Coordinating Committee

Cc: Dr Robindra Basu Roy.

Documents submitted for review:-

- SCC application form, version 2.0 – 12 November 2014
- Response letter – 12 November 2014
- Informed Consent Document, version 1.0 – 19 October 2014
- CV: Robindra Basu Roy

23 January 2015

Professor Beate Kampmann
Theme Leader, Vaccinology
MRC Unit, The Gambia
Fajara

Dear Professor Kampmann

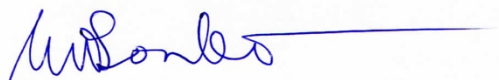
SCC 1405v2 Protection against mycobacterium tuberculosis infection: Learning from exposed but uninfected children

Thank you for submitting your response letter dated 19 January 2015 addressing the issues raised by the Gambia Government/MRC Joint Ethics Committee at its meeting held on the 28 November 2014.

I am happy to give Chair's approval. The project has now received full Ethics Committee approval and may proceed.

With best wishes

Yours sincerely



Mr Malamin Sonko
Chairman, Gambia Government/MRC Joint Ethics Committee

Cc: Dr Robindra Basu Roy

Documents submitted for review:-

- SCC approval letter – 13 November 2014
- SCC reply letter – 6 November 2014
- SCC application form, version 2.0 – 12 November 2014
- Response letter – 12 November 2014
- Informed Consent Document, version 2.0 – 19 January 2015
- CV: Robindra Basu Roy

The Gambia Government/MRC Joint Ethics Committee:

Mr Malamin Sonko, *Chairman*
Professor Ousman Nyan, *Scientific Advisor*
Ms Naffie Jobe, *Secretary*
Mrs Tulai Jawara-Ceesay
Dr Ahmadou Lamin Samateh
Dr Roddie Cole

Prof. Umberto D'Alessandro
Dr Stephen Howie
Dr Kalifa Bojang
Dr Ramatoulie Njie
Dr Momodou L. Waggeh
Dr Siga Fatima Jagne

Scientific Coordinating Committee

**MRC Unit: The Gambia, Fajara
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Webpage: <https://mrcportal.mrc.gm/Committees/SCC/SitePages/Home.aspx>



9 July 2015

Dr Robin Basu Roy
Academic Department of Paediatrics
Imperial college London

Dear Dr Roy

L2015.39, Re SCC 1405: Request for amendment

Thank you for submitting your letter dated 23 June 2015 for consideration by the SCC at its meeting held on 6 July 2015.

We are pleased to approve your request which will be forwarded to the Ethics Committee for further consideration at its meeting on 31 July 2015.

With best wishes

Yours sincerely

A handwritten signature in blue ink, appearing to read 'A Roca', written over a light blue horizontal line.

Dr Anna Roca
Acting Chair, Scientific Coordinating Committee

Documents submitted for review:-

- Request letter – 23 June 2015
- Informed Consent Document, version 2.0 – 23 June 2015
- SCC application form for SCC 1405

17 August 2015

Dr Robin Basu Roy
Academic Department of Paediatrics
Imperial college London

Dear Dr Roy

L2015.39, Re SCC 1405: Request for amendment

Thank you for submitting your letter dated 23 June 2015 for consideration by the Gambia Government/MRC Joint Ethics Committee at its meeting held on 31 July 2015.

Our Committee is happy to approve your proposed protocol amendment.

With best wishes

Yours sincerely



Mr Malamin Sonko
Chairman, Gambia Government/MRC Joint Ethics Committee

Documents submitted for review:-

- SCC approval letter – 9 July 2015
- Request letter – 23 June 2015
- Informed Consent Document, version 2.0 – 23 June 2015
- SCC application form for SCC 1405

The Gambia Government/MRC Joint Ethics Committee:

Mr Malamin Sonko, Chairman
Professor Ousman Nyan, Scientific Advisor
Ms Naffie Jobe, Secretary
Dr Roddie Cole
Dr Ahmadou Lamin Samateh
Mrs Tulai Jawara Ceesay

Prof. Umberto D'Alessandro
Dr Momodou L. Waggeh
Dr Kalifa Bojang
Dr Ramatoulie Njie
Dr Jane Achan
Dr Siga Fatima Jagne



**Imperial College Healthcare Tissue Bank
Department of Surgery and Cancer,
Hammersmith Hospital,
Du Cane Road,
London W12 0HS
Tel: +44 7711 701382
Email: gerry.thomas@imperial.ac.uk**

**ICHTB HTA licence: 12275
REC Wales approval: 12/WA/0196**

Professor Beate Kampmann
Academic Department of Paediatrics
2nd Floor Wright-Fleming Building, Room 220
St. Mary's Campus, Imperial College London
Norfolk Place
London W2 1NY
UK

15th November 2013

Dear Beate,

Re: Tissue Bank application number Project R13071; Protection from *Mycobacterium tuberculosis* infection.

I am pleased to confirm that the Tissue Management Committee of the ICHTB has approved your amended application for access.

In order to satisfy HTA tracking requirements, would you please ensure the samples you use are logged into your sub-collection on the online database, and then issued to your project on the same system as they are tested or used, along with any publications you produce, that should contain the acknowledgment wording as per the signed MTA.

Yours sincerely,

Professor Gerry Thomas
DI, Hammersmith HTA Research Licence.

PARTICIPANT INFORMATION SHEET

Version 3.1 Date 23rd June 2015

PAMI - Protection against *Mycobacterium tuberculosis* infection: Learning from TB-exposed children

SCC:	1405	Protocol:	
------	------	-----------	--

Sponsor: Medical Research Council / Department for International Development, UK

What is informed consent?

You are invited to let your children take part in a research study.

Before you decide, you need to understand why the research study is being done and what it will involve. Please take time to read the following information or get the information explained to you in your language. Listen carefully and feel free to ask if there is anything that is not clear or you do not understand. You may also wish to consult your spouse, family members, friends or others before deciding to let your child take part in the study.

Participating in a research study is not the same as getting regular medical care. The purpose of regular medical care is to improve one's health. The purpose of a research study is to gather information that may be useful in future for the whole population. It is your choice to take part and you can stop any time.

If you decide to allow your children to join the study, you will need to sign or put a thumbprint on a consent form saying you agree for your child to be in the study. You will receive a copy of this.

Why is this study being done?

Tuberculosis (TB) is still a common problem in The Gambia. Your children have already taken part in the MRC Reach4Kids study because there was an adult in the home with TB. We screened children in your household for TB by doing a skin test and a questionnaire. Any child who had signs of TB has already been investigated and possibly treated. Now, we want to come back to the other children who were screened but found well. We only wish to study one child in your compound whose skin test showed that they are infected with the bacteria that can cause TB although they were well, and two children that have no sign of infection. This study is being done to try to understand why one of the children was infected by the bacteria that can cause TB, whilst the others were not. This could help us to design a better vaccine against TB in the future. The overall results of the study will be made available to your community, but not any personal information.

What does this study involve?

The children who had a negative result with the initial skin test may need a repeat skin test 3 months later. If it has become positive, your child will be referred to the MRC Childhood TB Clinic.

If you agree they can take part in the study, these three children will also **each** need one blood sample collected (approximately 1 teaspoon in children under 5 years old and

two teaspoons in older children). After this and the repeat skin test, your children will continue to be followed up as part of the original Reach4Kids study.

The MRC Gambia and National Leprosy and Tuberculosis Program (NLTP) work together routinely and share information confidentially between them for the benefit of patients. During your TB care, the NLTP will have taken some blood tests including one for HIV. As part of this study, the results of your tests from NLTP will be shared with specific MRC Gambia health professionals, as they may have implications for your children. If your children would benefit from additional tests, they will be seen in the Reach4Kids clinic at MRC Gambia.

In case the investigator discovers your child is sick and decides that he/she cannot participate in the study because of that, he/she will receive immediate care at the study site and then be referred to the appropriate health facility.

If the research study needs to be stopped, you will be informed and your child will have the normal medical care.

What will happen to the samples taken in this study?

The blood samples will be initially analysed soon after they have been collected. Some will be stored for analysis later, either at the MRC or at collaborating laboratories outside of The Gambia.

What harm or discomfort can you expect in the study?

Your children will need to have a blood test, which can be associated with some discomfort. One of your children might need the skin test that was done as part of Reach4Kids to be repeated again.

What benefits can you expect in the study?

There is no direct benefit from participating in the study. However, if your child has changed from a negative skin test to a positive one, we will investigate your child for TB and organise the appropriate treatment, if that is necessary. The blood samples from both of your children might help us to learn how to make a better vaccine or treatment for tuberculosis.

Will you be compensated for your child's/ward's participation in the study?

You will not get paid for participation of your child in the study, but you will get either transport by MRC or get the costs for the transport reimbursed.

Are there other products or treatment?

No

What happens if you refuse to participate in the study or change your mind later?

You are free to let your child participate or not in the study and you have the right to stop his/her participating at anytime without giving a reason. This will not affect the medical care that your child would normally receive.

In case you decide to withdraw your child's participation during the study we will not work on your child's samples without your permission, but any information already generated from the samples will be kept. The study doctor may also ask for tests for your child's wellbeing.

Should any new information become available during the study that may affect your child's participation, you will be informed as soon as possible.

What compensation will be available if your child is injured during the study?

We will be responsible to provide for treatment caused by procedures of the research study.

If medical treatment is required as an emergency, please refer to your health centre or clinic and contact the field worker, Muhamed Sissoko, on 3707799 or contact Dr Robin Basu Roy on Ext 5038.

How your child's information will be kept and who will be allowed to see it?

All information that is collected about you and your child in the course of the study will be kept strictly confidential. You and your child's personal information will only be available to the study team members and might be seen by some rightful persons from the Ethics Committee, Government authorities and sponsor.

Who should you contact if you have questions?

If you have any queries or worries you can contact the field worker, Muhamed Sissoko, on 3707799, Professor Beate Kampmann, phone number 4495 442 Ext 3001 or Dr. Robin Basu Roy, Ext 5038; and you can always call the personal numbers of the study staff given to you.

If you have any concerns you can also contact staff at your health centre or clinic. Please feel free to ask any question you might have about the research study.

Who has reviewed this study?

This study has been reviewed and approved by a panel of scientists at the Medical Research Council and the Gambia Government/MRC Joint Ethics Committee, which consists of scientists and lay persons to protect your rights and wellbeing.

CONSENT / ASSENT FORM

Participant's Name _____

Participant's Identification Number: |_|_|_|_|_|_|_|_|_|_|_|_|_|_|_|_|

OR

(Printed name of parent)

(Printed name of guardian)

☐ I have read the written information **OR**

☐ I have had the information explained to me by study personnel in a language that I understand
and I

- confirm that my choice to let my child participate is entirely voluntarily,
- confirm that I have had the opportunity to ask questions about this study and I am satisfied with the answers and explanations that have been provided,
- understand that I grant access to data about my child to authorised persons described in the information sheet,
- have received time to consider to let my child take part in this study
- agree to allow my child take part in this study.

Tick as appropriate

I agree to further research on my child's samples as described in the information sheet

Yes ☐ No ☐

Participant's
parent/guardian
signature/thumbprint*

Date (dd/mmm/yyyy)Time (24hr)

Printed Name of Person
obtaining consent

I attest that I have explained the study information accurately in

_____ to, and was understood to the best of my knowledge by, the participant/parent/guardian and that he/she has freely given consent to participate *in the presence of the above named impartial witness (where applicable).

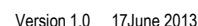
Signature of Person
obtaining consent

Date (dd/mmm/yyyy)Time (24hr)

**Only required if the participant is unable to read or write.*

A copy of this informed consent document has been provided for the participant.

A.3.1 Symptom screening questionnaire from MRC Program Grant



A. Background information

Subject Visit Status: 1 (1=Present, 2=Refused Participation, 3=Dead, 4=T/A, 5=Moved, 6=Others, 7=Clinic Recruit)

Ethnic group | ____ | (1=Mandinka, 2=Wolof, 3=Fula, 4= Serahula, 5=Jola, 6=Aku, 7=Serere, 8= Manjago, 9=other, specify -----)

A.3.3 Eligible Families Form

PAMI-Form 2: Eligible Families Form

Index Case_ID: [] **Screening Visit Date.** __/__/____ (dd/mm/yyyy)

If a family has met all of the inclusion and exclusion criteria on PAMI-Form 1 "Field Screening Criteria", use this form to record details of **ALL** children in an eligible family **EXCEPT** those with a TST of 6-9mm.

[illegible]

Field worker Name:

Date__ / __ / ____ (dd/mm/yyyy)

Field worker Signature:

Inclusion/Exclusion Criteria

Version 2.0

A.3.4 Record of blood samples taken

PAMI-Form 3: Phlebotomy Form

R4K ID: R _ _ _ / _ _ _ _	Clinic Visit Date. __/__/____ (dd/mm/yyyy)
Patients Initials: _ _ Age in years _ _ Age in months (if under 5 years) _ _	

Summary

When PAMI recruits are coming to clinic, there will be three children who already have study IDs from the Childhood TB Program Grant (SCC 1289).

There is a different bleeding schedule for children in PAMI. Only take the bloods samples below. The total volume is 10mls.

Each of the three children will need:

1. 1 x 10ml Lithium Heparin tube (green top – 7mls=filled to above word UK) → Dr. Robin/Basil
2. 1 x FBC (0.5ml) → Clinical Laboratories on paper form filled out by Dr. Robin
3. 1ml of blood into PAXGene reagent tube → Childhood TB Laboratory Team (Hadi)
4. Any leftover blood can go into a serum tube → Childhood TB Laboratory Team (Hadi)

Samples going to Childhood TB Laboratory Team should be accompanied by a laboratory check sheet as normal WITH PAMI CLEARLY WRITTEN ON THE TOP OF THE SHEET.

	Yes	No
1 X 10mL heparin tubes	☐	☐
1 X FBC	☐	☐
PAX gene reagent tube	☐	☐
1 X plain Serum tube	☐	☐

Please ensure all tubes are labeled with the date and study ID.

Clinic Staff Name:

Date __/__/____ (dd/mm/yyyy)

Clinic Staff Signature:

A.3.5 Characteristics of included families

PAMI-Form 4: Characteristics of included families

INDEX CASE ID: - -	Screening Visit Date: _ / _ / _ _ _ _
HIV STATUS INDEX CASE: (0=negative, 1=positive)	PAMI Date: _ / _ / _ _ _ _
	Info source: (TBCC=1, LTI=2)

HIGH EXPOSURE TST POSITIVE CHILD:

R4K ID: R | | | / | | | | Date of birth: _ / _ / _ _ _ _ (dd/mm/yyyy)
Sex: | | | (0=female, 1=male)

Proximity with the index case Where does this person sleep in relation to the index case? (This is before they found out that the case was positive for TB; note all contacts are those who sleep in the same house as the index case or the same house as the head of the household)	(1= sleeps in separate house [house of head of household], 2 = sleeps in same house but not room, 3 = sleeps in same room 4 = sleeps in same bed)
--	---

Relation to the case | | | state and check code list for code
Screening Mantoux test result: _____mm Date Read: | | | / | | | | | | | |
Chest X-ray Normal? | | | (1=yes; 2=no)
Diagnosis having attended clinic: _____

HIGH EXPOSURE TST NEGATIVE CHILD:

R4K ID: R | | | / | | | | Date of birth: _ / _ / _ _ _ _ (dd/mm/yyyy)
Sex: | | | (0=female, 1=male) Isoniazid? | | | (1=yes; 2=no)

Proximity with the index case	
-------------------------------	--

Relation to the case | | | state and check code list for code
Screening Mantoux test result: _____mm Date Read: | | | / | | | | | | | |
REPEAT Mantoux test result: _____mm Date Read: | | | / | | | | | | | |

LOW EXPOSURE TST NEGATIVE CHILD:

R4K ID: R | | | / | | | | Date of birth: _ / _ / _ _ _ _ (dd/mm/yyyy)
Sex: | | | (0=female, 1=male) Isoniazid? | | | (1=yes; 2=no)

Proximity with the index case	
-------------------------------	--

Relation to the case | | | state and check code list for code
Screening Mantoux test result: _____mm Date Read: | | | / | | | | | | | |
REPEAT Mantoux test result: _____mm Date Read: | | | / | | | | | | | |

Field Worker Name: _____ Date _ / _ / _ _ _ _
(dd/mm/yyyy)

Field Worker Signature: _____

A.4.1 Luminescence for all HEI vs HEU children

Mixed-effects REML regression Number of obs = **1044**

Group Variable	No. of Groups	Observations per Group		
		Minimum	Average	Maximum
pairstr	29	36	36.0	36
r4kidstr	58	18	18.0	18

	Wald chi2(13)	=	13286.37
Log restricted-likelihood = -418.02734	Prob > chi2	=	0.0000

logmeanbg tubesubrlu	Coef.	Std. Err.	z	P> z	[95% Conf. Interval]	
age						
2	.0245654	.0937061	0.26	0.793	-.1590952	.2082259
3	-.1270739	.0980328	-1.30	0.195	-.3192146	.0650669
3.groupcat	.1101415	.0775045	1.42	0.155	-.0417646	.2620476
time						
4	.7495844	.0492082	15.23	0.000	.653138	.8460308
24	.7779164	.0492082	15.81	0.000	.68147	.8743628
48	1.677269	.0492082	34.09	0.000	1.580823	1.773716
72	2.578679	.0492082	52.40	0.000	2.482232	2.675125
96	3.335775	.0492082	67.79	0.000	3.239328	3.432221
groupcat#time						
3 4	-.0829207	.069591	-1.19	0.233	-.2193165	.0534751
3 24	-.0242682	.069591	-0.35	0.727	-.1606639	.1121276
3 48	-.057133	.069591	-0.82	0.412	-.1935288	.0792628
3 72	-.0021058	.069591	-0.03	0.976	-.1385016	.13429
3 96	.036259	.069591	0.52	0.602	-.1001368	.1726548
_cons	5.991434	.1145422	52.31	0.000	5.766935	6.215933

Random-effects Parameters	Estimate	Std. Err.	[95% Conf. Interval]	
pairstr: Identity				
sd(_cons)	.3991	.0624083	.2937496	.5422334
r4kidstr: Identity				
sd(_cons)	.1961421	.031637	.14298	.2690707
sd(Residual)	.3245507	.0073459	.3104677	.3392724

A.4.2. Luminescence from households where HEI had concordant IGRA

```
. xtmixed logmeanbgtubesubrlu i.age i.groupcat##i.time || pairstr: || r4kidstr:, reml cov(uns)
```

Note: single-variable random-effects specification in `r4kidstr` equation; covariance structure set to `identity`

Performing EM optimization:

Performing gradient-based optimization:

Iteration 0: log restricted-likelihood = -396.8421

```
Iteration 1:  log restricted-likelihood = -396.8421
```

Computing standard errors:

Mixed-effects REML regression Number of obs = 900

Group Variable	No. of Groups	Observations per Group		
		Minimum	Average	Maximum
pairstr	25	36	36.0	36
r4kidstr	50	18	18.0	18

Log restricted-likelihood = **-396.8421** Wald chi2(13) = **10369.67**
 Prob > chi2 = **0.0000**

logmeanbgtubesubrlu	Coef.	Std. Err.	z	P> z	[95% Conf. Interval]	
age						
2	.0077354	.1092154	0.07	0.944	-.2063229	.2217937
3	-.0837125	.1137923	-0.74	0.462	-.3067413	.1393162
3.groupcat	.1065535	.087616	1.22	0.224	-.0651706	.2782777
time						
4	.7453635	.055007	13.55	0.000	.6375518	.8531753
24	.7778687	.055007	14.14	0.000	.6700569	.8856804
48	1.660861	.055007	30.19	0.000	1.55305	1.768673
72	2.545471	.055007	46.28	0.000	2.437659	2.653283
96	3.301243	.055007	60.01	0.000	3.193431	3.409055
groupcat#time						
3 4	-.0859024	.0777916	-1.10	0.269	-.2383712	.0665664
3 24	-.023966	.0777916	-0.31	0.758	-.1764348	.1285028
3 48	-.0708616	.0777916	-0.91	0.362	-.2233304	.0816072
3 72	-.0078756	.0777916	-0.10	0.919	-.1603444	.1445932
3 96	.0382656	.0777916	0.49	0.623	-.1142032	.1907344
_cons	5.977398	.1292737	46.24	0.000	5.724027	6.23077

Random-effects Parameters	Estimate	Std. Err.	[95% Conf. Interval]	
pairstr: Identity				
sd(_cons)	.4100032	.0704257	.2928042	.5741126
r4kidstr: Identity				
sd(_cons)	.2190468	.0374997	.1566085	.3063785
sd(Residual)	.3368477	.0082182	.3211192	.3533464

LR test vs. linear regression: $\chi^2(2) = 758.58$ Prob > $\chi^2 = 0.0000$

A.4.3 Luminescence from households where a LEU child was recruited

. xtmixed logmeanbgtubesubrlu i.age i.groupcat##i.time pairstr: r4kidstr:, reml cov(uns)				
Note: single-variable random-effects specification in r4kidstr equation; covariance structure set to identity				
Performing EM optimization:				
Performing gradient-based optimization:				
Iteration 0: log restricted-likelihood = -281.18519				
Iteration 1: log restricted-likelihood = -281.18519				
Computing standard errors:				
Mixed-effects REML regression Number of obs = 648				

A.4.4 12oxoLTB4 levels for all HEI vs HEU – medium only

```
. xtmixed logtwelve_oxoltb4 i.ageq i.group##i.time if bacteria==0|| pairstr: || r4kidstr, reml cov(uns)
```

Note: single-variable random-effects specification in `r4kidstr` equation; covariance structure set to `identity`

Performing EM optimization:

Performing gradient-based optimization:

```
Iteration 0: log restricted-likelihood = -45.896739
```

Iteration 1: log restricted-likelihood = -45.896738

Computing standard errors:

Mixed-effects REML regression Number of obs = 173

Group Variable	No. of Groups	Observations per Group		
		Minimum	Average	Maximum
paistr	29	5	6.0	6
r4kidstr	58	2	3.0	3

	Wald chi2(7)	=	21.70
Log restricted-likelihood = -45.896738	Prob > chi2	=	0.0029

logtwelve_oxoltb4	Coef.	Std. Err.	z	P> z	[95% Conf. Interval]	
ageq						
2	-.2074221	.2109665	-0.98	0.326	-.6209088	.2060646
3	-.3293038	.2197339	-1.50	0.134	-.7599742	.1013667
3.group	.0555694	.1528119	0.36	0.716	-.2439365	.3550753
time						
24	.1551173	.0389189	3.99	0.000	.0788376	.231397
96	.0414012	.0389189	1.06	0.287	-.0348785	.1176809
group#time						
3 24	-.1571542	.0554037	-2.84	0.005	-.2657435	-.0485649
3 96	.0026562	.0550397	0.05	0.962	-.1052195	.110532
_cons	12.65701	.2148236	58.92	0.000	12.23597	13.07806

Random-effects Parameters	Estimate	Std. Err.	[95% Conf. Interval]	
pairstr: Identity				
sd(_cons)	.5753473	.1144624	.3895721	.8497131
r4kidstr: Identity				
sd(_cons)	.4991779	.0719204	.3763711	.6620556
sd(Residual)	.1481989	.0099464	.1299321	.1690338

LR test vs. linear regression: $\chi^2(2) = 315.28$ Prob > $\chi^2 = 0.0000$

Note: LR test is conservative and provided only for reference.

A4.5 12oxoLTB4 levels for all HEI vs HEU – with BCG

```
. xtmixed logtwelve_oxolbt4 i.ageq i.group##i.time if bacteria==1|| pairstr: || r4kidstr, reml cov(uns)
Note: single-variable random-effects specification in r4kidstr equation; covariance structure set to identity
```

Performing EM optimization:

Performing gradient-based optimization:

Iteration 0: log restricted-likelihood = -54.695686

```
Iteration 1: log restricted-likelihood = -54.695683
```

Computing standard errors:

Mixed-effects REML regression Number of obs = 173

Group Variable	No. of Groups	Observations per Group		
		Minimum	Average	Maximum
pairstr	29	5	6.0	6
r4kidstr	58	2	3.0	3

	Wald chi2(7)	=	8.74
Log restricted-likelihood = -54.695683	Prob > chi2	=	0.2721

logtwelve_oxoltb4	Coef.	Std. Err.	z	P> z	[95% Conf. Interval]	
ageq						
2	-.1692428	.2138652	-0.79	0.429	-.588411	.2499253
3	-.2556231	.2226355	-1.15	0.251	-.6919806	.1807345
3.group	.010492	.1563041	0.07	0.946	-.2958584	.3168424
time						
24	-.0106151	.042062	-0.25	0.801	-.0930551	.0718249
96	.0442449	.042062	1.05	0.293	-.0381951	.1266849
group#time						
3 24	.026699	.0598776	0.45	0.656	-.0906589	.1440569
3 96	.0424677	.0594846	0.71	0.475	-.07412	.1590555
_cons	12.62782	.2159429	58.48	0.000	12.20458	13.05106

Random-effects Parameters	Estimate	Std. Err.	[95% Conf. Interval]	
pairstr: Identity				
sd(_cons)	.5587402	.1146351	.3737415	.8353115
r4kidstr: Identity				
sd(_cons)	.5093169	.0733342	.3840851	.6753808
sd(Residual)	.1601673	.0107498	.140425	.1826851

LR test vs. linear regression: $\chi^2(2) = 296.90$ Prob > $\chi^2 = 0.0000$

A.4.6 BCG-specific 12oxoLTB4 for all HEI vs HEU

```
. xtmixed adjtwelve_oxoltb4 i.ageq i.group##i.time || pairstr: || r4kidstr:, reml cov(uns)
```

Note: single-variable random-effects specification in `r4kidstr` equation; covariance structure set to `identity`

Performing EM optimization:

Performing gradient-based optimization:

```
Iteration 0: log restricted-likelihood = -2100.6854
```

```
Iteration 1: log restricted-likelihood = -2099.8211
```

```
Iteration 2: log restricted-likelihood = -2099.8209
```

Computing standard errors:

Mixed-effects REML regression Number of obs = 173

Group Variable	No. of Groups	Observations per Group		
		Minimum	Average	Maximum
pairstr	29	5	6.0	6
r4kidstr	58	2	3.0	3

	Wald chi2(7)	=	16.02
Log restricted-likelihood = -2099.8209	Prob > chi2	=	0.0250

adjtwelve_oxoltb4	Coef.	Std. Err.	z	P> z	[95% Conf. Interval]	
ageq						
2	21205.03	15375.3	1.38	0.168	-8929.997	51340.06
3	22575.68	15807.52	1.43	0.153	-8406.484	53557.84
3.group	-19082.48	20335.98	-0.94	0.348	-58940.27	20775.3
time						
24	-50016.45	19138.79	-2.61	0.009	-87527.79	-12505.11
96	8769.273	19138.79	0.46	0.647	-28742.07	46280.62
group#time						
3 24	78933.79	27194.58	2.90	0.004	25633.4	132234.2
3 96	11040.6	27066.34	0.41	0.683	-42008.45	64089.65
_cons	-18546.67	17992.54	-1.03	0.303	-53811.4	16718.06

Random-effects Parameters	Estimate	Std. Err.	[95% Conf. Interval]	
pairstr: Identity				
sd(_cons)	.7356976	2.726679	.0005152	1050.666
r4kidstr: Identity				
sd(_cons)	18509.76	12849.57	4747.797	72162.11
sd(Residual)	72878.35	4903.497	63874.4	83151.52

LR test vs. linear regression: $\chi^2(2) = 0.57$ Prob > $\chi^2 = 0.7509$

Note: LR test is conservative and provided only for reference.

A.4.7 PGE2 for all HEI vs HEU – medium only

```
. xtmixed logpg2 i.ageq i.group##i.time if bacteria==0 || pairstr: || r4kidstr:, reml cov(uns)
```

Note: single-variable random-effects specification in `r4kidstr` equation; covariance structure set to `identity`

Performing EM optimization:

Performing gradient-based optimization:

Iteration 0: log restricted-likelihood = -180.593

```
Iteration 1: log restricted-likelihood = -180.42268
```

```
Iteration 2: log restricted-likelihood = -180.41249
```

```
Iteration 2: log restricted-likelihood = -180.41245
Iteration 3: log restricted-likelihood = -180.4124
```

```
Iteration 4: log restricted-likelihood = -180.4124
```

Computing standard errors:

Mixed-effects REML regression Number of obs = 173

Group Variable	No. of Groups	Observations per Group		
		Minimum	Average	Maximum
pairstr	29	5	6.0	6
r4kidstr	58	2	3.0	3

	Wald chi2(7)	=	926.38
Log restricted-likelihood = -180.4124	Prob > chi2	=	0.0000

logpgge2	Coef.	Std. Err.	z	P> z	[95% Conf. Interval]	
ageq						
2	-.1120483	.1546316	-0.72	0.469	-.4151207	.1910241
3	.2130444	.1590726	1.34	0.180	-.0987322	.524821
3.group	.0113825	.184023	0.06	0.951	-.349296	.372061
time						
24	3.480337	.1596994	21.79	0.000	3.167332	3.793342
96	1.462314	.1596994	9.16	0.000	1.149309	1.775319
group#time						
3 24	-.0941485	.2270255	-0.41	0.678	-.5391104	.3508134
3 96	.3920604	.225849	1.74	0.083	-.0505956	.8347164
_cons	4.605283	.1700955	27.07	0.000	4.271902	4.938664

Random-effects Parameters	Estimate	Std. Err.	[95% Conf. Interval]	
pairstr: Identity				
sd(_cons)	.0664586	.3116285	6.78e-06	651.463
r4kidstr: Identity				
sd(_cons)	.2941362	.1009194	.1501404	.5762349
sd(Residual)	.6081172	.0407647	.533246	.6935008

```
LR test vs. linear regression:      chi2(2) =      5.85   Prob > chi2 = 0.0538
```

Note: LR test is conservative and provided only for reference.

A 4.8: 6ketoPGF1 α for all HEI vs HEU – medium only

```
. xtmixed sqrtsixketopgflalpha i.ageq i.group##i.time if bacteria==0|| pairstr: || r4kidstr:, reml cov(uns)
Note: single-variable random-effects specification in r4kidstr equation; covariance structure set to identity
```

Performing EM optimization:

Performing gradient-based optimization:

```
Iteration 0:  log restricted-likelihood = -616.04604
```

```
Iteration 1:  log restricted-likelihood = -616.04603
```

Computing standard errors:

Mixed-effects REML regression Number of obs = 173

Group Variable	No. of Groups	Observations per Group		
		Minimum	Average	Maximum
pairstr	29	5	6.0	6
r4kidstr	58	2	3.0	3

Log restricted-likelihood = -616.04603 Wald chi2(7) = 23.14
Prob > chi2 = 0.0016

sqrtsixketopgfi1alpha	Coef.	Std. Err.	z	P> z	[95% Conf. Interval]	
ageq						
2	-.0563476	3.433887	-0.02	0.987	-6.786643	6.673948
3	-.7230699	3.559538	-0.20	0.839	-7.699636	6.253496
3.group	7.908237	2.955743	2.68	0.007	2.115087	13.70139
time						
24	4.134187	1.76013	2.35	0.019	.6843952	7.583978
96	.7336191	1.76013	0.42	0.677	-2.716172	4.183411
group#time						
3 24	-10.27577	2.504727	-4.10	0.000	-15.18494	-5.366597
3 96	-6.72052	2.4892	-2.70	0.007	-11.59926	-1.841778
_cons	51.91872	3.433612	15.12	0.000	45.18896	58.64847

Random-effects Parameters	Estimate	Std. Err.	[95% Conf. Interval]	
pairstr: Identity				
sd(_cons)	6.41435	1.877594	3.614022	11.38451
r4kidstr: Identity				
sd(_cons)	8.011499	1.352626	5.754409	11.1539
sd(Residual)	6.702376	.4497518	5.876386	7.644467

LR test vs. linear regression: $\chi^2(2) = 88.22$ Prob > $\chi^2 = 0.0000$

Note: LR test is conservative and provided only for reference.

A.4.9: 6ketoPGF1 α for all HEI vs HEU – with BCG

```
. xtmixed sqrtsrtsixetopgflalpha i.ageq i.group##i.time if bacteria==1|| pairstr: || r4kidstr:, reml cov(uns)
Note: single-variable random-effects specification in r4kidstr equation; covariance structure set to identity
```

Performing EM optimization:

Performing gradient-based optimization:

```
Iteration 0: log restricted-likelihood = -610.70867
```

```
Iteration 1: log restricted-likelihood = -610.70866
```

Computing standard errors:

Mixed-effects REML regression Number of obs = **173**

Group Variable	No. of Groups	Observations per Group		
		Minimum	Average	Maximum
pairstr	29	5	6.0	6
r4kidstr	58	2	3.0	3

	Wald chi2(7)	=	7.37
Log restricted-likelihood = -610.70866	Prob > chi2	=	0.3919

sqrtsixketopgfialpha	Coef.	Std. Err.	z	P> z	[95% Conf. Interval]	
ageq						
2	.0380722	3.669774	0.01	0.992	-7.154552	7.230696
3	.4609754	3.817906	0.12	0.904	-7.021983	7.943934
3.group	.5344629	2.937681	0.18	0.856	-5.223285	6.292211
time						
24	-2.855901	1.58845	-1.80	0.072	-5.969206	.2574034
96	-2.898544	1.58845	-1.82	0.068	-6.011849	.2147608
group#time						
3 24	3.134626	2.260686	1.39	0.166	-1.296237	7.565488
3 96	.8997986	2.246408	0.40	0.689	-3.503079	5.302676
_cons	56.38612	3.760244	15.00	0.000	49.01618	63.75607

Random-effects Parameters	Estimate	Std. Err.	[95% Conf. Interval]	
pairstr: Identity				
sd(_cons)	9.133299	1.903293	6.070792	13.74074
r4kidstr: Identity				
sd(_cons)	8.25953	1.335447	6.016284	11.3392
sd(Residual)	6.048637	.4059287	5.303136	6.898939

LR test vs. linear regression: $\chi^2(2) = 136.63$ Prob > $\chi^2 = 0.0000$

Note: LR test is conservative and provided only for reference.

A.4.10 BCG-specific 6ketoPGF1 α for all HEI vs HEU

```
. xtmixed adjsixketopgf1alpha i.ageq i.group##i.time || pairstr: || r4kidstr, reml cov(uns)
```

Note: single-variable random-effects specification in **r4kidstr** equation; covariance structure set to **identity**

Performing EM optimization:

Performing gradient-based optimization:

```
Iteration 0: log restricted-likelihood = -1397.9748
Iteration 1: log restricted-likelihood = -1397.6511
Iteration 2: log restricted-likelihood = -1397.6378
Iteration 3: log restricted-likelihood = -1397.6377
```

Computing standard errors:

Mixed-effects REML regression	Number of obs	=	173
-------------------------------	---------------	---	-----

Group Variable	No. of Groups	Observations per Group		
		Minimum	Average	Maximum
pairstr	29	5	6.0	6
r4kidstr	58	2	3.0	3

	Wald chi2(7)	=	17.52
Log restricted-likelihood = -1397.6377	Prob > chi2	=	0.0143

adjsixketopgflalpha	Coef.	Std. Err.	z	P> z	[95% Conf. Interval]	
ageq						
2	-33.39178	233.0709	-0.14	0.886	-490.2024	423.4188
3	89.0181	241.6905	0.37	0.713	-384.6866	562.7228
3.group	-876.6803	273.107	-3.21	0.001	-1411.96	-341.4003
time						
24	-775.6848	257.6937	-3.01	0.003	-1280.755	-270.6144
96	-427.9828	257.6937	-1.66	0.097	-933.0532	77.08766
group#time						
3 24	1483.508	366.2684	4.05	0.000	765.6356	2201.381
3 96	874.8414	364.434	2.40	0.016	160.5639	1589.119
_cons	586.3195	268.4892	2.18	0.029	60.09039	1112.549

Random-effects Parameters	Estimate	Std. Err.	[95% Conf. Interval]	
pairstr: Identity				
sd(_cons)	453.4081	126.1383	262.8359	782.1571
r4kidstr: Identity				
sd(_cons)	193.7611	276.2791	11.84567	3169.374
sd(Residual)	981.2685	65.73569	860.5288	1118.949

LR test vs. linear regression: $\chi^2(2) = 10.09$ Prob > $\chi^2 = 0.0064$

Note: LR test is conservative and provided only for reference.

A.4.11: 6ketoPGF1 α for compounds where HEI had positive IGRA – medium only

```
. xtmixed sqrtsixketopgflalpha i.ageq i.group##i.time if bacteria=0|| pairstr: || r4kidstr:, reml cov(uns)
```

Note: single-variable random-effects specification in **r4kidstr** equation; covariance structure set to **identity**

Performing EM optimization:

Performing gradient-based optimization:

```
Iteration 0: log restricted-likelihood = -529.04027
```

Iteration 1: log restricted-likelihood = -529.04024

Computing standard errors:

Mixed-effects REML regression Number of obs = **149**

Group Variable	No. of Groups	Observations per Group		
		Minimum	Average	Maximum
pairstr	25	5	6.0	6
r4kidstr	50	2	3.0	3

	Wald chi2(7)	=	27.30
Log restricted-likelihood = -529.04024	Prob > chi2	=	0.0003

sqrtsixketopgfi1alpha	Coef.	Std. Err.	z	P> z	[95% Conf. Interval]	
ageq						
2	-.1456136	3.728644	-0.04	0.969	-7.453621	7.162394
3	-.9459603	3.850515	-0.25	0.806	-8.492832	6.600911
3.group	8.198625	3.140972	2.61	0.009	2.042433	14.35482
time						
24	4.695222	1.895212	2.48	0.013	.980674	8.40977
96	1.299354	1.895212	0.69	0.493	-2.415194	5.013902
group#time						
3 24	-11.93785	2.699796	-4.42	0.000	-17.22935	-6.646345
3 96	-8.910705	2.680235	-3.32	0.001	-14.16387	-3.657541
_cons	52.11754	3.694702	14.11	0.000	44.87606	59.35902

Random-effects Parameters	Estimate	Std. Err.	[95% Conf. Interval]	
pairstr: Identity				
sd(_cons)	6.82168	2.056162	3.778555	12.31564
r4kidstr: Identity				
sd(_cons)	8.19193	1.491177	5.733793	11.7039
sd(Residual)	6.700588	.4860302	5.812602	7.72423

LR test vs. linear regression: $\chi^2(2) = 80.35$ Prob > $\chi^2 = 0.0000$

Note: LR test is conservative and provided only for reference.

A.4.12 BCG-specific 6ketoPGF1 α for compounds where HEI had positive IGRA

```
. xtmixed adjsixketopgflalpha i.ageq i.group##i.time || pairstr: || r4kidstr:, reml cov(uns)
```

Note: single-variable random-effects specification in `r4kidstr` equation; covariance structure set to `identity`

Performing EM optimization:

Performing gradient-based optimization:

```
Iteration 0:  log restricted-likelihood = -1197.5719
```

```
Iteration 1: log restricted-likelihood = -1197.332
```

```
Iteration 2:  log restricted-likelihood = -1197.3247
```

Iteration 3: log restricted-likelihood = -1197.3247

Computing standard errors:

Mixed-effects REML regression Number of obs = 149

Group Variable	No. of Groups	Observations per Group		
		Minimum	Average	Maximum
pairstr	25	5	6.0	6
r4kidstr	50	2	3.0	3

	Wald chi2(7)	=	19.23
Log restricted-likelihood = -1197.3247	Prob > chi2	=	0.0075

adjsixketopgflalpha	Coef.	Std. Err.	z	P> z	[95% Conf. Interval]	
ageq						
2	86.37568	246.8646	0.35	0.726	-397.4701	570.2214
3	79.10551	254.4616	0.31	0.756	-419.6301	577.8411
3.group	-1057.481	296.6989	-3.56	0.000	-1639	-475.9617
time						
24	-897.472	284.1433	-3.16	0.002	-1454.383	-340.5614
96	-457.8524	284.1433	-1.61	0.107	-1014.763	99.05816
group#time						
3 24	1683.225	404.1736	4.16	0.000	891.0598	2475.391
3 96	1017.682	401.8393	2.53	0.011	230.0911	1805.272
_cons	704.0003	282.9588	2.49	0.013	149.4113	1258.589

Random-effects Parameters	Estimate	Std. Err.	[95% Conf. Interval]	
pairstr: Identity				
sd(_cons)	395.4171	149.9633	188.0331	831.5276
r4kidstr: Identity				
sd(_cons)	204.92	302.9632	11.30131	3715.696
sd(Residual)	1004.598	72.71484	871.7274	1157.721

LR test vs. linear regression: $\chi^2(2) = 5.43$ Prob > $\chi^2 = 0.0663$

Note: LR test is conservative and provided only for reference.

A.4.13 6ketoPGF1 α for compounds where LEU child was recruited – medium only

```
. xtmixed sqrtsixketopgflalpha i. ageq i. group##i. time if bacteria==0 || pairstr: || r4kidstr, reml cov(uns)
```

Note: single-variable random-effects specification in **r4kidstr** equation; covariance structure set to **identity**

Performing EM optimization:

Performing gradient-based optimization:

Iteration 0: log restricted-likelihood = -384.86164

```
Iteration 1: log restricted-likelihood = -384.86164
```

Computing standard errors:

Mixed-effects REML regression Number of obs = 108

Group Variable	No. of Groups	Observations per Group		
		Minimum	Average	Maximum
pairstr	12	9	9.0	9
r4kidstr	36	3	3.0	3

	Wald chi2(10)	=	21.29
Log restricted-likelihood = -384.86164	Prob > chi2	=	0.0192

sqrtsixketopgflalpha	Coef.	Std. Err.	z	P> z	[95% Conf. Interval]	
ageq						
2	7.191606	4.913751	1.46	0.143	-2.439169	16.82238
3	4.192718	5.125538	0.82	0.413	-5.853153	14.23859
group						
3	4.965013	5.157598	0.96	0.336	-5.143693	15.07372
4	16.22927	5.210387	3.11	0.002	6.017102	26.44144
time						
24	3.99467	3.298421	1.21	0.226	-2.470118	10.45946
96	3.222198	3.298421	0.98	0.329	-3.242589	9.686985
group#time						
3 24	-9.154878	4.664672	-1.96	0.050	-18.29747	-.0122879
3 96	-7.686728	4.664672	-1.65	0.099	-16.82932	1.455861
4 24	-4.831725	4.664672	-1.04	0.300	-13.97432	4.310864
4 96	-9.275086	4.664672	-1.99	0.047	-18.41768	-.1324965
_cons	46.91785	4.925795	9.52	0.000	37.26347	56.57223

Random-effects Parameters	Estimate	Std. Err.	[95% Conf. Interval]	
pairstr: Identity				
sd(_cons)	7.279935	2.797448	3.427976	15.46028
r4kidstr: Identity				
sd(_cons)	9.655711	1.865152	6.612433	14.09961
sd(Residual)	8.079449	.7032257	6.812307	9.582291

A.4.14 BCG-specific 6ketoPGF1 α for compounds where LEU child was recruited

```
. xtmixed adjsixketopgflalpha i.ageq i.group##i.time || pairstr: || r4kidstr:, reml cov(uns)
```

Note: single-variable random-effects specification in `r4kidstr` equation; covariance structure set to `identity`

Performing EM optimization:

Performing gradient-based optimization:

```
Iteration 0:  log restricted-likelihood = -846.51568
```

Iteration 1: log restricted-likelihood = -846.09537

Iteration 2: log restricted-likelihood = -846.09248

Iteration 3: log restricted-likelihood = -846.09248

Computing standard errors:

Mixed-effects REML regression Number of obs = 108

Group Variable	No. of Groups	Observations per Group		
		Minimum	Average	Maximum
pairstr	12	9	9.0	9
r4kidstr	36	3	3.0	3

	Wald chi2(10)	=	19.55
Log restricted-likelihood = -846.09248	Prob > chi2	=	0.0338

adjsixketopgflalpha	Coef.	Std. Err.	z	P> z	[95% Conf. Interval]	
ageq						
2	-677.0007	330.4204	-2.05	0.040	-1324.613	-29.38864
3	-116.5153	333.917	-0.35	0.727	-770.9806	537.95
group						
3	-403.2146	527.4277	-0.76	0.445	-1436.954	630.5248
4	-1105.984	529.6253	-2.09	0.037	-2144.031	-67.93769
time						
24	-293.34	503.4913	-0.58	0.560	-1280.165	693.4849
96	-774.7525	503.4913	-1.54	0.124	-1761.577	212.0724
group#time						
3 24	833.1042	712.0443	1.17	0.242	-562.477	2228.685
3 96	1005.607	712.0443	1.41	0.158	-389.9745	2401.188
4 24	232.3625	712.0443	0.33	0.744	-1163.219	1627.944
4 96	643.855	712.0443	0.90	0.366	-751.7262	2039.436
_cons	895.9299	408.7716	2.19	0.028	94.75236	1697.107

Random-effects Parameters	Estimate	Std. Err.	[95% Conf. Interval]	
pairstr: Identity				
sd(_cons)	1.58e-08	.	.	.
r4kidstr: Identity				
sd(_cons)	378.6989	247.298	105.3034	1361.902
sd(Residual)	1233.297	107.3445	1039.873	1462.699