Investigating the influence of BCG and hepatitis B vaccine on neonatal immune responses

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Abstract

Neonatal infectious diseases result in an estimated 40% of neonatal deaths worldwide and contribute significantly to chronic morbidity. Childhood disease-specific immunisation is irrefutably linked to the decline in deaths from targeted infections over the last century. However, neonatal immunisation strategies are limited, in part, due to impairments in their adaptive immune function. Vaccine-induced protection from severe forms of tuberculosis (TB) with the Bacille Calmette-Guérin vaccine (BCG) and perinatally-acquired hepatitis B infection with the hepatitis B vaccine (HBV) are two exceptions, with these vaccines commonly administered to neonates worldwide.

Evidence for heterologous ('non-specific') effects (NSEs) of various vaccines used in childhood, most notably for BCG, is increasing. This refers to the immunomodulatory capabilities of vaccines to influence immune outcomes beyond inducing protective immunity against the vaccine's specific targeted disease. There is limited evidence for these effects in neonates, particularly for HBV.

The research reported in this thesis aimed to explore the influence of BCG, HBV and concurrent administration of both vaccines at birth on the neonatal immune responses to unrelated antigens compared with unvaccinated babies in a randomised control trial (RCT): The Early Life Vaccine and Immunity Study (ELVIS).

Neonatal blood samples from 128 participants, collected seven days after randomisation, were stimulated with various unrelated antigens for 20 hours. Cytokine responses, measured in the supernatants by an enzyme-linked immunosorbent assay (ELISA) method, were analysed using non-parametric statistical tests to determine differences in median responses between the four groups: BCG alone, HBV alone, concurrent BCG and HBV and the unvaccinated control group.

I found minimal differences in the median in vitro cytokine responses to all stimulants between the four groups. However, both vaccines independently influenced cytokine-stimulant responses. Effects on responses were strongest for BCG-vaccinated babies, but only decreased interferon gamma (IFN-γ) responses to the Toll-like receptor (TLR) ligand resiquimod (R848) and monocyte chemoattractant protein-1 (MCP-1) responses to heat-inactivated *E. coli* were significantly different from controls. Combined vaccines tended to induce similar cytokine-stimulant response patterns as BCG alone, although for some cytokine-stimulant pairs, the BCG-induced effects were mitigated by HBV and vice versa.

This study adds to the evidence for NSEs of vaccines in neonates. It is the first study to investigate the influence of HBV immunisation on immune responses to unrelated antigens, finding no statistically significant differences in median cytokine responses compared with controls. The finding that concurrent vaccination with HBV and BCG at birth induced the same cytokine-stimulant response pattern as BCG alone, suggested that cytokine responses to unrelated stimulants are driven by BCG. This is also the first study to show that in neonates concurrent HBV and BCG vaccination at birth weakens the NSEs of BCG for certain cytokine-stimulant pairs.

Further research into neonatal vaccine NSEs are warranted. Future studies should explore and further investigate the clinical relevance of certain cytokine-stimulant response signatures identified in my thesis and the mechanism for these observations in neonates. These results will direct research on how we could potentially exploit any beneficial vaccine NSEs to provide protection against infection in the very young.

Declaration

I declare that this thesis

- Comprises only my original work towards the Master of Philosophy degree except where indicated in the preface;
- Due acknowledgement has been made in the text to all material used;
- The thesis is fewer than 50 000 words, exclusive of tables, bibliography and appendices

Lianne Cox

Preface

Contributions

This thesis presents predominantly my work, under the supervision and guidance of my supervisors, advisory committee members and laboratory staff. Many people contributed to various aspects of the study and their contributions are detailed below.

Under the guidance of my primary supervisor, Prof Nigel Curtis, I designed the ELVIS trial. All funding for the ELVIS trial came from Murdoch Children's Research Institute (MCRI) Theme funding.

I was solely responsible for writing and preparing the study protocol and the full ethics applications and amendments to both the Mercy Human Research Ethics Committee (HREC) and the Royal Children's Hospital (RCH) HREC. I prepared the parent/guardian information and consent form (PGCIF) and all amendments.

I designed the data collection questionnaires, set up the electronic project and maintained the Research Electronic Data Capture (REDCap) database forms for all clinical data collection in the study. The lab database was set up and maintained by laboratory staff.

I was the trial coordinator for the study at the recruiting site, and prepared and submitted all the regulatory documents for the study with guidance from Ms Kaya Gardiner.

The ELVIS trial took place at the same hospital and at the same time as the Melbourne Infant Study BCG for Allergy and Infection Reduction (MIS BAIR) study that was supported by several research nurses. An exclusion criterion for involvement in the larger trial was intended travel to an area of high TB prevalence. The targets for my recruitment included these expectant mothers. Research nurses - Ms Brooke O' Neill, Ms Karen Bellamy, Ms Clare Brophy, Ms Clare Morrison, Ms Kate Wall, Ms Julie-Anne Quinn, Ms Monica Owlad and Ms Veronica Abruzzo - assisted with recruitment by providing information to these expectant mothers, occasionally consenting and randomising participants to the ELVIS trial. When available, Ms Kate Wall, Ms Karen Bellamy and Ms Clare Brophy occasionally vaccinated babies with BCG. However, I recruited, consented, randomised and vaccinated most of patients into the study.

I was completely responsible for participant coordination in the trial including identifying participants, arranging hepatitis B surface antigen (HBsAg) testing of mothers in, or shortly after, labour, liaising with birth suite and postnatal staff on study procedures, following up maternal HBsAg results before randomisation and ensuring appropriate vaccines were available, prescribed and administered after randomisation. Randomisation took place seven days a week for the duration of the study. I was also solely responsible for the coordination of participants post randomisation including scheduling study appointments, calling participants 24 hours before appointments to remind them of the study visit and arranging vaccine availability at their appointment.

I conducted almost all the seven-day study visits and performed the phlebotomy for >100 of the 128 participants included in the analysis. Dr Dan Casalaz, Dr Freya Harewood, Dr Joshua Osowicki, Dr Hayley Du Plessis and Ms Judith Spotswood collected neonatal blood samples when I was unavailable.

As part of the coordination role, I was responsible for organising timely packaging and transport of all blood samples back to the laboratory.

The blood samples were processed, and the stimulation assays performed by laboratory staff- Ms Susie Germano, Ms Rhian Bonnici, Dr Ben Forbes and Ms Snezana Spillane. The cytokine assays were performed by Ms Susie Germano.

I did the data cleaning and checking of data used in the analyses, assisted by Ms Susie Germano for the lab database and assisted by Ms Clare Morrison for the clinical database. I performed the statistical analyses, interpretation of data, the design of all graphs and figures and the presentation of all data in the thesis. I researched, prepared and wrote the introduction, literature review, methods, results, discussion and conclusions in entirety as presented. My supervisors, Nigel Curtis, Katie Flanagan and Roy Robins-Browne all reviewed the thesis before submission and provided constructive feedback that improved my work.

Scholarships

At the start of my candidature I received an International Fee Remissions Scholarship to undertake my studies. This scholarship was later replaced by an Australian Postgraduate Award both of which I am incredibly grateful to have received.

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As I write this, I'm reflecting over the previous years where the ELVIS trial was part of my life. It was a time incredibly rich in experiences both positive and negative and at times the 'work-life-study-parenting' balance seemed unachievable. I'm very grateful to many people who supported me through this time and made this final stage possible.

I am so grateful to my primary supervisor Nigel Curtis, who has supported me throughout my studies, and genuinely understood the competing demands throughout my candidature. I am thankful for his mentorship on life as a clinician, researcher and parent. It has been a privilege to learn from someone so experienced and successful in their research career. His guidance and advice in all aspects of my research from trial design, ethics applications, statistics, data analyses, good scientific writing and publishing have been very much appreciated. Nigel's optimism, encouragement and ability to streamline challenges during difficult times was incredibly helpful. His inspiring introduction to the world of medical research has definitely motivated me to seek out research opportunities within my clinical practice.

I would also like to express my thanks to my other supervisors, Roy Robins-Browne and Katie Flanagan for their feedback over the years. Roy, who is an experienced researcher, gave me helpful advice when facing tough decisions. He is a good listener and is able to weigh up the pros and cons of the situation and provide expert advice. He also provided valuable comments on the final content of the thesis. I was grateful for the advice I received from Katie who has a particular interest and experience in lab-based immune response research. Her advice on statistical analyses and insight on visualisation of my data was appreciated. Aside from the project, Katie supported and encouraged me throughout the years and has been a fantastic female role model.

I also wanted to thank my temporary supervisor, Clare Collins, who provided support and advice in the running of the trial at the Mercy Hospital for Women, Heidelberg. Clare also provided valued mentorship in the difficult year running the trial and her kindness will not be forgotten.

I am also incredibly grateful to the members of my advisory committee. I would like to thank Susan Donath, as my advisory chair, for her advice, insights, frankness, understanding and fairness along the journey. I am also thankful for the support, advice and feedback of the other members at various times, Christel Zufferey, Tom Connell, Kirsten Perrett, Kaya Gardiner and Nicole Messina.

I would also like to extend a special acknowledgment to Kaya Gardiner, our research lab study coordinator, who has supported me through the entire journey. She has provided advice on all aspects of my research: editing, proofreading, data handling, statistics software and keeping track of various aspects of the study. She has been a confidant and I thank her for her friendship, kindness, patience and support both at work and beyond. I would not have made it to this stage without this fellow curly girl.

I am also indebted to the research nurses – Brooke O' Neill, Clare Brophy, Clare Morrison, Gill Ormond, Hannah Elborough, Judith Spotswood, Julie-anne Quinn, Faith Reilly, Karen Bellamy, Kate Wall, Marie Gentile-Andrit, Monica Owlad, Natalie Papagiorcopu, Simone Hamilton and Veronica Abruzzo – who were part of the team at some point during my studies. I thank them not only for their help at the Mercy Hospital, Heidelberg, but for their support, encouragement, companionship, lunchtime conversation and inevitable friendship.

This project could not have been completed without the help from our research laboratory staff. I wanted to thank Frances Oppedisano, our research laboratory manager, who has always been kind, friendly and welcoming. At the start of my studies, Christel Zufferey was a post-doctoral fellow in our research lab. Having absolutely no exposure to a laboratory before, Christel showed me the ropes and taught me basic laboratory procedures. She patiently explained the techniques and provided advice at the very beginning of trial design. She was extremely kind and supportive, and I am grateful for her friendship. I would also like to thank Laure Pittet, another of our research lab post-doctoral fellows for her advice on the structure of the thesis and calm, encouraging words. I am indebted to the laboratory staff who processed the blood and performed the stimulation assays on my samples including Ben Forbes and Snezana Spillane but especially Rhian Bonnici and Susie Germano who did the bulk of that work. I am also grateful for the advice from our current post-doctoral fellow, Nicole Messina, who readily answered my ever-growing list of questions. I particularly wanted to thank Susie Germano who completed the cytokine assays and several other laboratory procedures on my samples. Susie is incredibly knowledgeable and skilled and provided useful advice over many years. She has supported and encouraged me through what seemed like endless challenges and I am very grateful for her friendship and advice on matters in life in general – and of course for her cakes!

Of course, without the study participants, none of this could have been possible. I am thankful and grateful to the 242 families enrolled into the study. I am even a bit surprised by their commitment to the study, many being first-time parents, exhausted and sleep deprived, who brought their babies back to hospital at only one week of age for a blood test. The families were generous with their time and it was an overwhelmingly positive experience interacting with families as a clinician researcher. I must also acknowledge the clerical staff at the Mercy Hospital for Women, Heidelberg, who were very accommodating of our presence.

The work outlined in this thesis was possible through funding from my Australian Postgraduate Award and theme funds from the Murdoch Children's Research Institute.

I would also like to thank the Clinical Epidemiology and Biostatistics Unit (CEBU) for providing helpful advice on the statistical analyses in the study, especially Susan Donath and Kate Francis.

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Several members of the University paediatric administration team provided advice and support of a practical nature throughout my studies. I am grateful to Kate Bride, Hiroko Miyano, Carmen Van Tiggel, Gus Fraser and Pam Morison for the assistance over the years and Poh Chua, who helped with referencing issues on several occasions.

I was fortunate to be enrolled in my studies at the same time as some amazing students in our research lab, some becoming inspirational role models. I thank all of them for sharing their ups and downs, supporting each other and friendship offered. I thank Vanessa Clifford, Bridget Freyne, Freya Harewood, Paola Villanueva, Samantha Bannister and Eva Sudbury for the positive and supportive environment during my studies. I also thank PhD students outside of my research group who encouraged me along the way, Laila Ibrahim, Elliot Long, Amanda Gwee, Josh Osowicki and Megan Chapman.

I am also grateful for having supportive clinical directors including Stuart Lewena, Martin Wright and Dan Casalaz, who provided opportunities to work part time and complete specialist training concurrently with my studies. Many of my work colleagues at the Mercy Hospital for Women, Heidelberg were encouraging and optimistic during the running of the trial and I thank Gillian Opie, Andrew Watkins and Clare Collins who provided advice, encouragement and practical support.

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To my family, I dedicate this all to you. I thank my parents for their confidence in me, their early morning and late-night words of encouragement and the reminders of what is truly important. I am very grateful to my husband, Patrick, who supports almost all the crazy ideas I have and who has supported me through two house moves, two babies, two specialist examinations, two interstate moves, six training posts, a clinical diploma and ELVIS! I thank him for the weekends, too numerous to count, when he drove me to the hospital and cared for our son so I could randomise a participant. Finally, I thank my sons, Ross and Jenson, for always having a smile ready for me when I finished work and for putting life's struggles into perspective.

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Abbreviations and Acronyms

AE	Adverse event
4PL	Four Parameter Logistic
ALRTI	Acute lower respiratory tract infection
ASIA	Autoimmune syndrome induced by adjuvants
ASQ	Ages and Stages Questionnaires
BCG	Bacille Calmette-Guérin vaccine
bDMARDS	Biological Disease-Modifying Anti-Rheumatic Drugs
B. pertussis	*Bordetella pertussis*
C. albicans	*Candida albicans*
CD	Cluster of differentiation
CEBU	Clinical Epidemiology & Biostatistics Unit
CFU	Colony forming units
CI	Confidence interval
CMV	Cytomegalovirus
CONSORT	Consolidated Standards of Reporting Trials
DT	Diphtheria toxin
DTP	Diphtheria- tetanus-pertussis vaccine
DTaP	Diphtheria- tetanus- acellular pertussis vaccine
DTwP	Diphtheria- tetanus- whole cell pertussis vaccine
E. coli	*Escherichia coli*
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
ELISPOT	Enzyme-linked immunosorbent spot
ELVIS	Early Life Vaccines and Immunity Study
FGF	Fibroblast growth factor
Flt-3L	Fms-related tyrosine kinase 3 ligand
GBS	Group B streptococcus
G-CSF	Granulocyte-colony stimulating factor
GM-CSF	Granulocyte macrophage-colony stimulating factor
GRO	Growth-regulated oncogene
HBsAg	Hepatitis B surface antigen
HBsAb	Hepatitis B surface antibody
HBV	Hepatitis B vaccine
Hib	*H. influenzae* group b vaccine
H. influenzae	*Haemophilus influenzae* type b
HIV	Human immunodeficiency virus
HPV	Human papillomavirus
HR	Hazards ratio
HREC	Human Research and Ethics Committee
HSV	Herpes simplex virus
IFN-γ	Interferon-gamma
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IL	Interleukin
IPV	Inactivated poliovirus vaccine
IU	International unit

IVIG	Intravenous immunoglobulin
K-Wallis	Kruskal-Wallis
LBW	Low-birth-weight
L. monocytogenes	*Listeria monocytogenes*
LPS	Lipopolysaccharide
MAC	Mycobacteria avium complex
MCP-1	Monocyte chemoattractant protein 1
MCRI	Murdoch Children's Research Institute
MDC	Macrophage-derived chemokine
MHW	Mercy Hospital for Women
MIF	Macrophage migration inhibitory factor
MIG	Monokine induced by IFN-γ
MIS BAIR	Melbourne Infant Study: BCG for Allergy and Infection Reduction
MRR	Mortality rate ratio
M. tuberculosis	*Mycobacterium tuberculosis*
M. ulcerans	*Mycobacterium ulcerans*
MV	Measles vaccine
NOD-2	Nucleotide-binding and oligomerization domain- 2
NSE	Non-specific effect
NSEs	Non-specific effects
NHL	Non-Hodgkin lymphoma
NK	Natural killer
NTM	Non-tuberculous mycobacteria
OPV	Oral polio virus vaccine
OR	Odds ratio
PAM3CSK4	(S)-(2,3-bis(palmitoyloxy)-(2-RS)-propyl)-N-palmitoyl-(R)-Cys-(s)-Ser-(s)-Lys4-OH, trihydrochloride
PBMCs	Peripheral blood mononuclear cells
PCV-7	7 valent pneumococcal conjugate vaccine
PCV-13	13 valent pneumococcal conjugate vaccine
PDGF	Platelet derived growth factor
PEPG	Peptidoglycan
PGICF	Parent/Guardian Information and consent form
PHA	Phytohemagglutinin
PMA	Phorbol 12-myristate 13-acetate
PPD	Purified protein derivative
R848	Resiquimod
RANTES	Regulated on activation normal T cell expressed and secreted
REDCap	Research Electronic Data Capture
RPMI	Roswell Park Memorial Institute 1640 medium
RCH	Royal Children's Hospital (Melbourne)
RCT	Randomised controlled trial
RSV	Respiratory syncytial virus
SAGE	Strategic Advisory Group of Experts
S. aureus	*Staphylococcus aureus*
Str. pneumoniae	*Streptococcus pneumoniae*
SD	Standard deviation
SSI	*Statens Serum Institute*
STARD	Standards for Reporting Diagnostic Accuracy Studies

T1DM	Type 1 diabetes mellitus
TB	Tuberculosis
TGF	Transforming growth factor
TH1	T helper 1
TH2	T helper 2
TMB	Tetramethyl Benzidine
TNF-α	Tumour Necrosis Factor-alpha
TST	Tuberculin skin test
UK	United Kingdom
VEGF	Vascular endothelial growth factor
WHO	World Health Organization

Chapter 1: Introduction

Despite advances in health practices over the last decades, infections remain a major cause of death in children aged 0-28 days (neonates) worldwide. Most of these deaths occur in low and middle-income countries (1). For babies born in high-income countries, those who survive severe infections in this vulnerable period often suffer from the long-term health implications related to chronic morbidity and rely on health care resources long term.

Immunisation has successfully decreased mortality from specific targeted infections and is one of the greatest medical advances of all time. The neonatal immune system is unique both in structure and function compared with that of adults and older children (2) and therefore the ability to generate effective immune responses to the many available vaccines in their current design is impaired, limiting their use at birth (3).

Bacille Calmette-Guérin vaccine (BCG) and hepatitis B vaccine (HBV) are two exceptions and provide protection against severe forms of tuberculosis (TB) and perinatally acquired hepatitis B infection, respectively. Both are therefore two commonly administered neonatal vaccines. BCG is routinely recommended in 156 countries, particularly those with high tuberculosis prevalence, with a global coverage rate of nearly 90% that is fairly uniform across World Health Organization (WHO) defined regions (4, 5). A birth dose of HBV is routinely recommended in 108 countries with a global coverage rate of 42%. However, there are significant differences in the HBV birth dose immunisation coverage across the WHO regions, with the Western Pacific region's coverage approaching that of BCG at 83% (5).

Various vaccines used in childhood have immunomodulatory capabilities that influence immune outcomes outside of the vaccine's specific targeted disease (6). Researchers refer to these effects as the 'non-specific effects (NSEs)', 'heterologous' or 'off-target' vaccine effects. Several factors may influence these NSEs, including whether the vaccine is a live vaccine or not (7). Live vaccines, including BCG, measles, oral polio vaccine and smallpox vaccines, have shown trends towards beneficial NSEs on all-cause mortality (8-10), whereas non-live vaccines, including inactivated polio (IPV) and the combination diphtheria-tetanus-pertussis (DTP) vaccines, have shown trends towards detrimental NSEs on overall infant survival in observational studies (9, 11).Researchers have also observed that these NSEs persist until a different vaccine is given and can be influenced by concurrent immunisation (7).

The largest body of evidence for non-specific beneficial effects of vaccines is for BCG. In children, BCG vaccination promotes overall child survival by decreasing mortality due to infections other than tuberculosis (9, 12). BCG also protects and/or augments immune responses in childhood against infections caused by viruses (13, 14), protozoa (15) and non-tuberculous mycobacteria (NTM) (16). With respect to neonates, early BCG in low-birth-weight (LBW) babies decreases all-cause mortality between 30%-72% in high disease burden settings (17-19).

Less is known about the immunomodulatory capability of HBV, a non-live vaccine, containing recombinant hepatitis B surface antigen (HBsAg). The only report focussed on possible NSEs of HBV on immunity was an observational study in an area of high disease burden where HBV was administered to children older than 6 months of age. This study, in keeping with results from studies with other non-live vaccines, reported a negative effect on overall survival, especially for girls (20). A retrospective case study in Brazil found that childhood vaccinations, including HBV, decrease childhood visceral leishmaniasis (15).

In response to data from clinical observational studies, researchers have started to investigate NSEs of vaccines at the cellular level. Immune cell responses to infections, infectious antigens and vaccines can be measured by quantifying the production of cytokines that play a role in coordinating immune responses and eliminating infections. Accordingly, cytokine responses are the most common outcome measure in laboratory studies of immune responses.

Few studies have investigated the influence of neonatal vaccines on cytokine responses to stimulants that are unrelated to the vaccine within the neonatal period. One two-group randomised control study (RCT) described alterations in certain cytokine responses to unrelated stimulants after combined neonatal BCG and HBV, with the control group receiving HBV alone (21). To our knowledge, there are no research data regarding the effect of neonatal HBV or the combined effects of neonatal BCG and HBV on immune responses to unrelated stimulants in the neonatal period, or beyond.

The following research questions aimed to address a current gap in the literature i.e., the influence of isolated and combined neonatal BCG and HBV on quantifiable cellular immune responses to unrelated infective stimulants within the neonatal period.

1.1 Research question

What is the effect of:

a. an isolated birth dose of BCG
b. an isolated birth dose of HBV
c. a concurrent birth dose of BCG and HBV

on neonatal immune responses to in-vitro stimulation by various unrelated infective stimulants?

1.2 Research aim

The primary aim of this study was to determine the influence of immunisation at birth with BCG, HBV or concurrent BCG and HBV on neonatal immune responses to unrelated stimulants. This was achieved by comparing cytokine concentrations in supernatants 20 hours after stimulation by various innate and pathogen-related stimulants in four groups of infants (BCG, HBV, BCG+HBV, Control) on day 7 (± 4) after randomisation.

1.3 Rationale for this research study

Neonatal morbidity and mortality from infectious diseases is of global concern. Research into strategies to enhance defences against infection in this population is ongoing. Results from studies investigating the beneficial NSEs of vaccines, in particular BCG, have gained interest as a possible strategy to exploit these effects to improve protection against infections in the very young (22).

We need more studies with higher levels of evidence and in more clinical settings for these observed effects, as most of research to date is (i) from observational studies with many potential confounders, or (ii) in developing countries with high disease burdens. The neonatal period is a time when few vaccines are administered and therefore immune outcomes are not confounded by administration of other vaccines.

Where vaccine policies around the world differ in their recommended birth vaccination schedule for BCG and HBV, it would be useful to know whether there are any NSEs for these two vaccines in isolation, as well as their combined effect on neonatal immune responses to unrelated infective stimulants. Research has shown that NSEs from live vaccines, like BCG, differ from those of non-live vaccines, like HBV. It is plausible that the addition of HBV may negate some of the beneficial effects of BCG already described in the literature.

In Australia, where the incidence of TB is very low, BCG is recommended for protection against severe forms of TB for children under the age of 5 years who are travelling to areas where TB prevalence is high (23). There is no standard as to when BCG should be administered before travel, other than a recommendation that it be administered at least one month prior to intended travel. BCG is safely administered at birth to infants in countries where TB is endemic and a recent report of modelled scenarios found an increase in global TB associated deaths with delay in BCG vaccination in high TB-burden countries (24).

As all Australian infants are offered HBV as soon as possible after, and within the first 10 days of birth, we were presented with a unique and ethical opportunity to study the NSEs of both HBV and BCG on neonatal immunity without changing the standard care these neonates would have received. This opportunity allowed the design and implementation of a four-group RCT with two single and a combined vaccine test groups, and a nil vaccine control group. This design would not be possible in countries with high TB prevalence or in countries which do not routinely provide a birth dose of HBV to all neonates.

1.4 Overview of thesis structure

This thesis comprises a further four chapters. Chapter 2 provides a review of relevant literature. I then present the original research I undertook during my candidature. Chapter 3 details the design of the clinical randomised control trial and describes the laboratory methods used to derive the data to complete my research aim. Chapter 4 presents the results of the study. In chapter 5, I discuss the findings of the study and place these in context with similar studies, offering suggestions for future research in this area.

Chapter 2: Background literature review

2.1 Introduction

This chapter reviews the current literature on the non-specific effects (NSEs) of Bacille Calmette-Guérin vaccine (BCG) and hepatitis B vaccine (HBV) in children and neonates. NSEs of vaccines encompass effects that are unrelated to protection against the vaccine's target infection - including immunity to non-targeted infections, effects on non-infectious conditions and immunomodulatory therapeutic effects on infectious and non-infectious conditions.

Heterologous immunity resulting from BCG is not a novel concept, with references in the literature dating back to the 1930s and 1940s (25, 26). More recently, researchers published the observation that BCG immunisation of West African infants was associated with better infant survival likely due to protection against diseases other than tuberculosis (TB) (27). This publication led to renewed interest in the NSEs of BCG - particularly in children - and a subsequent surge of studies in this field. The World Health Organization (WHO) Strategic Advisory Group of Experts (SAGE) reviewed studies on the NSEs of vaccines in 2014, acknowledged beneficial BCG-induced NSEs and called for more research as the available evidence was insufficient to recommend any changes to the immunisation schedule.

Most evidence on the NSEs of vaccines over the last two decades has come from studies in West Africa (6, 7, 9, 28-33). However, during my candidature, two rigorous large-scale randomised control trials (RCTs) investigating the NSEs of BCG in children were established in low-disease burden countries: the Danish 'Calmette Study' and the Australian 'Melbourne Infant Study BCG for Allergy and Infection Reduction (MIS BAIR)' trials, respectively.

The evidence on NSEs of vaccines from studies in animals have been extensively reviewed (34) and will not be discussed further here . Several limitations in the design of these studies caused difficulties in the assessment of their relevance for human studies.

In this chapter, I will first review the evidence for NSEs of BCG in children and provide a brief overview of the potential mechanisms for these effects and the factors known to influence them. I will then review the evidence for NSEs of HBV in children and the evidence for NSEs of both vaccines used in combination.

2.2 Bacille Calmette-Guérin

BCG, introduced in the 1920s, is commonly administered for specific protection against TB. It was discovered by Calmette and Guerin and developed from a live attenuated strain of a related bovine sub-species of *Mycobacterium tuberculosis (M. tuberculosis), Mycobacterium bovis* (*M. bovis*).The original strain was sent to different locations but differing culture protocols resulted in different strains with unique antigenicity and efficacy against *M. tuberculosis* (35). Here, I review the evidence for the NSEs of BCG with relevance to children and neonates, grouped by clinical outcomes or laboratory immunological parameters.

2.2.1 Clinical evidence for non-specific effects of BCG

Studies investigating the NSEs of BCG include clinical effects on mortality and morbidity and evidence for BCG as a therapeutic immunomodulator for infectious and non-infectious conditions.

Evidence for BCG-induced non-specific effects on childhood mortality

Cohort studies

Cohort studies, which collectively included several thousand participants, mostly in West Africa, have investigated the effects of BCG on childhood mortality. These studies were heterogenous in design, particularly for factors now known to impact the NSEs of vaccines and thus had a high risk of bias. All studies were conducted in countries with high infectious disease burdens and with a long-standing use of other vaccines, resulting in herd immunity. These factors make it difficult to determine the effects of BCG on protection from unrelated infections. Few studies adjusted results for periods when only BCG was administered. Outcome measures varied between studies, some reporting mortality rate ratios (MRR) and others hazard ratios (HR). These limitations make direct comparisons difficult or impossible. Nevertheless, several studies published since 2000 (Table 2.1) showed that BCG in childhood decreases all-cause infant mortality presumably from diseases other than TB in African countries (12, 27, 36-39), Southern India (40) and Papua New Guinea (41). None of these studies reported outcomes exclusively in neonates, some actively excluding them from their analyses, although many beneficial effects of BCG on mortality appear to be strongest in younger children.

The conclusions of some studies have been criticised for potential introduction bias, in particular in relation to treatment allocation and care, and for drawing conclusions from health record data and verbal autopsy reports that may be unreliable as to the cause of death (42). Although beneficial effects on infant survival have been reported with BCG in these settings, the results are not generalisable. Further studies are needed in different populations and contexts to assess these effects. Ideally these studies should be randomised trials with a clinically and ethically feasible control group.

Non-randomised trials

Non-randomised cohort trials in North America in the 1930s and 1940s reported beneficial effects of BCG on all-cause mortality in children (43, 44), but these studies were heterogenous in design and difficult to interpret.

Randomised controlled trials

Stronger evidence for the influence of BCG on childhood mortality has come from studies in low-birth-weight (LBW) babies in Guinea-Bissau (Table 2.2). Here, three RCTs showed that BCG vaccination is associated with a 17%-59% reduction in all-cause mortality from diseases other than TB up to one year of age (17-19). Although the authors did not report the details of other vaccinations the infants received, the beneficial effect of BCG appeared to be strongest in the youngest children, possibly due to fewer vaccine interactions during that period, which are potential confounders (18). Biering-Sorensen et al. (19) showed an 83% reduction in mortality in the youngest participants within 3 days of enrolment, although the actual numbers of neonatal deaths was very small (one death in 148 babies receiving early BCG compared with five deaths in 151 babies in the control group). Although encouraging,

collectively including over 6,500 infants, these trials were limited to one country where the benefit of early BCG on survival occurred in a defined LBW population. RCTs in other areas that include infants and neonates, of normal and large-for-gestational weights are needed.

Meta-analyses

A WHO-instigated review of 18 studies concluded that BCG administered in early life reduced average infant mortality by 30% (95% CI [confidence interval]-1% to 51%) in RCTs, and to 53% (95% CI 31% to 68%) in observational studies (9). A subsequent meta-analysis of three RCTs in BCG-vaccinated LBW babies in Guinea-Bissau showed a reduction in infant mortality of 16% (95% CI 0% to 29%) and in neonatal mortality of 38% (95% CI 17% to 54%) (18). Taken together, these analyses indicate a protective effect of BCG on infant survival. What these studies weren't designed to show were the biological mechanisms and clinical relevance in different settings for the protective effects of BCG on infant survival. Further studies, particularly in neonates, where benefits appear to be strongest, should be prioritised.

Table 2.1: Cohort studies since 2000 on the influence of BCG on all-cause mortality

Epidemiological cohort studies					
Author, year, location (reference)	**Age at BCG immunisation**	**Other vaccines**	**Age at outcome**	**Outcome**	**Comment**
Kristensen, 2000, Guinea-Bissau (27)	Median age 31 days	Birth: OPV 6,10 and 14 weeks: DTP, OPV 9 months: MV	0-6 months	BCG-vaccinated compared with BCG-unvaccinated had a 45% reduction in mortality rate (MRR 0.55 [95% CI 0.36-0.85]). *Adjusted results by age:* birth to <2 months old had a 57% reduction in mortality rate (MRR 0.43 [95% CI 0.36-0.85]). >31 days and <2 months had an 81% reduction in mortality rate (MRR 0.19 [95% CI 0.04-0.95]).	Participants: 15,351 women and their children born 1990-1996. Results also adjusted for DPT and cluster. Children weighing < 2500 g do not receive routine BCG, assumed babies > 31 days weighed > 2500 g. Strongest effects were seen in younger infants when results adjusted for age.
Vaugelade, 2004, Burkina-Faso (12)	Mean age 4.8 months	6, 10 and 14 weeks: DTP 9 months: MV	Under 2 years	BCG-vaccinated compared with BCG-unvaccinated had a 63% reduction in mortality rate (MRR 0.37 [95% CI 0.29-0.48]).	Participants: 9,085 children born 1985-1993. Results adjusted for birth season and other factors.
Roth, 2004, Guinea-Bissau (39)	BCG at birth or at any time after birth	DTP MV	Up to 6 months	BCG-vaccinated compared with BCG-unvaccinated children: 0-6-month old children had a 75% reduction in mortality rate (MRR 0.26 [95% CI 0.12-0.56]).	Participants: 7,138 children born 1989-1999. Reported BCG effects in children with LBW. Immunisation schedule not stated.
			Up to 12 months	0-12-month-old children had a 69% reduction in mortality rate (MRR 0.31 [95% CI 0.15-0.64]). *Adjusted results for BCG within the first week of life:* 0-6-month old children had an 85% reduction in mortality rate (MRR 0.14 [95% CI 0.03-0.63]). 0-12-month-old children had an 83% reduction in mortality rate (MRR 0.17 [95% CI 0.05-0.56]).	

Author, year, location (reference)	Age at BCG immunisation	Other vaccines	Age at outcome	Outcome	Comment
Moulton, 2005, Southern India (40)	Median age close to 30 days	Birth: OPV 6, 10 and 14 weeks: DTP, OPV	6 months	BCG-vaccinated compared with BCG-unvaccinated had a 28% reduction in deaths (HR 0.72 [95% CI 0.52-0.99] p = 0.04).	Participants: 10,274 infants. Babies were given either Vitamin A or placebo in the first 2 days of life. Excluded neonates who died within the first week of life. Censored for gender and Vitamin A as a vaccine effect modifier. Results adjusted for season and other characteristics.
Elguero, 2005, Senegal (37)	2 month of age	2,4 and 6 months: DTP, IPV 9 months: MV	Up to 2 years	BCG/DTP vaccinated compared with BCG/DTP unvaccinated had a 30% reduction in mortality rate (MRR 0.7 [95% CI 0.5-0.97] p = 0.03).	Participants: 7,796 vs 3573 (unvaccinated). BCG was given with DTP at 2 months of age. Similar results when adjusted for ages up to 6, 12 and 18 months of age.
Lehmann, 2005, Papua New Guinea (41)	Half by age 28 days, 80% by 6 months	Birth: HBV 1-2, 2-4 months: HBV, DTP, IPV, *Clostridium perfringens* type C vaccine 3-6 months: DTP, IPV, *Clostridium perfringens* type C vaccine 3-59 months: 23 valent pneumococcal vaccine 6-9 months: MV	29 days to 5 months 6-11 months 12-23 months	Infants 29 days to 5 months: BCG-vaccinated compared with BCG-unvaccinated had an 83% reduction in deaths (HR 0.17 [95% CI 0.09-0.34]). No beneficial effect of BCG at older age groups.	Participants: 3,502 vs 546 (unvaccinated). Vaccine schedule varied during the study period. Excluded neonates. Analysed results for sex-effects and deaths from ALRI and acute abdominal causes. Results adjusted for other vaccines and other factors.

Author, year, location (reference)	Age at BCG immunisation	Other vaccines	Age at outcome	Outcome	Comment
Aaby, 2006, Malawi (36)	Median age 16 days	Birth (or soon after): OPV 6, 10 and 14 weeks: DTP, OPV 9 months: MV	8 days to 8 months	BCG-vaccinated compared with BCG-unvaccinated had a 55% reduction in mortality rate (MRR 0.45 [95% CI 0.16-1.23]).	Participants: 17,000 born between 1998-2002. Excluded neonates who died within the first week of life, none received vaccinations. Analysed results for sex-effects.
Nankabirwa, 2015, Uganda (38)	Median age 4 days	Birth: OPV 6, 10 and 14 weeks: DTP, HBV, Hib, OPV 9 months: MV	1 month to 1 year 1 year to 5 years	1 month to 1 year: BCG-vaccinated compared with BCG-unvaccinated had a 53% reduction in deaths (HR 0.47 [95% CI 0.14-1.53]). 1 year to 5 years: BCG-vaccinated compared with BCG-unvaccinated had a 74% reduction in deaths (HR 0.26 [95% CI 0.14-0.48]).	Participants: 819 eligible pregnant women followed till children 5 years of age. Excluded neonates. Results adjusted for age at immunisation and others. Analysed results for sex-effects.

Abbreviations: ALRI = acute lower respiratory infections; CI = confidence interval; DTP = diphtheria-tetanus-pertussis vaccine; g = grams; HBV = hepatitis B vaccine; Hib = *Haemophilus influenzae* type b vaccine; HR = hazards ratio; IPV = inactivated polio vaccine; LBW = low-birth-weight (<2500 grams); MRR = mortality rate ratio; MV = measles vaccine; OPV = oral polio vaccine; RCT= randomised control trial

Table 2.2: Randomised control trials and meta-analyses for the effect of BCG on all-cause infant mortality

Randomised control trials					
Author, year, location (reference)	**Groups**	**Other vaccines**	**Age at outcome**	**Outcome**	**Comments**
Aaby, 2011, Guinea-Bissau (17)	1168 BCG at birth (early group) 1,152 Delayed BCG (controls)	DTP	Up to 12 months	Early BCG reduced infant mortality by 17% (MRR 0.83 [95% CI 0.65-1.08]). *Adjusted results by age:* Early BCG reduced neonatal mortality by 45% (MRR 0.55 [95% CI 0.34-0.89]). Early BCG reduced mortality from birth to 2 months by 27% (MRR 0.73 [95% CI 0.5-1.05]). Early BCG reduced mortality from birth to 6 months by 22% (MRR 0.78 [95% CI .058-1.06]).	All participants LBW. Same sample for a Vitamin A trial, but no interaction between BCG and Vitamin A reported. BCG Danish strain used. Analysed for sex-related differences. Vaccines other than DTP not stated.
Biering-Sorensen, 2012, Guinea- Bissau (19)	51 BCG at birth (early group) 54 Delayed BCG (controls)	After 6 weeks of age: DTP, OPV	Up to 12 months	Early BCG reduced infant mortality by 59% (MRR 0.41 [95% CI 0.14-1.18]). *Adjusted results by age:* Early BCG reduced mortality: within the first 3 days of enrolment (before 4 weeks of age) by 83% (MRR 0.17 [95% CI 0.02-1.35]). from birth to 4 weeks by 72% (MRR 0.28 [95% CI 0.06-1.37]). from birth to 2 months by 73% (MRR 0.27 [95% CI 0.07-0.98]). from birth to 6 months by 54% (MRR 0.46 [95% CI 0.15-1.35]).	All participants LBW. Vaccines other than DTP and OPV not stated. BCG strain not stated.

Author, year, location (reference)	Groups	Other vaccines	Age at outcome	Outcome	Comments
Biering-Sorensen, 2017, Guinea-Bissau (18)	2083 BCG at birth (early group) 2089 Delayed BCG (controls)	6,10 and 14 weeks: DTP, HBV, Hib 9 months: yellow fever vaccine	Up to 4 weeks Up to 12 months	Early BCG reduced neonatal mortality by 30% (MRR 0.70 [95% CI 0.47-1.04]). Early BCG reduced infant mortality by 12% (MRR 0.88 [95% CI 0.67-1.13]).	All participants LBW. BCG Denmark strain. Other vaccines not stated but assumed MV at 9 months and OPV at birth. There was no sex-related influence on BCG effects on neonatal or infant mortality.

Metanalyses

Author, year, location (reference)	Studies	Other vaccines	Age at outcome	Outcome	Comments
Higgins, 2016 (9)	5 clinical trials 8 cohort studies 1 case control study	In all studies and variable vaccine and timing in relation to each other	Variable range 1 month to 5 years	BCG reduced average all-cause mortality by 30% compared with BCG-unvaccinated children (average RR 0.70 [95% CI 0.49-1.031]) from the analysis on the trials data. BCG reduced average all-cause mortality by 53% compared with BCG-unvaccinated children (average RR 0.47 [95% CI 0.32-0.69]) from the analysis of 8 cohort studies and 1 case-control study.	Assessment of risk of bias in studies using a standardised tool. 4 cohort studies were excluded due to very high risk of bias. BCG strains varied among the studies. Age of BCG administration varied between studies.
Biering-Sorensen, 2017, Guinea-Bissau (18)	3 RCTs from Guinea-Bissau (see above)			Early BCG associated with 38% reduction in neonatal mortality and 16% reduction in infant mortality.	All participants LBW.

Abbreviations: ALRI = acute lower respiratory infections; CI = confidence interval; DTP = diphtheria-tetanus-pertussis vaccine; g = grams; HBV = hepatitis B vaccine; Hib = *Haemophilus influenzae* type b vaccine; HR = hazards ratio; IPV = inactivated polio vaccine; LBW = low-birth-weight (<2500 grams); MRR = mortality rate ratio; MV = measles vaccine; OPV = oral polio vaccine; RCT= randomised control trial; RR = relative risk

Evidence for BCG-induced effects on childhood morbidity

Several studies have investigated the influence of BCG on the outcomes of non-infectious diseases, infections with non-tuberculous mycobacteria (NTM) and various viruses, protozoa and infections unrelated to TB (Tables 2.3 and 2.4). The non-infectious conditions that have been investigated in this context include childhood malignancy, allergy, growth and psychomotor development (Table 2.5).

Cross-protective effects of BCG against non-tuberculous mycobacteria (NTM)

The influence of BCG on immunity to NTM in adults and children has been comprehensively reviewed (45). Cohort studies in Sweden, the Czech Republic and Finland showed a protective effect of BCG from infections with NTM in children (16, 46-48).

For Buruli ulcer disease, which is caused by *Mycobacterium ulcerans* (*M. ulcerans*), there are few studies exclusively in children. In one of the largest case control studies from three different countries, however, more than half of the sample comprised children (49). Unfortunately, this study has several limitations: (i) children were more common amongst cases than controls, (ii) BCG status was assessed by the presence of a scar, which is not a reliable measure of vaccination status, and (iii) various BCG strains were used. A higher level evidence from a RCT in Uganda showed a benefit from BCG for infection with *M*. *ulcerans*, which waned over time (50). This study included adults and randomised participants to BCG immunisation at a time they would not routinely have received the vaccine. The effects of other vaccines and unmeasured confounders could therefore not be assessed. Therefore, evidence for a protective effect of BCG from *M. ulcerans* in children remain inconclusive.

BCG protects against leprosy, caused by *M. leprae,* as shown in a RCT exclusively in children exposed to *M. leprae* in an endemic area (51). Setia et al. (52) did a meta-analysis of seven trials, five cohort studies and 14 case-control studies on the role of BCG in leprosy prevention. Five of the seven trials and two of the case-control studies analysed paediatric-specific data. Analysis of the observational studies indicated that BCG was most protective for children under 10 years, offering 76% protection against leprosy (summary estimate 0.24 [95% CI 0.11-0.53]). For those under 15 years, however, protective efficacy was not significantly different from those older than 15. Overall, the studies differed in design, BCG strain used and in population exposures to leprosy that potentially influenced results. Nevertheless, these studies together provide useful information on the protective effects offered by BCG against leprosy, especially in children.

BCG effects on immunity to unrelated infections and hospitalisation

Few studies have investigated the effects of prior BCG vaccination on specific or laboratory-confirmed unrelated (i.e., non-mycobacterial) infections (Table 2.3). Three cohort studies reported BCG influences on respiratory syncytial virus (RSV) (13), visceral leishmaniasis (15) and *Plasmodium falciparum* antigenaemia (53).

Stensballe et al. (13) studied measles-unvaccinated children under the age of 5 years in Guinea-Bissau and showed that BCG vaccination of infants was protective against acute lower respiratory tract infections (ALRTIs) caused by respiratory syncytial virus (RSV). The results of the primary analysis did not reach statistical significance. When the data were

analysed for other variables, however, BCG vaccination offered statistically significant protection against RSV ALRTIs in the rainy season, especially for girls.

Childhood vaccination, including BCG, was associated with a decrease in the incidence of visceral leishmaniasis in children under 5 years in Brazil (15). During this cohort study, the overall incidence of this disease decreased, due to several other public health interventions to increase child health at the time.

Hollm-Delgado et al. (53) investigated the influence of vitamin A and immunisations, including BCG, on the risk of *Plasmodium* infections in children aged 6 to 59 months in four Sub-Saharan countries. They reported that children who were vaccinated with BCG were more likely to have *Plasmodium falciparum* (*P. falciparum*) antigenaemia, measured by the rapid *P. falciparum* histamine rich protein-2 test in capillary blood samples, than BCG-unvaccinated children. There was no association with BCG immunisation and *Plasmodium* parasitaemia, but the absolute numbers were small (Table 2.3).

Several potential confounders and risks of bias were common to all cohort studies in these settings. For example, all infants received other vaccines during the study periods, making any analysis of isolated effects of BCG immunisation impossible. In addition, there is little evidence of BCG effects on specific or laboratory -confirmed unrelated infections in children, specifically RCTs, which could eliminate some sources of bias.

Table 2.3: Studies of BCG effects on specific infections other than tuberculosis

Infection	Author, year, location (reference)	Study design	Age at BCG immunisation	Age at outcome	Outcome	Comment
Non-tuberculous mycobacteria						
Extrapulmonary	Romanus, 1995, Sweden (47)	Retrospective and prospective cohort 390 cases	Mostly given in neonatal period	Under 15yrs	BCG decreased incidence of extrapulmonary NTM infections	BCG Sweden/Denmark strain. Hospital record review for BCG vaccination status. Results reported according to period of birth.
MAC lymphadenitis	Trnka, 1994, The Czech Republic (46)	Retrospective cohort 27 cases	At birth	Children	BCG reduced incidence of MAC lymphadenitis	BCG Russia strain.
Lymphadenitis	Katila, 1987, Finland (16)	Retrospective cohort 33 cases	Neonatal	1-13 years	BCG reduced the risk of NTM lymphadenitis	BCG Sweden/Denmark strain. BCG status from record review.
NTM infection	Kontturi, 2018, Finland (48)	Retrospective cohort 97 cases	Assumed at birth	Under 5 years	BCG decreased the incidence of NTM infection in children	BCG strain not stated. Estimated BCG coverage from BCG vaccine policies.
Buruli ulcer (*M. ulcerans*)	Phillips, 2015, DRC, Ghana and Togo (49)	Retrospective case control 401 cases 826 controls	At birth or within 12 months	Adults and children median age 13yrs	BCG offered protection from Buruli ulcer disease	Various BCG strains used in the different countries. Results were stratified for country, sex and age. BCG status assessed by presence of scar. Cases were younger than controls.
Buruli ulcer	Smith, 1976, Uganda (50)	RCT 2775 BCG compared with 2764 no BCG (control) followed up for 1 years	At any age if no evidence of BCG vaccination at start of trial	Adults and children	BCG vaccination reduced the risk of Buruli ulcer disease by 47%	Effect was only seen for patients with <2 mm TST reaction. Effect reduced with time.

Infection	Author, year, location (reference)	Study design	Age at BCG immunisation	Age at outcome	Outcome	Comment
Leprosy (*M. leprae*)	Stanley, 1981, Uganda (51)	RCT 8085 BCG compared with 8065 no BCG (control) Followed up for 8 years	At any age if no evidence of BCG vaccination at start of trial	Children up to 10 years	BCG vaccination was associated with an 81% reduction in leprosy compared with controls	BCG Denmark strain. The protective effect was seen throughout the study period. All participants were relatives or close contacts of patients with leprosy. Randomised process was each alternate child.
Virus						
RSV	Stensballe, 2005, Guinea-Bissau (13)	Case control 17/155 cases compared with 9/155 controls not BCG-vaccinated	Median age within the 1st month	Under 5 years	Infants with RSV-ALRTI were more likely to be BCG-unvaccinated (OR 2.6 [95% CI 0.93-7.29])	All infants who received MV were excluded. Results adjusted for other vaccines, season and other factors. Results analysed for influence of sex and presence of BCG scar.
Protozoa						
Leishmaniasis	Lima, 2018, Brazil (15)	Retrospective cohort	Not stated	Reporting for children under the age of 5 years	Decrease in incidence of visceral leishmaniasis associated with childhood vaccinations including HBV, polio and measles	Several vaccinations in children under 5 years old included in the analysis.
Malarial antigen	Hollm-Delgado, 2015, 4 countries in Sub-Saharan Africa (53)	Retrospective cohort 2102 children vaccinated compared with 9 unvaccinated tested positive from 6047 infants	Not stated	6-59 months	BCG-vaccinated children were more likely to have *Plasmodium falciparum* antigenaemia Adjusted RR 4.06 {95% CI 2.00-8.28])	Several potential confounder information collected. Other vaccines used. BCG effects were stronger at younger ages, longer duration since vaccination and if received in the wet season.

Abbreviations: CI = confidence interval; DRC = Democratic Republic of Congo; MAC = *Mycobacterium avium* complex; mm = millimetre; MV = measles vaccine; NTM = non-tuberculous mycobacteria; OR= odds ratio; RCT = randomised control trial; RR = relative risk; RSV = respiratory syncytial virus; TST = tuberculin skin testing

Four observational studies, including one case-control study and three retrospective cohort studies, as well as a RCT investigated BCG effects on general disease morbidity and hospitalisation (Table 2.4). The observational studies are heterogeneous in design and in the measure of reported outcomes. Three of these studies suggested a protective effect of BCG on ALTRI, not due to TB (13, 54, 55).

For children under the age of 5 years, a cohort study in Guinea-Bissau showed that BCG decreased the risk of ALRTIs in children with a more pronounced effect in girls and those vaccinated before 3 months of age where BCG offered a 26% (95% CI 15-35%) protection against ALRTI (13). Hollm-Delgado et al. (55) analysed health records and BCG status from >50,000 children, in over 30 countries over a 25-year period and showed that BCG vaccination at any time during childhood reduced suspected ALRTIs. A study in Spanish children (54) showed neonatally administered BCG decreased hospitalisations from respiratory illnesses, other than TB, by almost 42% in children up to 14 years of age; and for children under one year, the hospitalisation rate for sepsis was decreased by 53%.

By contrast, a study from Greenland (56) showed no effect of BCG vaccination on hospitalisation rates for infectious diseases or respiratory tract infections other than TB in children aged 3 months to 3 years. However, on further analysis of the study data, BCG vaccination decreased hospitalisation from infectious causes by 28% (95% CI -6 to 51%) for babies aged 3 days to 3 months (57).

RCTs designed to investigate the influence of BCG on morbidity and hospitalisation rates for children in high disease burden countries, with poor resources and high TB prevalence are not feasible for ethical reasons. Therefore, the only evidence for BCG effects on infections in a RCT comes from the Danish Calmette Study (58), where no association was found between neonatal BCG and parent-reported infections or rates of hospitalisation (59, 60). Only hospitalisation due to injuries were excluded. Secondary analysis of the data showed that in infants of BCG-vaccinated mothers, neonatal BCG vaccination was associated with a 35% reduction in parent-reported infections in the first 3 months of life and a 19% reduction in hospitalisation (HR 0.81 [95% CI 0.60-1.09]) (59, 60). Reasons for the discrepancy between the observational studies in high disease burden countries and Denmark are uncertain. However, results from the Calmette trial suggest that antenatal priming with BCG may influence BCG NSEs in neonates (59).

To summarise, BCG vaccine was designed for protection against TB. Accordingly, studies showing protective effects of BCG against NTM in children have biologic plausibility through cross-protective immunity to mycobacterial antigens. By contract, there is no clinical evidence for BCG effects on non-mycobacterial, laboratory confirmed infections in children, apart from the observational studies showing protection from leishmaniasis, laboratory confirmed malarial antigenaemia and RSV (13, 15, 53) none of which have been independently confirmed. More RCTs in different settings and studies investigating mechanisms than may explain the non-specific beneficial effects of BCG are needed. At the time of writing, there were no published studies on the effects of BCG on infectious disease morbidity in neonates - the most vulnerable group for negative outcomes from infections, although available evidence suggested possible benefit for children less than 5 years of age.

BCG effects on non-infectious conditions

The influence of BCG on allergic disease, childhood malignancy, growth and psychomotor development has been investigated (Table 2.5).

Allergic disease

A meta-analysis of several observational studies and two RCTs describe the evidence for and against NSEs of BCG on allergic disease in children.

Arnoldussen et al. (61) analysed 17 studies and found a 31% (95% CI 0.7%-60%) increase in sensitisation to common allergens as measured by specific immunoglobulin E (IgE) and no appreciable overall difference in rhinoconjunctivitis in those vaccinated with BCG compared with BCG-unvaccinated controls (OR 1.07 [95% CI 0.89-1.28]). These authors also reported a 16% (95% CI -9%-36%) reduction in eczema/atopic dermatitis and a 27% (95% CI 5%-44%) reduction in asthma in those vaccinated with BCG compared with unvaccinated controls. For asthma, when results were adjusted to include higher quality studies, the protective effect of BCG was lower. The studies they analysed were heterogeneous and there was insufficient evidence from pooled analyses for BCG effects on food allergy. Sub-analysis for those with an increased risk of allergic disorders did not change the outcomes (61). Higher level evidence from RCTs are needed, especially for food allergy and severe allergic disorders in childhood.

More recently, analyses of data from the Danish Calmette trial showed that neonatal BCG decreased atopic dermatitis in children with an atopic predisposition by 16% (95% CI 5%-26%) (62), but no beneficial or deleterious effects were found on recurrent wheeze (63), suspected food allergy or allergic sensitisation, as measured by IgE blood testing (64). Unfortunately, the number of participants in the latter cohort was too low to detect differences between the groups. This trial also had potential for reporting bias as it was not possible to blind participants to their study allocation. Nevertheless, it is the largest trial to date, including thousands of children, investigating neonatal BCG effects on a range of conditions (58). Together, the evidence for BCG effects on allergic disorders provided by these studies in children remains inconclusive and further results are expected soon from the MIS BAIR trial (65). At the time of writing there were no studies investigating the effect of BCG on neonatal allergic disorders, such as cow milk protein allergy.

Childhood malignancy

The first reports from epidemiological studies on BCG having a protective effect on mortality from childhood leukaemia were published in the 1970s. Researchers claimed a nearly 50% reduction in leukaemia mortality in BCG-vaccinated children under 15 years of age (66). A targeted review and meta-analysis on the effect of early vaccination on leukaemia was done in 2017 (67). In a sub-analysis investigating the effect of early BCG on leukaemia, 12 studies, including two trials and ten observational studies were analysed. The collective results showed a 27% (95% CI -0.08%-50%) reduction in childhood leukaemia for those vaccinated with BCG compared with those who were unvaccinated. Further analysis showed similar results for those vaccinated before one year of age and for those vaccinated later (67). The studies included were largely observational, heterogeneous in design with several unmeasured confounders that may have biased the outcome.

Hodgkin lymphoma is rare in young children but is the most common malignancy in adolescents. Non-Hodgkin lymphoma (NHL), by contrast, is more common in younger children. A recent meta-analysis investigating the effect of BCG on lymphoma risk included 11 studies and reported no association of BCG on Hodgkin lymphoma, but a potential increased risk of NHL in those vaccinated with BCG (OR 1.2 [95% CI 1.10-1.43]) compared with those BCG-unvaccinated (68). Salmon et al. (69) previously found no association between BCG and either lymphoma type in Canadian lymphoma sufferers. However, in a sub-analysis of children under 18 years, BCG vaccination increased the risk of Hodgkin lymphoma (HR [hazard ratio] 2.26, [95% CI 1.39-3.69]) in that cohort.

These results are inconclusive, and more studies are needed to determine the effects, if any, of BCG on childhood malignancies. Biological plausibility for BCG effects on malignancy are incompletely understood, and further trials could investigate potential mechanisms of the observed effects, assuming they are borne out.

Growth, haemoglobin concentration and psychomotor development

One cohort study and one trial reported the effects of BCG on children's growth and psychomotor development, respectively.

Berendsen et al. (70) investigated the effect of BCG on stunting and haemoglobin concentration in children under age 5 years. Overall there was no association between BCG and stunting in Sub-Saharan children (OR 1.00 [95% CI 0.98-1.03]). Timing of BCG vaccination appeared to be an important factor, with those given BCG in the first 6 weeks of life being less stunted than those who were BCG-unvaccinated, but those administered BCG later in infancy were more stunted than BCG-unvaccinated children. A sub-analysis by age showed that neonates vaccinated with BCG were less stunted than those BCG-unvaccinated (OR 0.92 [95% CI 0.89-0.94]). The association with BCG and haemoglobin concentration followed a similar pattern to the timing of vaccination (70), with children vaccinated with BCG early having higher haemoglobin concentrations compared with BCG-unvaccinated infants. When vaccinated later, the children have lower haemoglobin concentrations compared with BCG-unvaccinated infants. As with all observational studies, there is potential for bias and unmeasured confounders, due in part to the failure to measure the isolated effect of BCG vaccine on the stipulated outcome of interest.

Results from the Danish Calmette trial showed no effect of neonatal BCG on infant psychomotor development in children at 12 months of age as measured by Ages and Stages Questionnaires (ASQ) (64, 71). Similar results were reported when analysed at other time points and with respect to sex and prematurity.

In summary, evidence for the effects of BCG immunisation on all these non-infectious disease outcomes – allergic disease, malignancy, stunting, haemoglobin concentration and psychomotor development – are currently inconclusive. At the time of writing, there were no neonatal studies for any non-infectious disease outcome and limited evidence for any plausible mechanisms.

Table 2.4: Studies of BCG and general infection and hospitalisation in children

Author, year, location, (reference)	Study design	Age at vaccination	Age at outcome	Outcome	Comment
Observational studies					
Stensballe, 2005, Guinea-Bissau (13)	Case-control study 36/386 cases compared with 17/386 BCG-unvaccinated controls	Median age within the 1st month	Under 5 years	Infants with ALRTI were more likely to be BCG-unvaccinated (OR 2.73 [95% CI 1.37-5.44])	All infants who received MV were excluded. Results adjusted for other vaccines, season and other factors. Results analysed for influence of sex and presence of BCG scar. ALRTI was diagnosed clinically.
Hollm-Delgado, 2014, 33 countries (55)	Retrospective cohort 58,021 survey participants BCG-vaccinated compared with BCG-unvaccinated	Median age was 1 month	Under 5 years	BCG decreased suspected ALRTI by 17-37% overall	The order of DTP and BCG and vaccine strain modified the effect. No country used Hib or pneumococcal vaccines. Suspected ALRTI was diagnosed clinically. Results adjusted for several variables.
Haahr, 2016, Greenland (56)	Retrospective cohort 19,363 records reviewed BCG-vaccinated compared with BCG-unvaccinated	Neonatal	3 months to 3 years	No differences in hospitalisation rates: for infectious diseases IRR 1.07 (95% CI 0.96-1.2) or for respiratory tract infections IRR 1.1 (95% CI 0.98-1.24)	Other vaccines in the schedule include OPV, Pertussis, DTP, Hib, MMR. Results adjusted by sex and ethnicity.
de Castro, 2015, Spain (54)	Retrospective cohort 464,611 hospital admission records reviewed BCG-vaccinated compared with BCG-unvaccinated	Neonatal	Up to 14yrs	Neonatal BCG significantly decreased hospitalisation due to respiratory illness, other than TB, in all age groups with a total PF of 41.4% (95% CI 40.3-42.5) Neonatal BCG significantly decreased hospitalisation rate due to sepsis with a total PF of 52.8% (95% CI 43.8-60.7) in children under 1 year old	Results analysed by age groups. Disease categories identified by hospital codes. Results reported as preventive fractions, calculated as 1-odds ratio. Other vaccines given according to Spanish schedule during the study.

Author, year, location, (reference)	Study design	Age at vaccination	Age at outcome	Outcome	Comment
Randomised control trial: The Danish Calmette Study (58)					
Kjaergaard, 2016, Denmark (59)	2129 BCG-vaccinated compared with 2133 BCG-unvaccinated	7 days	Under 3 months	No benefit of neonatal BCG on parent-reported infections at either time point <3 months: IRR 0.87 (95% CI 0.72-1.05)	BCG Denmark strain. Other vaccines as per Danish immunisation schedule during study period used.
			3-13 months	3-13 months: IRR1.02 (95% CI 0.97-1.07)	Telephone interviews for data collection. BCG may have reduced infections in children aged 0-3 months whose mothers had BCG vaccination by 35% IRR 0.62 (95% CI 0.39-0.98) with number needed to vaccinate of 14.
Stensballe, 2017, Denmark (60)	2129 BCG-vaccinated compared with 2133 BCG-unvaccinated	7 days	Up to 15 months	No beneficial effect from BCG on rate of all-cause hospitalisation for disease HR 1.05 (95% CI 0.93-1.18)	BCG Denmark strain. Other vaccines as per Danish immunisation schedule during study period used. Hospitalisations were identified using the national register. Hospitalisation due to injuries excluded. Trend for lower risk of hospitalisation in infants whose mothers received BCG (effect modification between groups; p =0.05).

Abbreviations: ALRTI = acute lower respiratory tract infection; CI = confidence interval; DTP = diphtheria-tetanus-pertussis vaccine; Hib = *Haemophilus influenzae* type b vaccine; HIV = human immunodeficiency virus; HR = Hazard ratio; IRR = incidence rate ratio; MMR = measles, mumps, rubella vaccine; OPV = oral polio vaccine; OR = odds ratio; PF = preventive fraction; RCT= randomised control trial; TB = tuberculosis

Table 2.5: Evidence for and against non-specific effects of BCG on non-infectious conditions

Disease	Author, year, Location	Study design	Age BCG administered	Age at outcome	Outcome	Comment
Allergic disease (reference)						
Allergic disease (61)	Arnoldussen, 2011, High and low-income countries	Meta-analysis of 1 RCT, 6 retrospective and 1 prospective cohort studies, 7 cross-sectional studies and 2 case control studies.	Under age 5 years	Varied	For BCG-vaccinated compared with BCG-unvaccinated controls: Allergic sensitisation as measured by specific IgE levels (5 studies): OR 1.31 (95% CI 1.07-1.60) Eczema/atopic dermatitis (9 studies): OR 0.84 (95% CI 0.64-1.09) Asthma (13 studies) OR 0.73 (95% CI 0.56-0.95) Rhinoconjunctivitis (9 studies) OR 1.07 (95% CI 0.98-1.29)	Only one RCT included. Heterogenous studies.
Asthma/wheeze (63)	Thostesen, 2017, Denmark	RCT 2129 BCG-vaccinated compared with 2133 BCG-unvaccinated	Within 7 days of birth	Up to 13 months	For BCG-vaccinated compared with BCG-unvaccinated controls: Recurrent wheeze: RR 1.07 (95% CI 0.89-1.28)	Telephone interview/physician reviews to assess recurrent wheeze. No effect modifiers apart from an increased risk of recurrent wheeze in children who had no older siblings.
Allergic sensitisation and suspected food allergy (64)	Thostesen, 2017, Denmark	RCT Sensitisation: 743 BCG-vaccinated compared with 627 BCG-unvaccinated Food allergy 2109 BCG-vaccinated compared with 2079 BCG-unvaccinated (controls)	Within 7 days of birth	13 months	BCG-vaccinated compared with controls Sensitisation: RR 0.94 (95% CI 0.64-1.36) Food allergen sensitisation RR 0.97 (95% CI 0.68-1.39)	Various common allergens tested for sensitisation Analysed the correlation between allergic disease and food sensitisation

Disease	Author, year, Location	Study design	Age BCG administered	Age at outcome	Outcome	Comment
Atopic dermatitis (62)	Thostesen, 2018, Denmark	RCT 2052 BCG-vaccinated compared with 1952 BCG-unvaccinated (controls)	Within 7 days of birth	Up to 15 months	BCG-vaccinated compared with controls RR 0.9 (95% CI 0.8-1.00)	Neonatal BCG decreased atopic dermatitis in children with atopic predisposition RR 0.84 (95% CI 0.74-0.95).
Malignancy (reference)						
Leukaemia (67)	Morra, 2017, Various	Meta-analysis of 2 clinical trials and 10 observational studies,	Childhood	Childhood	BCG-vaccinated compared with controls OR 0.73 (95% CI 0.50-1.08)	Isolated effects of BCG given early not possible due to other vaccines given during follow up periods. Observational studies have many confounders and potential for bias. Limited studies prevent a meta-analysis of leukaemia subtypes.
Lymphoma (68)	Salmon, 2020, Various	Meta-analysis of 2 clinical trials and 9 observational studies	Varied and not available for all included studies	Varied, one case-control study included adolescent age group	Hodgkin lymphoma: BCG-vaccinated compared with controls in 7 studies: Fixed effect summary OR 1.10 (95% CI 0.93-1.3) Non-Hodgkin lymphoma: BCG-vaccinated compared with controls in 11 studies: OR 1.2 (95% CI 1.01-1.43)	Results statistically underpowered. Marked heterogeneity among studies. Only 4 studies reported age of BCG vaccination and in too few a number to allow meta-analysis.

Disease	Author, year, Location	Study design	Age BCG administered	Age at outcome	Outcome	Comment
Infant growth and psychomotor development (reference)						
Infant Growth and haemoglobin concentration (70)	Berendsen, 2016, 33 Sub-Saharan countries	Retrospective cohort study and cross-sectional data review BCG-vaccinated compared with BCG-unvaccinated controls	Before age 12 months	Up to 5 years	Stunting: BCG-vaccinated compared with controls OR 0.76 (95% CI 0.74-0.77). Haemoglobin concentration: Higher concentrations seen with BCG vaccination <1 month, but lower if given after 7 months compared with BCG-unvaccinated controls.	Results controlled for several variables. Effect of BCG timing showed less stunting when BCG given earlier in life. When given <1 month of age OR 0.92 (95%CI 0.89-0.94). When BCG given later in life the odds were higher or stunting compared with BCG-unvaccinated controls.
Psychomotor Development (72)	Kjaergaard, 2016, Denmark	RCT 2129 BCG-vaccinated compared with 2133 BCG-unvaccinated	Within 7 days	Up to 13 months	BCG does not negatively influence ASQ outcomes in term babies. The mean difference in ASQ points between vaccinated and unvaccinated children at 12 months was -0.7 (95% CI -3.7-2.4) p = 0.67.	Excluded preterm babies < 32/40. Measured development using ASQ. Results adjusted for age and prematurity.

Abbreviations: ASQ = Ages and Stages Questionnaires; CI = confidence interval; IgE = immunoglobulin E class; OR = odds ratio; RCT = randomised control trial; RR = relative risk

Evidence for BCG as a therapeutic immunomodulator

Further evidence for NSEs of BCG come from studies that investigated its therapeutic immunomodulatory effect. A few studies in children have shown that BCG can successfully treat human papillomavirus (HPV) verrucae infections (14), and as a component in the treatment for paediatric acute non-lymphoblastic leukaemia (73).

Studies in adults of the immunomodulatory properties of BCG have shown beneficial effects with topical treatment of infections unrelated to TB, including leishmaniasis (74, 75) and HPV (76, 77). Success with systemic BCG has also been shown in adults in the management of herpes simplex virus (HSV) (78) (Table 2.6).

For non-infectious conditions, in adults, beneficial BCG immunomodulatory properties have been exploited in the treatment of various malignancies including melanoma (79) and bladder cancer, where BCG is now well established as a therapeutic adjuvant treatment for certain grades of these cancers (80, 81).

The therapeutic utility of BCG has been extensively reviewed in relation to type 1 diabetes mellitus (T1DM). Yu-Chen et al. (82) performed a meta-analysis of the four RCTs comparing the glycaemic control of participants with T1DM randomised to receive BCG or not. All four studies included children and adults ranging in age from 10 to 36 years. The authors reported a tendency for improved glycaemic control in those receiving BCG, but the difference was not statistically significant. The limited number of trials and paediatric participants did not allow for a meaningful analysis of the effects of BCG on T1DM in children.

To summarise, there are few studies in children describing BCG as a therapeutic immunomodulator. The conditions treated have been varied and the mechanisms to explain any effects are not known.

Table 2.6: Studies of the use of BCG as an immunomodulatory treatment for infectious diseases

Disease (reference)	**Author, year,**	**Study design, Location**	**Therapy**	**Age**	**Outcome**
Cutaneous leishmaniasis (74)	Convit, 1987	RCT, Venezuela	Topical BCG	Adults	BCG as effective as standard chemotherapy
Human papilloma virus (14, 76)	Salem, 2013	RCT, Egypt	Topical BCG	3-14yrs	BCG group better outcome compared with placebo controls
	Metawea, 2005	RCT, Egypt	Topical BCG	Adults	High success rate with BCG compared with placebo controls
Herpes simplex virus (78)	Anderson, 1974	Case-control, USA	Intradermal BCG vaccination	Adults	BCG immunisation decreased and occasionally stopped recurrent genital herpes reactivation

Abbreviation: RCT = randomised control trial

2.2.2 In-vitro measurement of the non-specific immunological effects of BCG

Most laboratory studies report on multiple immune outcomes simultaneously. Commonly, studies investigating immune function have measured antibody titres and cytokine/chemokine responses to certain stimulants, as proxies for B-cell and innate/T-cell immune function, respectively. The most common cytokines investigated in these studies have been TNF-α and IFN-γ (31). Methods used to measure cytokines include enzyme-linked immunoassays (ELISA), bead-based arrays and multiplex arrays; the last with the advantages of investigating multiple cytokines in a single sample and a requirement for smaller blood volumes. Cellular staining and assays of gene expression have the added advantage of being able to identify the cell type responsible for cytokine production, as many immune cells are polyfunctional with respect to this. However, these tests are seldom performed in studies of cytokine responses. Because of this, the cells of origin are presumed based on previous work describing the predominant cytokine produced by various types of immune cells. Technological advances have permitted sophisticated interrogation of immunity using systems biology tools including genomics, transcriptomics, proteomics and metabolomics allowing increasing complexity of research questions (83, 84).

Here, I review the evidence for the NSEs of BCG in response to stimulation of immune cell with unrelated stimulants. The studies are grouped by whether they measured cytokines or antibodies in children and neonates (Table 2.6).

Evidence for BCG's non-specific effects on cytokine responses to unrelated stimulants

At the time of writing, there were seven studies, including five RCTs and two nested case-control studies reporting on the influence of BCG on cytokine responses to unrelated antigens in children (Table 2.7). Together, the studies measured several different cytokines/chemokines, but there was only one cytokine, IFN-γ, that was common to all studies. Stimulant choice also varied between studies and there was no common stimulant for all studies.

Three of the studies investigated the cellular origin of the cytokines produced in an attempt to classify BCG effects acting on either the adaptive or innate arm of immunity (85-87). The other studies presumed or did not report the cellular origin of the cytokines.

Ota and colleagues were among the first to investigate the influence of BCG on cytokine responses to unrelated stimulants in Gambian children in a prospective RCT (88). They showed that BCG at birth enhanced presumed T helper 1 (TH1) and T helper 2 (TH2) responses to unrelated stimulants as early as 2 months of age. Similar findings were observed by Libraty et al. (85) in a nested case control study of 51 infants in the Philippines, where neonatal BCG enhanced TH1-mediated IFN-γ responses to vaccine antigens after 2-3 months. These findings were supported by a RCT in The Gambia confirming that a TH1-derived IFN-γ response to an unrelated stimulant was enhanced following BCG administration at 6 weeks of age. Interestingly, these authors also showed that these effects were measurable 1 week after vaccination (87). In contrast, a RCT in Denmark of 158 babies showed no differences in presumed T-cell-derived cytokine responses to unrelated stimulants at 3 or 13 months of age in babies given BCG at birth (89).

In an exploratory case control study on innate immune responses in 11 United Kingdom (UK)-born infants given BCG at 6 weeks of age, Smith et al. (86) showed that BCG vaccination either enhanced or reduced cytokine responses compared with BCG-unvaccinated controls, depending on the stimulant. They found that BCG enhanced natural killer (NK) cell expression of IL-12p40 cytokine in response to stimulation with Pam3CSK4 (a synthetic triacylated lipopeptide and a TLR2/TLR1 ligand that mimics the structure of lipopeptides in bacterial cell walls). Jensen et al. (90) showed that BCG was associated with an increase in monocytes, but did not investigate whether increases in any cytokine responses to unrelated stimulants originated from innate immune cells, though stimulants used in their study were known innate cell simulants. Nissen et al. (89) reported no differences in presumed innate immune responses to unrelated stimulants in 80 infants vaccinated with BCG at birth compared with 78 BCG-unvaccinated babies 4 days after randomisation.

These studies report discrepant results that may be explained by differences in study design. Table 2.7 shows the variation in the age of participants receiving BCG, age of participants when outcomes were measured, other vaccines administered before outcomes were measured, stimulants used, cytokines measured and geographic location. Only one trial included an unvaccinated control group (89). Therefore, results reported in most studies may be due to the interplay of various vaccines in childhood rather than an effect of BCG alone. Few modifications for factors affecting immune responses were analysed and, when performed, were inconsistent between the trials. The results of these studies suggest that BCG-induced effects on cytokine responses to unrelated stimulants depend on the stimulant and may either enhance or reduce responses. However, there were insufficient matched cytokine-stimulant pairs across the studies to allow for comparison. From these results, it is not possible to attribute BCG NSEs to changes in either the adaptive or innate immunity arm. Only three of these RCTs reported outcomes for neonates (21, 89, 90), and their findings were inconsistent. More RCTs are needed that include an investigation of the cellular origin of the cytokines measured.

Evidence for BCG's non-specific effects on antibody responses to unrelated stimulants

Researchers have also investigated the non-specific influence of BCG on B-cell immune function in children by measuring immunoglobulin G (IgG) antibody production to unrelated stimulants (Table 2.8). Of six studies in children, three were RCTs, one was a non-randomised trial and two were nested cohort studies. The unrelated stimulants were other vaccine antigens.

In a landmark RCT investigating the influence of BCG on antibody production, Ota et al. (88) showed that BCG at birth enhanced antibody production to unrelated vaccine antigens in Gambian children aged 2 months and 4.5 months. They suggested that this effect was influenced by the timing of BCG in relation to other vaccines. In nested cohort studies in our laboratory in Melbourne, Ritz et al. (91) and Zimmerman et al. (92) confirmed that the non-specific enhanced or reducing effects of BCG on antibody production to unrelated vaccine antigens depends on the vaccine antigen, even though the immunisation schedules in Australia and The Gambia countries were quite different, both in terms of when BCG and other vaccines are administered, as well as the BCG strain that was used (Table 2.8).

Other RCTs in high disease-burden countries failed to show an effect of BCG on antibody production to unrelated vaccine antigens (87, 93). A study in South African infants included some immunised with BCG Denmark at 6 weeks of age and measured antibody levels at 14, 24 and 52 weeks of age. The study in The Gambia included infants immunised with BCG Russia at 6 weeks of age and measured antibodies at 7 and 18 weeks of age. The immunisation schedules in these two studies differed between each other and also between The Gambian infants in the earlier study by Ota et al. (88). Similarly, a nested control study in Denmark (94), where BCG Denmark was administered at birth, found no effect of BCG on antibody production to unrelated vaccines at 13 months of age. Once again, the immunisation schedule used in this study differed from those in the other studies (Table 2.8).

Differences in study design prevents direct comparison of their outcomes. For example, immunisation schedules differ around the world, resulting in different comparative control groups. Moreover, the age of participants receiving BCG, and their age when the outcome was measured differed, as did the BCG strain they received and the vaccine antigens and modifiers that were assayed (Table 2.8). None of the studies reported outcomes for neonates.

In all the studies, infants in the BCG and control groups showed protective antibody levels to the vaccine antigens that were measured. Because BCG effects were seen in some studies, but not in others, however, the overall question regarding possible effects of BCG on antibody responses to unrelated stimulants remain unresolved.

A summary of the progression of studies investigating BCG NSEs with clinical and laboratory outcome measures are shown in Figure 2.1.

Table 2.7: Studies investigating the influence of BCG on cytokine responses to unrelated stimulants in children

Author, year location (reference)	Study design	Age at outcome	Stimulants	Cytokines	Results	Comments
Neonates only						
Jensen, 2015, Guinea-Bissau (90)	RCT in LBW babies 260 BCG at birth compared with 207 BCG at 6 weeks (unvaccinated control)	4 weeks	Negative control: RPMI Positive control: PMA BCG-related stimulants: PPD BCG unrelated stimulants: LPS Pam3CSK4 CLO-75	IL-1ß IL-5 IL-6 IL-10 IL-17 IFN-γ TNF-α	BCG enhanced cytokine responses: Statistically significant for IL-1ß, IL-6, TNF-α and IFN-γ to Pam3CSK4. BCG had no effect on IL-5 or IL10 responses to any simulants. BCG was not associated with a reduced response to any cytokines. BCG increased cytokine responses in unstimulated samples (RPMI).	BCG Denmark strain. All babies received OPV at birth. BCG effect on white blood cell differential count: Overall BCG was associated with borderline increase in monocytes. Cell of origin of cytokine presumed, but stimulants known to stimulate innate cells. 24 hr stimulations in whole capillary blood. Cytokines measured by multiplex assay.
Freyne, 2018, Australia (21)	RCT in 212 babies Numbers for each stimulant varied between 69-212 BCG in the first week of life compared with BCG-unvaccinated (controls)	3-22 days	Negative control: RPMI BCG-related stimulants: BCG *M. tuberculosis* BCG unrelated stimulants: GBS *S. aureus* *Str. pneumoniae* *E. coli* *H. influenzae* *L. monocytogenes* *C. albicans* LPS PEPG Pam3CSK4 R848	IFN-γ TNF-α IL-1ß IL-6 MIF MIG MCP-1 IL-8 MIP-1α MIP-1ß IL-1RA IL-10	BCG reduced most responses: strongest for IL6, IL-10, MIP-1α, MIP- 1ß and MCP-1 to PEPG and R848 and IL- 1RA, MCP-1 and MIP-1ß to *S. pneumoniae, E. Coli, H. influenzae, L. monocytogenes* and *C. albicans.* BCG enhanced IL-6, IL-1ß and IL-1RA in unstimulated samples (RPMI).	BCG Denmark strain. All babies received HBV at birth. BCG interacted with sex for specific cytokine-stimulant responses. Maternal BCG status: evidence for an interaction with infant BCG status for certain cytokine-stimulant pair responses. Timing of vaccine: BCG effects were stronger in late vaccine group (>48 hrs post birth). 20 hr stimulation in whole venous blood. Cytokines measured by multiplex assay.

Author, year location (reference)	Study design	Age at outcome	Stimulants	Cytokines	Results	Comments
Neonates and infants						
Nissen, 2018, Denmark (89)	RCT in 158 babies 80 BCG within 7 days of life compared with 78 BCG-unvaccinated (controls)	4 days 3 months and 13 months post randomisation	Negative control: RPMI Positive control: PHA BCG-related stimulants: BCG BCG unrelated stimulants: *S. pneumoniae* *E. coli* *C. albicans* LPS	TNF-α IL-1ß IL-6 IFN-γ IL-17 IL-22 IL-10	Overall: no effect of BCG on cytokine responses to unrelated stimulants at all time points. BCG vaccination tended to reduce IL-10 responses to all stimulants except BCG.	BCG Denmark strain. All babies received additional combination vaccines: DTaP-IPV-Hib or DTaP-IPV-Hib-HBV and PVC-13 at 3, 5 and 12 months. Data analysed for influence of age at randomisation, sex, maternal BCG status, time from vaccination to day 4 bleeding. Babies vaccinated with BCG day 2-7 had higher responses in certain cytokine-stimulant pairs in the 4-day samples. Cytokines cell of origin presumed innate immune cell derived: I.e., TNF-α, IL-1ß and IL-6 from monocytes and IFN-γ, IL-17, IL-22 and IL-10 from lymphocytes. 24 hr stimulation in whole venous blood for TNF-α, IL-1ß and IL-6, 96 hr stimulation for other stimulants. Cytokines measured by ELISA.
Infants only						
Ota, 2002, The Gambia (88)	Prospective RCT in 151 babies Group 1: 35 BCG at birth Group 2: 34 BCG at 2 months Group 3: 34 BCG at 4.5 months	2 months 4.5 months	Negative control: RPMI Positive control: PHA BCG-related stimulant: PPD BCG unrelated stimulants: TT HBsAg	IFN-γ IL-5 IL-13	Babies vaccinated with BCG had enhanced cytokine responses to HBsAg at both time points. These were most marked for babies vaccinated with BCG at birth. BCG at 2 months did not significantly increase IFN-γ responses. Babies vaccinated with BCG had enhanced cytokine responses to TT. The pattern was dependent on time of vaccination with BCG.	BCG Pasteur strain. All babies received other vaccines as per recommended The Gambian schedule: OPV at birth, 1 and 2 months HBV at birth, 2 and 4 months DTP at 2, 3 and 4 months. Presumed cytokine cell of origin: TH1 (IFN-γ)/ TH2 (IL-5 and IL-13). 48 hr stimulation for PHA, 6-day stimulation for other stimulants in PBMCs. Cytokines measured by ELISA.

Author, year location (reference)	Study design	Age at outcome	Stimulants	Cytokines	Results	Comments
Libraty, 2014, Philippines (85)	Nested case control in 51 babies 38 BCG at birth-2 weeks of age compared with 13 BCG at 6 weeks of age (controls)	8-12 weeks	Negative control: RPMI Positive control: PHA BCG unrelated stimulants: TT HBsAg IPV	IFN-γ	Neonatal BCG is associated with enhanced IFN-γ responses to TT and IPV at 2-3 months post BCG vaccination No difference in IFN-γ response to HBsAg between the groups BCG at birth increased frequency of CD4-T cells that produced IFN-γ and TNF-α at 2-3 months of age.	BCG strain not stated. All babies received OPV, DTP at approximately 6 weeks of age. 48 hr stimulation for all stimulants except PHA (18-24 hrs) in PBMCs. IFN-γ measured by ELIspot.
Darboe, 2017, The Gambia (87)	RCT in 131 infants 67 BCG at 6 weeks compared with 64 BCG at 18 weeks	7 weeks 18 weeks	Negative control: RPMI Positive control: PMA BCG-related stimulants PPD BCG unrelated stimulants: *Str. pneumoniae* *E. coli* *L. monocytogenes* LPS CLO-75	IFN-γ TNF-α IL-2 IL-4 IL-10 IL-12(p70) IL-17	No effect of BCG on TNF-α response from CD4 or CD8 T cells and innate cells to any stimulants. 7 weeks: BCG enhanced IFN-γ responses from CD8 T cells to *C. albicans*. 12 weeks: BCG reduced TH1 cytokines BCG influenced responses for girls and boys differently for certain stimuli. More changes were seen at 18 weeks compared with at 7 weeks.	BCG Russia strain. Both groups received OPV and HBV at birth followed by routine vaccines as per The Gambian schedule: Hib, HBV, DTP, PCV-13, OPV at 8,12 and 16 weeks. Intracellular cytokine staining and flow cytometry Cytokine data were analysed for influence of sex and time from vaccination. 16 hr stimulation for all stimulants in PBMCs. Cytokines measured by multiplex assay.

Author, year location (reference)	Study design	Age at outcome	Stimulants	Cytokines	Results	Comments
Smith, 2017, UK (86)	Nested case control in 21 babies 11 BCG at 6 weeks of age compared with 10 babies BCG-unvaccinated (controls)	4 months post BCG vaccination (approximately 5.5 months of age)	Negative control: RPMI BCG-related stimulants *M. tuberculosis* BCG unrelated stimulants: *S. aureus* *E. coli* *C. albicans* LPS Pam3CSK4	IL-1α IL-1ß IL-1RA IL-2 to IL-10 IL-12p40 IL-12p70 IL-13 to IL-17 EGF Eotaxin Flt-3L FGF-2 Fractalkine G-CSF GM-CSF GRO IFN-α2 IFN-γ IP-10 MCP-1 MCP-3 MIP-1α MIP-1ß MDC PDGF-AA PDGF-AB/BB RANTES sCD40L sIL-2Ra TNF-α TGF-α TNF-ß VEGF	BCG enhanced more cytokine-stimulant responses but also reduced responses in other cytokine-stimulant pairs to unrelated stimulants. Each stimulant produced a distinct cytokine response signature.	BCG Denmark strain. All babies received routine recommended vaccines as per UK schedule at time of study, but vaccines and vaccine coverage were not stated. Peripheral blood Leucocytes harvested for intracellular staining of monocytes and NK cells for flow cytometry. No BCG effect on monocyte expression in response to unrelated stimulants. BCG enhanced NK cell expression in response to Pam3CSK4 stimulation with subsequent increase in IL-12p40 secretion. 48 hr stimulation for all stimulants in venous blood. Cytokines measured by multiplex assay.

Abbreviations: *C. albicans* = *Candida albicans*; CD = cluster of differentiation; DTP = diphtheria, tetanus, pertussis vaccine; DTaP = diphtheria, tetanus acellular pertussis vaccine; *E. coli* = *Escherichia coli*; EGF = epidermal growth factor; ELISA = enzyme linked immunosorbent assay; ELIspot = enzyme linked immunosorbent spot; FGF = fibroblast growth factor; Flt-3L = Fms-related tyrosine kinase3 ligand; GBS = group B streptococcus, *Streptococcus agalactiae*; G-CSF = granulocyte-colony stimulating factor; GM-CSF = granulocyte macrophage-colony stimulating factor; GRO = growth-regulated oncogene; HBsAg = hepatitis B surface antigen; HBV = hepatitis B vaccine; Hib = *Haemophilus influenzae* type b vaccine; *H. influenzae* = *Haemophilus influenzae*; hrs = hours; IFN-γ = interferon γ; IL = interleukin; IPV = inactivated poliovirus vaccine; LBW = low-birth-weight; *L. monocytogenes* = *Listeria monocytogenes*; LPS = lipopolysaccharide; MCP-1 = monocyte chemoattractant protein 1; MDC = Macrophage-derived chemokine; MIF = macrophage migration inhibitory factor; MIG = monokine induced by IFN-γ; MIP = macrophage inflammatory protein; MMR = measles, mumps rubella vaccine; *M. tuberculosis* = *Mycobacterium tuberculosis*; OPV = oral polio vaccine; Pam3CSK4 = (S)-(2,3-bis(palmitoyloxy)-(2-RS)-propyl)-N-palmitoyl-(R)-Cys-(s)-Ser-(s)-Lys4-OH,trihydrochloride; PBMCs = peripheral blood mononuclear cells; PCV-13 = 13-valent pneumococcal conjugate vaccine; PDGF = platelet derived growth factor; PEPG = peptidoglycan; PHA = phytohemagglutinin; PMA = phorbol 12-myristate 13-acetate; PPD = purified protein derivative from *M. tuberculosis*; R848 = resiquimod; RANTES = regulated on activation normal T cell expressed and secreted; RCT = randomised control trial; RPMI = Roswell Park Memorial Institute medium; *S. aureus* = *Staphylococcus aureus*; *Str. pneumoniae* = *Streptococcus pneumoniae*; TGF = transforming growth factor; TH = T helper; TNF-α = tumour necrosis factor α; TT = tetanus toxoid; VEGF = vascular endothelial growth factor

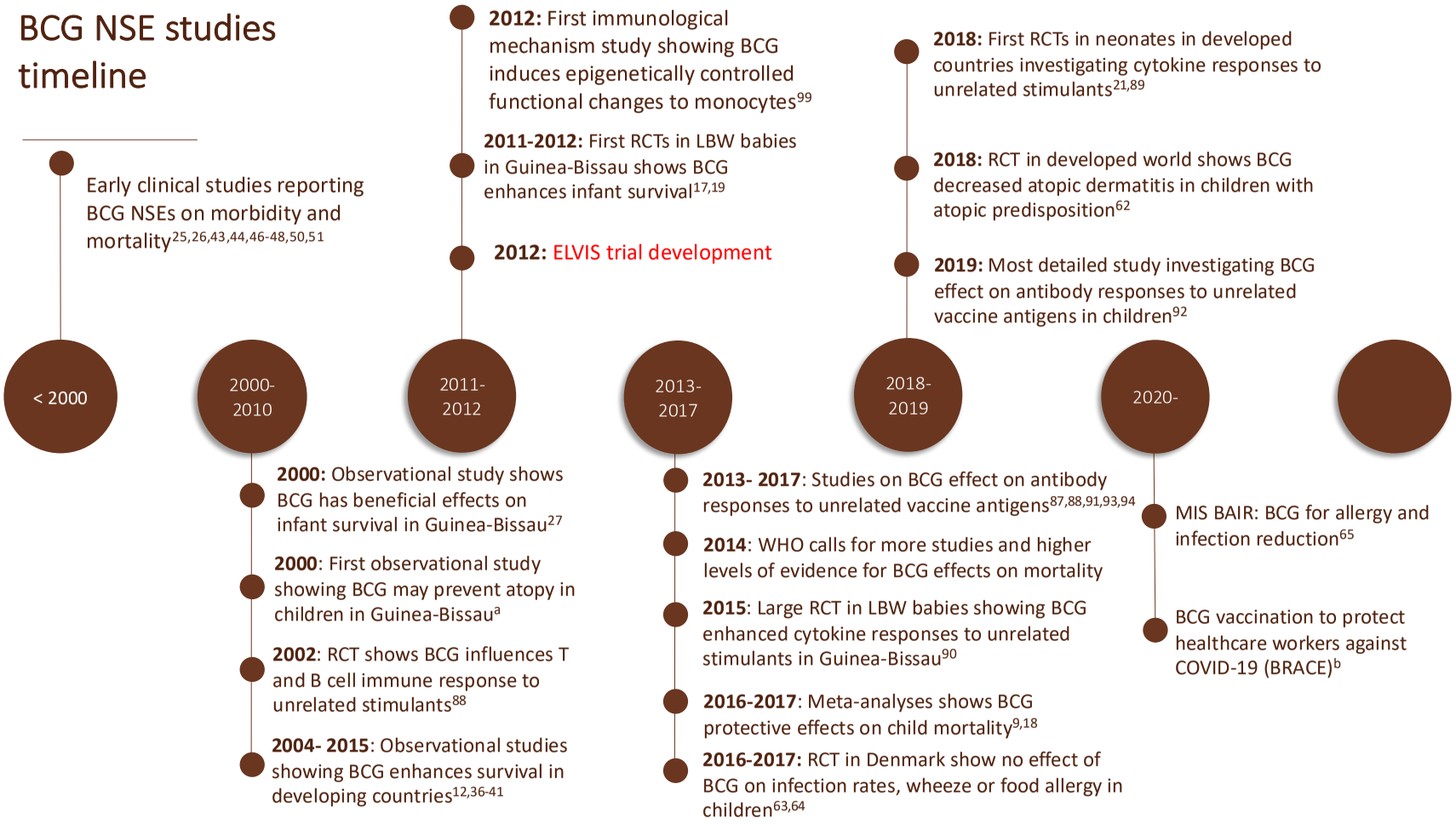
Table 2.8: Studies investigating the influence of BCG on antibody responses to unrelated vaccine antigens in children

Author, year location (reference)	Study design	Age at outcome	Vaccine antigens	Results	Comments
Ota, 2002, The Gambia (88)	Prospective RCT in 151 babies Group 1: 35 BCG at birth Group 2: 34 BCG at 2 months Group 3: 34 BCG at 4.5 months	2 months 4.5 months	OPV TT DT HBsAg Measured by ELISA	BCG at birth (but not 2 months) enhanced antibody responses to HBsAg. BCG enhanced the antibody response to OPV, but only if given at boosting (not at birth). BCG had no effect on TT and DT antibody responses.	BCG Pasteur strain. All babies received other vaccines as per recommended The Gambian schedule: OPV at birth, 1 and 2 months HBV at birth, 2 and 4 months DTP at 2, 3 and 4 months. The increased antibody response to HBsAg was associated with the enhanced cytokine responses to HBsAg in BCG-vaccinated babies.
Ritz, 2013, Australia (91)	Non-randomised study in 108 infants 56 BCG at birth compared with 52 BCG-unvaccinated (controls)	7 months	*Str. Pneumoniae*[a] *H. influenzae* type b TT HBsAg Measured by ELISA	BCG enhanced antibody responses to TT pneumococcal and Hib polysaccharide antigens. The BCG effect was strongest for antibody responses to the pneumococcal antigens. BCG reduced antibody responses to HBsAg.	Various BCG strains (Denmark/Russia/Japan) used. All babies received other vaccines as per routine Australian schedule: HBV at birth HBV, PCV-7, DTaP-IPV-Hib and oral rotavirus vaccine at 2, 4 and 6 months: Sub-analysis showed no difference in antibody responses with different BCG strains.
Hesseling, 2016, South Africa (93)	RCT in 180 infants 106 BCG at birth compared with 74 BCG at 14 weeks (controls)	14 weeks 24 weeks 52 weeks	PT and FHA *H. influenzae* type b polysaccharide TT HBsAg Measured by ELISA	BCG at birth did not influence antibody levels to vaccine antigens. Trend toward higher antibody responses to HBsAg at 14 weeks for babies vaccinated with BCG at birth.	BCG Denmark strain. All babies received other vaccines as per routine South African schedule: OPV at birth DTwP, Hib, HBV and OPV at 6, 10 and 14 weeks: MV at 9 months
Nissen, 2017, Denmark (94)	Nested cohort study in 300 infants 178 BCG Denmark within 7 days compared with 122 BCG-unvaccinated (controls)	13 months	*Bordetella pertussis* *Corynebacterium diphtheriae* *Clostridium tetani* *H. influenzae* type b *Str. Pneumoniae*[b] Measured by multiplex immunoassay	No effect of BCG at birth on antibody levels to vaccine antigens.	BCG Denmark strain. All babies received other vaccines as per routine Danish schedule: DTaP-IPV-Hib at 3, 5 and 12 months. Higher antibody responses to PT and Hib in those vaccinated at day 2-7 compared with day 0-1. No effect from sex or maternal BCG.

Author, year location (reference)	Study design	Age at outcome	Vaccine antigens	Results	Comments
Darboe, 2017, The Gambia (87)	RCT in 131 infants 67 BCG at 6 weeks compared with 64 BCG at 18 weeks (controls)	7 weeks 18 weeks	Polio: types:1 and 3 HBsAg DT TT PT Measure by ELISA/ multiplex immunoassay	No effect from BCG on antibody responses to vaccine antigens.	BCG Russia strain. All babies received other vaccines as per routine The Gambian schedule: OPV and HBV at birth Hib, HBV, DTP, PCV-13, OPV at 8, 12 and 16 weeks.
Zimmermann, 2019, Australia (92)	Nested cohort study in 91 infants at 7 months 45 BCG within 10 days of birth compared with 46 BCG-unvaccinated (controls) And 147 infants at 13 months 78 BCG within 10 days of birth compared with unvaccinated (controls)	7 months 13 months	*Corynebacterium diphtheriae* *Clostridium tetani* Pertussis: PT, FHA, PRN Polio; types1, 2, 3 *H. influenzae* type b *Str. Pneumoniae*[c] *Neisseria meningitidis* type c Measles Mumps Rubella Measured by multiplex immunoassay	BCG-vaccinated babies had enhanced antibody responses to pneumococcal, tetanus and diphtheria vaccine antigens at 7 and 13 months of age.	BCG Denmark strain. All babies received other vaccines as per routine Australian schedule: HBV at birth DTaP, HBV, IPV, Hib, PCV-13 and oral rotavirus at 6 weeks, 4 months and 6 months MMR, *Neisseria meningitidis* type c and Hib at 12 months.

Antibody responses measured as immunoglobulin G titres against the vaccine antigen.
[a] included serotypes 4, 6B, 9V, 14, 18C, 19F, 23F and11A
[b] included serotypes 4, 6B, 9V, 14, 18C, 19F and 23F
[c] included serotypes 1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A,19F and 23F
Abbreviations: DT = diphtheria toxin; DTP = diphtheria, tetanus, pertussis vaccine; DTaP = diphtheria, tetanus acellular pertussis vaccine; DTwP = diphtheria, tetanus, whole cell pertussis vaccine; ELISA = enzyme linked immunoassay; FHA = filamentous haemagglutinin antigen of *Bordetella pertussis*; HBV = hepatitis B vaccine; HBsAg = hepatitis B surface antigen; Hib = *haemophilus influenzae* type b vaccine; IPV = inactivated poliovirus vaccine; LBW = low-birth-weight; Men C = meningococcal C vaccine; MMR = measles, mumps rubella vaccine; MV = measles vaccine; OPV = oral polio vaccine; PCV-7 = 7-valent pneumococcal conjugate vaccine; PCV-13 = 13-valent pneumococcal conjugate vaccine; PRN = pertactin; PT = pertussis toxoid; RCT = randomised control trial; *Str, pneumoniae* = *Streptococcus pneumoniae*; TT = tetanus toxoid

Figure 2.1: Progression of studies on the non-specific effects of BCG showing the progression of clinical and laboratory outcomes



References in superscript: [a] = reference (95), [b] = reference (96)
Abbreviations: ELVIS = Early Life Vaccines and Immunity Study; LBW = low-birth-weight; MIS BAIR = Melbourne Infant Study BCG for Allergy and Infection Reduction; NSE = non-specific effect; RCT = randomise control trial; TB = tuberculosis; WHO = World Health Organization

2.2.3 Possible mechanisms for the non-specific effects of BCG

These in-vitro studies of BCG NSEs on cellular immune responses to unrelated antigens described previously (Chapter 2.2.2) provide some insight into potential mechanisms for the observations. These mechanisms are still incompletely defined and are likely to involve several complex interactions that are influenced by factors in the host, vaccine, environment and pathogen.

For structurally related pathogens i.e., mycobacteria other than *M. bovis*, such as *M. tuberculosis, M. leprae* and *M. ulcerans,* BCG can induce immunity and thereby confer clinical protection from tuberculosis, leprosy (97) and Buruli ulcer disease (50). This is called 'cross protection'. For unrelated pathogens, the potential mechanisms of BCG NSEs are less clear. Possible mechanisms include BCG-induced effects on the innate and adaptive immune system and changes to cellular metabolism through epigenetic re-programming.

Effects of BCG on the innate immune system

In animal studies, researchers reported that that murine macrophages pre-treated with BCG can defend their host against *C. albicans* (98). Two decades later, Kleinnijenhuis and colleagues confirmed that BCG vaccination protected mice from death due to *C. albicans* by enhancing monocyte-derived cytokine responses, independent of T or B cell-mediated immunity (99). They also demonstrated that survival of BCG protected mice was partially dependent on natural killer (NK) cells, another population of innate immune cells (100).

In adult volunteers, Kleinnijenhuis's team found that monocytes were increased and functionally changed by BCG vaccination, with increased cytokine responses to unrelated stimulants, an effect that lasted up to a year (99, 101). They also showed that the change to monocytes induced by BCG vaccination, occurred at the level of transcription due to epigenetic alterations that were dependent on NOD-2 receptor signalling pathways. They called this "innate immune training" and in doing so, shifted the traditional view that the innate immune system lacks the ability to produce memory responses to previously encountered pathogens (99). Further evidence for NSEs of BCG on monocytes was provided by a study involving 30 adult males, which showed that BCG induces changes in monocyte-derived cytokine responses to unrelated stimulants one-month after vaccination. These changes were the result of epigenetic reprogramming and not due to a change in monocyte quantity (102). Though the process of BCG-induced epigenetic reprogramming is incompletely understood, it is evident that autophagy, a process required for physiological homeostasis that allows controlled cell degradation, is required for BCG training of monocytes (103). In a prospective cohort study of 18 adult volunteers, BCG vaccination caused NK cells to produce more pro-inflammatory cytokines after stimulation with unrelated antigens compared with unvaccinated controls (100). A study of both mice and human adult blood showed that metabolic pathways involving glycolysis and glutamine influence the BCG-induced changes in monocytes through epigenetic modification (104).

A RCT in Gambian children showed that BCG slightly increased total monocyte numbers 4 weeks after BCG vaccination (90). A study of 21 UK-born infants 4 months after BCG vaccination, however, showed no difference in monocyte numbers compared with BCG-unvaccinated infants, although blood from vaccinated infants stimulated with Pam3CSK4 showed increased NK cell numbers as well as increased NK cell-derived cytokines (86).

Darboe et al. (87) also reported no difference in innate cell-derived cytokine responses to unrelated antigens in 7-week old BCG-vaccinated infants compared with controls.

Changes in the adaptive immune system

Studies in adults have shown that BCG induces changes in the adaptive immune system. Both TH1 and TH17 cytokine responses to unrelated antigens are altered by BCG vaccination after one month (102) and up to one year later (101). Several studies in children have shown BCG-induced changes in TH1, Th2 and TH17 cytokine responses to unrelated antigens (21, 85, 87, 88, 90). Changes were seen from as early as 1 week post vaccination and in the neonatal period (87).

Studies in children investigating the effect of BCG vaccination on adaptive immune responses to unrelated antigens, where the cytokines are proven to originate from adaptive immune cells are few. Darboe et al. (87) reported no differences in T-cell-derived cytokine responses to unrelated antigens in 7-week old BCG-vaccinated infants compared with controls in a RCT in The Gambia. Birk et al. (105) showed no effect of BCG on lymphocyte subsets at 4 days of age in a cohort of Danish babies in the Calmette Study vaccinated with BCG at birth compared with unvaccinated controls in a subset of infants in a RCT. However, they showed that BCG-vaccinated babies had a higher proportion of effector memory T cells at 3 months of age compared with babies who did not receive the vaccine.

Studies investigating these mechanisms are underrepresented for children and neonates, partly due to the limitations in accessing adequate blood volumes to perform these tests. The development of the immune system in early life prevents comparison with studies from adult populations. With technological advances, testing in low blood volumes may allow more research in the mechanisms involved in vaccine-induced NSEs to provide potential targets to exploit any beneficial effects.

2.2.4 Clinical correlates of BCG-induced changes in laboratory outcomes

The clinical relevance of the findings from studies investigating BCG-induced NSE with laboratory outcome measures is unknown. The level of protective antibody titres induced by vaccines for their targeted diseases are well described. The clinical utility of boosting antibody titres to prolong protection against infection has clear benefits. However, the clinical correlates of BCG-induced changes in cytokine responses to unrelated stimulants is unknown. At the time of writing, only one published study had investigated the influence of BCG on cytokine responses to unrelated antigens with clinical outcomes (106). In this study, the researchers used data from a RCT and failed to show any predictive cytokine signature in LBW babies, aged 6 weeks and vaccinated at birth, that correlated with infant mortality. Babies who died younger than 6 weeks were not assessed. Therefore, a correlation between cytokine responses to unrelated stimulants in neonates with neonatal mortality remain unknown.

2.2.5 Factors influencing the non-specific effects of BCG

The non-specific effects of BCG vaccine appear to be influenced by several factors and often described as the vaccine effect modifier. The extent to which these factors affect immune responses, the interactions between factors and mechanisms are still poorly understood. Most of the evidence for BCG NSE modifiers is from secondary analyses of data from both observational studies and trials. A summary of factors influencing BCG non-specific effects in children is described below (Table 2.9).

Table 2.9 Factors influencing the non-specific effects of BCG

Factors influencing the non-specific effects of BCG
Infant factors
Sex
Presence of a BCG scar
Age at vaccination
Maternal factors
BCG vaccination
Environmental factors
Season
Other factors
Order and timing of vaccinations
Vitamin and micronutrient supplementation

Infant factors

Sex

Several studies investigating BCG NSEs, report that BCG influences outcomes differently for boys and girls (36, 40, 107-109). When found, these sex differences appear to depend on other modifying factors, such as the timing of BCG (110) and diphtheria-tetanus-pertussis vaccine (DTP) administration (108, 109, 111). The evidence for and against sex-differential NSEs after receiving BCG comes largely from secondary analyses of data from cohort studies of all-cause mortality in high-disease burden countries (Table 2.10). One of the earliest of this type was a cohort study that reported enhanced survival in BCG-vaccinated girls compared with BCG-vaccinated boys in West African girl-boy twin pairs (107). Several further cohort studies reported conflicting results (Table 2.10). These studies are difficult to assess in terms of the isolated effect of BCG on sex-related mortality as other vaccines were usually administered in the follow up period. In this regard, Aaby et al. (111) did a meta-analysis of observational studies on the effect of DTP on mortality in BCG-vaccinated infants and found that mortality in girls was higher when they received DTP after BCG.

Results from RCTs are limited to a few trials in West Africa that generally found stronger BCG protective effects on mortality for boys (17-19). Biering-Sorensen et al. (110) analysed data from three RCTs that investigated isolated BCG effects on neonatal mortality and found that the different protective effects of BCG in boys and girls depended on the timing of BCG vaccination. BCG given to boys within the first week of life enhanced neonatal survival three-fold compared with unvaccinated boys (MRR 0.36 [95% CI 0.20-0.67]) and the protective effect decreased when BCG was given later in the neonatal period. However, in girls, BCG administered in the first week of life had a marginal protective effect, with a 15% reduction in neonatal mortality (MRR 0.85 [95% CI 0.46-1.54]) compared with unvaccinated girls. The protective effect of BCG was significantly increased in girls vaccinated with BCG in weeks two to four (MRR 0.56 [95% CI 0.32-1.00]).

The sex-related effects of BCG are not limited to mortality, as the protective effect of BCG immunisation on ALRTIs with RSV was more pronounced in girls than in boys (OR for not being BCG-vaccinated 4.60 [95% CI 0.85-24.8] for girls and 1.08 [95% CI 0.35-3.32] for boys (13). By contrast, no sex influences on BCG effects on morbidity were reported in the Danish Calmette Study (59).

In RCTs investigating the NSE of BCG on cytokine responses to unrelated antigens in children in vitro, some authors have analysed their data for any influence of sex on the results (21, 87, 89, 90). One of these reported a trend towards stronger BCG NSEs in females that reached statistical significance for IL-1β responses to Pam3CSK4 (90). Freyne et al. (21) from our laboratory found an interaction between sex and BCG vaccination on macrophage migration inhibitory factor (MIF) responses to several unrelated antigens. In this study, BCG-vaccinated boys tended to have lower responses than unvaccinated boys, whereas BCG-vaccinated girls had higher responses compared with unvaccinated girls. Darboe et al. (87) reported reduced IL-10 and IL-17 responses to unrelated stimulants in BCG-vaccinated girls 3 months after vaccination, but not in BCG-vaccinated boys. By contrast, Nissen et al. (89) found no influence of sex on cytokine responses to unrelated antigens in BCG-vaccinated infants up to 13 months of age. In a single study, sex was also not shown to influence BCG effects on antibody production to unrelated antigens (94).

Overall, these studies are inconclusive on the interaction between sex and BCG NSEs. Further evidence from RCTs is needed. Studies that investigate associations of biological mechanisms with clinical outcomes would be most informative.

Presence of a BCG scar

In a cohort study in Guinea-Bissau, Garly et al. (112) reported enhanced infant survival in BCG-vaccinated infants with a BCG scar compared with BCG-vaccinated infants without a scar (MRR 0.41 [95% CI 0.25-0.67]). In another cohort study, Roth et al. (113) reported similar findings from Uganda where BCG-vaccinated infants with a scar survived longer than those who did not. The presence of a BCG scar in BCG-vaccinated infants is also associated with greater protection against ALRTIs compared with BCG-vaccinated infants without a scar (OR of having no scar 1.54 [95%CI 0.86-2.75]) (13).

Age at vaccination

From cohort studies in Guinea-Bissau, Kristensen et al. (27) found no effect on infant mortality rates for children vaccinated early (at the first study visit between 0-6 months of age) or later. On the other hand, Biering-Sorensen et al. (18) reported enhanced survival for infants vaccinated with BCG at birth compared with approximately 6 weeks of age from a meta-analysis of three RCTs in the same country. Recently, RCTs in neonates investigating BCG effects on cytokine and antibody responses to unrelated antigens showed differences in babies vaccinated with BCG within the first 24 hours of life compared with those who received BCG at days 2-7 (21, 89, 94). When found, BCG NSEs on cytokine responses to certain unrelated stimulants were stronger in babies vaccinated after 48 hours compared with earlier vaccination (21, 89). Similarly, higher antibody responses to certain unrelated vaccine antigens were found in infants vaccinated after 48 hours compared with those BCG-vaccinated earlier (94).

Table 2.10 Evidence for and against sex-differential non-specific effects of BCG from epidemiological cohort studies

Author, year, location (reference)	Age at BCG immunisation	Other vaccines	Age at outcome	Outcome	Comment
Aaby, 2004, Guinea-Bissau and Senegal (107)	Birth	Birth: OPV 6, 10 and 14 weeks: DTP, OPV 9 months: MV	1-17 months	The female: male MRR was 0.25 (95% CI 0.05-0.93) if BCG was the last vaccine given and 7.33 (95% CI 2.2-38.3) if DTP was the last vaccine given.	Examined differences in mortality in girl-boy twin pairs. Investigated mortality by the last vaccine given before death.
Vaugelade, 2004, Burkina-Faso (12)	Mean age 4.8 months	6, 10 and 14 weeks: DTP 9 months: MV	Under 2 years	BCG-vaccinated boys compared with BCG-unvaccinated boys had a 66% reduction in mortality rate (MRR 0.34 [95% CI 0.23-0.49]). BCG-vaccinated girls compared with BCG unvaccinated girls had a 59% reduction in mortality rate (MRR 0.41 [95% CI 0.29-0.58]).	The adjusted mortality rates under 2 years of age were not statistically different between the sexes vaccinated with BCG compared with BCG-unvaccinated infants or between both sexes combined.
Moulton, 2005, Southern India (40)	Median age close to 30 days	Birth: OPV 6, 10 and 14 weeks: DTP, OPV	6 months	Girls who received both DTP and BCG were more likely to die compared to girls who received only BCG or DTP (HR = 2.4 [95% CI 1.2-5.0]. No difference for boys who received both DTP and BCG compared to boys who received only BCG or DTP (HR = 1.1 [95% CI 0.48-2.4].	Censored Vitamin A as a vaccine effect modifier.
Aaby, 2006, Malawi (36)	Median age 16 days	Birth (or soon after): OPV 6, 10 and 14 weeks: DTP, OPV 9 months: MV	8 days to 8 months	BCG-vaccinated girls compared with BCG unvaccinated girls had a 37% reduction in mortality rate (MRR 0.63 [95% CI 0.20-2.01]). BCG-vaccinated boys compared with BCG unvaccinated boys had a 28% reduction in mortality rate (MRR 0.72 [95% CI 0.26-1.96]).	BCG-vaccinated girls compared with BCG-vaccinated boys had a 20% reduction in mortality rate (MRR 0.80 [95% CI 0.3-2.1]).

Author, year, location (reference)	Age at BCG immunisation	Other vaccines	Age at outcome	Outcome	Comment
Chan, 2007, Philippines (108)	58% by 11 weeks of age	6, 10 and 14 weeks: DTP, OPV 9 months: MV	Up to 30 months	In BCG-vaccinated infants: DTP reduced deaths in males by 68% HR = 0.32 (95% CI 0.14-0.73) DTP reduced deaths in females by14% HR 0.86 (95% CI 0.18-4.23)	In those vaccinated with BCG, DTP was associated with reduced child deaths. DTP protective effects were strongest in males, no harmful effect of DTP for females.
Aaby, 2015, Senegal (109)	Median age 43 days 44% coverage at 12 months	DTP, MV Details not stated	0-23 months	The female: male MRR for those unvaccinated was 0.79 (95% CI 0.64-0.96) BCG-vaccinated first was 0.48 (95% CI 0.12-1.98) BCG and first DTP vaccinated together was 1.43 (95% CI 0.76-2.70) DTP first, not yet BCG was 0.95 (95% CI 0.2- 4.49)	Infant survival compared for different vaccine sequences- included MV. With increasing DTP coverage, female mortality increases above that for males.
Nankabirwa, 2015, Uganda (38)	Median age 4 days	Birth: OPV 6, 10 and 14 weeks: DTP, HBV, Hib, OPV 9 months: MV	1 month to 5 years	BCG-vaccinated girls compared with BCG unvaccinated girls had a 48% reduction in deaths (HR 0.52 [95% CI 0.10-2.79]). BCG-vaccinated boys compared with BCG unvaccinated boys had a 48% reduction in deaths (HR 0.34 [95% CI 0.05-2.18]). Sex did not influence the BCG effect on child deaths at any age group	Excluded neonates. Results adjusted for age at immunisation and others.

Abbreviations: CI = confidence interval; DTP = diphtheria-tetanus-pertussis vaccine; g = grams; HBV = hepatitis B vaccine; Hib = *Haemophilus influenzae* type b vaccine; HR = hazards ratio; IPV = inactivated polio vaccine; LBW = low-birth-weight (<2500 grams); MRR = mortality rate ratio; MV = measles vaccine; OPV = oral polio vaccine; RCT= randomised control trial

Table 2.11 Evidence for and against sex-differential non-specific effects of BCG from randomised control trials and meta-analyses

Randomised control trials					
Author, year, location (reference)	**Groups**	**Other vaccines**	**Age at outcome**	**Outcome**	**Comments**
Aaby, 2011, Guinea-Bissau (17)	1168 BCG at birth (early group) 1,152 Delayed BCG (controls)	DTP	Up to 12 months	No statistical difference between the sexes. Early BCG reduced mortality in girls by 49% (MRR 0.51 [95% CI 0.25-1.06]). Early BCG reduced mortality in boys 41% (MRR 0.59 [95% CI 0.32-1.11]).	All participants LBW BCG Danish strain used. Vaccines other than DTP not stated. Limited power to detect sex differences.
Biering-Sorensen, 2012, Guinea- Bissau (19)	51 BCG at birth (early group) 54 Delayed BCG (controls)	After 6 weeks of age: DTP, OPV	Up to 12 months	BCG effect was stronger in boys, but overall no statistical interaction for early BCG and sex (p = 0.21). Early BCG reduced mortality in boys by 79% (MRR 0.21 [95% CI 0.04-1.03]). Early BCG reduced mortality in girls by 19% (MRR 0.81 [95% CI 0.19-3.36]).	All participants LBW Vaccines other than DTP and OPV not stated. BCG strain not stated.
Biering-Sorensen, 2017, Guinea-Bissau (18)	2083 BCG at birth (early group) 2089 Delayed BCG (controls)	6,10 and 14 weeks: DTP, HBV, Hib 9 months: yellow fever vaccine	Up to 4 weeks Up to 12 months	There was no sex-related influence on BCG effects on neonatal or infant mortality. Early BCG reduced neonatal mortality in girls by 24% (MRR 0.76 [95% CI 0.45-1.28]). Early BCG reduced neonatal mortality in boys by 37% (MRR 0.63 [95% CI 0.35-1.14]).	BCG Denmark strain. Other vaccines not stated but assumed MV at 9 months and OPV at birth.

Metanalyses					
Author, year, location (reference)	**Studies**	**Other vaccines**	**Age at outcome**	**Outcome**	**Comments**
Aaby, 2016, various (111)	Seven cohort studies	Various, all DTP	Various	In seven studies of infants vaccinated with BCG: DTP increased mortality in girls MRR 2.54 (95% CI 1.68-3.86) In ten studies of BCG-vaccinated infants: The female: male mortality ratio was 2.45 (95% CI 1.48-4.06) times higher after DTP compared with before. In 15 studies where infants received DTP after BCG: The mortality was1.53 (95% CI 1.21-1.93) times higher in girls	Meta-analysis of observational studies primarily to assess DTP effects on female mortality. Limitations: potential confounders from other vaccines, all in high-disease burden countries.
Biering-Sorensen, 2018, Guinea-Bissau (110)	3 RCTs from LBW boys Guinea-Bissau receiving Early BCG at birth compared with delayed BCG at 6 weeks of age		Up to 4 weeks	Within the first week of enrolment: Early BCG reduced mortality in boys by 64% MRR 0.36 (95% CI = 0.16-0.8) compared with unvaccinated boys between 1-3 days and by 63% MRR 0.37 (95% CI 0.14-0.94) between 4-7 days. For girls the BCG effect on mortality at these time points were much smaller MRR 0.84 (95% CI 0.41-1.73) Form day 8-28: The BCG protective effect on mortality for boys was small MRR 0.91 (95% CI 0.51-1.69) The BCG protective effect on mortality for girls was significant MRR 0.56 (95% CI 0.31-1.00)	The effect of BCG differed for boys and girls. The influence of sex on BCG effects reached statistical significance (p = 0.05) in the first week of life. Stronger protective effects from BCG were seen in boys in the first week of enrolment and for girls in the second week.

Abbreviations: CI = confidence interval; DTP = diphtheria-tetanus-pertussis vaccine; g = grams; HBV = hepatitis B vaccine; Hib = *Haemophilus influenzae* type b vaccine; HR = hazards ratio; LBW = low-birth-weight (<2500 grams); MRR = mortality rate ratio; MV = measles vaccine; OPV = oral polio vaccine; RCT = randomised control trial

Maternal factors

BCG vaccination

In a sub-analysis of data from a RCT investigating the influence of BCG vaccination at birth on morbidity in Danish infants, Kjaergaard et al. (59, 60) reported that those BCG-vaccinated whose mothers had also received BCG vaccination, experienced 38% (95% CI 2-61%) fewer infections unrelated to TB between 0 to 3 months of age and were 17% (95% CI -0.13-39%) less likely to be admitted to hospital, compared with BCG-vaccinated children whose mothers had not received BCG. Freyne et al. (21) from our group reported an influence of maternal BCG status on cytokine responses to unrelated antigens in BCG-vaccinated infants, with an increase in IFN-γ in response to *Str. pneumoniae* and MIP-1α in response to peptidoglycan (PEPG). However, no such association was found in a similar study in Denmark (89).

Environmental factors

Season

Though it has been shown that season of birth influences the specific cytokine responses to *M. tuberculosis* in BCG-vaccinated infants in Malawi, The Gambia and the UK (114, 115), there is limited evidence for the influence of season on BCG NSEs. Jensen et al. (90) reported an interaction between the season of birth of babies BCG-vaccinated in Guinea-Bissau and infant cytokine responses to unrelated antigens, insofar as IL-17 responses were significantly higher to Pam3CSK4 and lipopolysaccharide (LPS) in infants vaccinated with BCG in the dry season compared to those vaccinated during the wet season.

Other factors

Time since BCG vaccination

Some studies suggest that the beneficial effect on child survival conferred by BCG vaccination wanes over time, with most benefit observed for neonates (17-19, 27) and young children (41). A waning effect was also observed in a study with a 14-year follow-up period, where BCG immunisation was associated with decreased morbidity and fewer hospitalisations for sepsis in children under one year of age, but not in older children (54).

Order and timing of vaccinations

The first modulators of BCG-related NSEs to be reported in observational cohort studies were the order and timing of vaccinations. A cohort study in India showed that concurrent vaccination with BCG and DTP, or BCG after DTP, was associated with 85% lower mortality rate in children up to 5 years of age (MRR 0.15 [95% CI 0.03-0.70]) compared with DTP after BCG (116). In Senegal, in an area of low vaccine coverage, BCG and concurrent DTP was associated with 31% lower infant mortality rate up to one year of age (MRR 0.69 [95% CI 0.53-0.89]) compared with unvaccinated children or children receiving either of those vaccines alone (109). When infants received the first dose of DTP after BCG the infant mortality rate was higher (MRR 1.34 [95% CI 0.80-2.23]) than in unvaccinated infants. Similarly, concurrent BCG, DTP and inactivated polio vaccine (IPV) was associated with a 28% lower infant mortality (MRR 0.72 [95% CI 0.5-1.04]) compared with unvaccinated Senegalese infants (117). Later, the same research group showed an increase in mortality if the first dose of DTP was given after BCG (MRR 1.78 [95% CI 1.03-3.03]) in infants up to 9 months of age compared with infants receiving these vaccines concurrently (118).

These studies suggest that the beneficial effect of BCG on infant survival is negated by subsequent, but not concurrent vaccination with DTP. Aaby et al. (119) hypothesised that live vaccine NSEs are negated by subsequent administration of non-live vaccines, such as DTP. Unfortunately, results from observational studies are limited by potential confounders, but RCTs in high-disease burden countries investigating vaccine NSE may not be feasible.

Few laboratory-based studies have investigated the issue of timing of BCG in relation to other vaccines, although Ota et al. (88) found that concurrent vaccinations with BCG altered cytokine and antibody responses to unrelated vaccine antigens in West African children.

Worldwide, vaccination schedules have been designed with a focus on protection from targeted diseases. In several countries where disease burdens are high and access to health care is limited, children may receive vaccinations out of their recommended sequence. These observations of the influence of timing of vaccines and their potentially negative NSEs on immunity warrant further exploration through appropriately designed RCTs.

Vitamin and micronutrient supplementation

In a RCT in Guinea-Bissau, Benn et al. (120) reported that high dose vitamin A supplementation was associated with a 14% decrease in mortality (MRR 0.86 [95% CI 0.48-1.54]) when BCG was the last vaccine given compared with BCG-vaccinated babies who did not receive vitamin A supplementation.

2.3 Hepatitis B vaccine (HBV)

Monovalent HBV is used in neonates to protect against vertical transmission of hepatitis B virus and thus protects against chronic hepatitis B infection which is the major risk factor for hepatocellular carcinoma later in life. It is a safe and well-tolerated vaccine, comprising recombinant HBsAg adsorbed onto aluminium hydroxide.

2.3.1 Clinical evidence for non-specific effects of hepatitis B vaccine

Autoimmunity

The hepatitis B vaccine contains alum (a hydrated double sulphate salt of aluminium) that historically had caused concerns regarding the development of immune dysregulatory conditions. In 2011, a syndrome called autoimmune inflammatory syndrome induced by adjuvants (ASIA) was described, mainly in adults, implicating alum, including HBV. However, at the time of writing, there remains no evidence in support of these claims (121, 122).

A paediatric case-control study investigated autoantibody production in 210 six-year-old children vaccinated at birth with HBV (123). The investigators grouped the children into those who were seroprotected from hepatitis B with hepatitis B surface antibody (HBsAb) titres ≥10 IU/L (200 children) and those who were not seroprotected where HBsAb titres were < 10 IU/L (10 children). They found no difference in seven autoantibody subtypes between the groups. There was a family history of autoimmunity in 18% of the children with autoantibody production and authors concluded that genetic susceptibility, rather than vaccination was the major factor determining autoantibody production (123). The study's major limitation is the lack of an unvaccinated control group.

Malignancy

Hepatitis B infection has been reported to increase the risk of non-Hodgkin lymphoma (NHL) (124). At the time of writing, only one study has investigated the influence of HBV on development of NHL in adolescents and adults in Taiwan (125). This cohort study compared incidence rates of NHL in those born before and after the implementation of universal HBV at birth. They showed a significantly higher incidence of NHL in adolescents aged 12.5 years to 20 years born before universal HBV vaccination (1.85 [95% CI 1.02-3.3]) compared with those born after (0.74 per 100,000 person years [95% CI: 0.41-1.34]) (p = 0.03) (125). By contrast, a meta-analysis of four studies investigating the influence of HBV on childhood leukaemia found no association between the two (OR 1.00 [95% CI 0.85-1.16]) (67). These case-control studies were all from high-income countries with different HBV immunisation schedules and differed in respect to the collection and analysis of HBV data (126-129). Ma et al. (127) were the only researchers to sight the vaccination records of participants, whereas the other studies received vaccination histories from parent interviews with the potential of recall bias and error in reporting. Ma et al. (127) were also the only researchers to report on the number of HBV doses and risk of leukaemia, and reported a non-significant increase in the risk of leukaemia in children vaccinated with more than three doses of HBV in infancy (OR 1.41 [95% CI 0.9-2.2]) compared with matched controls. The combination of HBV with other vaccinations were not included in the analyses in any of the studies, nor was age at HBV vaccination. Further studies are needed where accurate vaccination records are viewed and more detailed data collection and analysis are performed, with a focus on the number of HBV doses in infancy and its association with the development of acute leukaemia.

The number of HBV doses in infancy may rise as universal birth doses of HBV are introduced in several more countries to target chronic hepatitis B infection prevention.

Together, these studies investigating the association of HBV and malignancy found no negative HBV-induced effects and a possible beneficial effect on NHL in an endemic area. Further studies in different locations are needed to investigate protective effects of HBV on NHL and other malignancies in children.

Mortality

Only four cohort studies have reported on HBV effects on childhood mortality, but none reported effects in the neonatal period. One study investigated HBV as part of a combination vaccine and therefore the individual effect of HBV cannot accurately be reported (130). Another study reported increased mortality in children receiving HBV, that was more significant in girls (20). Yet another study reported mortality rates in children aged 2 to 8 months in relation to HBV, but the number of deaths were so few (4 deaths out of 59 children) in the groups that the significance of the results are unknown (131). The final study reported a non-significant benefit of HBV on childhood mortality (41).

In Chapter 2.2.5, I discussed the influence of the effect of giving non-live vaccines, such as DTP, before or after BCG which may influence the beneficial NSEs of BCG. Garly et al. (20) investigated whether HBV, a non-live vaccine, given at 7.5 months of age influenced mortality in girls in Guinea-Bissau . In this nested cohort study, three observations suggested that HBV was associated with increased mortality; (i) they found an increased mortality rate in children after receiving HBV compared with those who had not yet received HBV ([MRR] 1.62 95% CI 1.09-2.41, p = 0.03), (ii) the MRR for HBV-unvaccinated children aged 7.5 months compared with HBV vaccinated children was 1.81 (95% CI 1.19-2.75) and (iii) the MRR for 9-12 month old children vaccinated with measles vaccine and HBV compared with those vaccinated with measles vaccine alone was 5.08 (95% CI 0.67-38.3). Fisker et al. (130) recently investigated the effect of vaccine sequence on childhood mortality in Guinea-Bissau. HBV was given as part of a combination vaccine, “Penta3”. The mortality rate for infants who had not received “Penta3” prior to 9 months had a higher mortality (7%) compared to those vaccinated in sequence, with HR of 2.26 (95% CI 1.17-4.38). This negative effect on survival was more pronounced in girls in both studies.

One study, outside West Africa, investigated the effects of HBV on childhood mortality up to 2 years of age. In this retrospective cohort study in the Papua New Guinea highlands, Lehmann et al. (41) reviewed surveillance data from children born between 1989 and 1994. They reported a non-significant beneficial effect on infant survival (HR 0.69 [95% CI 0.28-1.71]). The authors did not analyse data for sex-related HBV influences on mortality.

Apart from the study by Garly et al. (20), none of these studies were primarily designed to test the hypothesis whether HBV increases infant mortality. Therefore, differences in mortality should be interpreted with caution. Whether the changes were due to HBV administration itself or the order it was given in relation to live vaccines remains unknown. There is potential for selection and reporting bias. Immunisation schedules for these vaccines differed in the trials and from current practices. Other concurrent vaccines and previous vaccination status of the infants were also not considered, making it impossible to

detect an isolated effect from HBV on mortality. Further studies using current vaccine schedules, assessing the duration of any NSEs and investigating a biological explanation for any observations are needed.

2.3.2 In-vitro investigations of non-specific effects of hepatitis B vaccine

The only report of a laboratory outcome for HBV effects on immune responses to a stimulant other than HBsAg comes from an exploratory study in adult volunteers (132). Lee et al. (132) hypothesised that if there are vaccine-induced NSEs, what the researchers called universal bystander effects, on memory B cells, then vaccination with one antigen would stimulate antibody production to other unrelated antigens on subsequent exposure. The study included 97 volunteers, 19 of whom received a vaccination, 56 healthy controls and 22 with proven respiratory infections. The researchers confirmed the presence of circulating memory B cells to a range of antigens, including HBsAg, RSV, tetanus and influenza, in the healthy controls due to previous exposure or vaccination. Only one participant was vaccinated with HBV, making it impossible to draw any conclusions from the result. However, the researchers reported an increase in antibody secreting cells specific to HBsAg 6 days after HBV administration but there was no evidence for an increase in antibody secreting cells to the other antigens.

At the time of writing, there were no studies in children or neonates investigating isolated HBV influences on antibody or cytokine responses to an antigen other than HBsAg. There is insufficient evidence for HBV-induced NSEs on antibody or cytokine responses in any age group. Further studies in children, investigating clinically important antigens, including HBsAg are warranted.

2.4 BCG and HBV

Infant BCG and HBV policies differ around the world partly depending on prevalence of TB and endemic hepatitis B infection in a particular region. Routinely, neonates can receive any combination of no birth vaccines, intradermal BCG with or without intramuscular HBV or isolated intramuscular HBV, limiting comparisons between studies in countries with different recommendations for these two vaccines.

Two Brazilian studies have investigated whether the route of administration of HBV in babies vaccinated concurrently with intradermal BCG and HBV influenced immune responses to HBsAg and mycobacterial antigens (133, 134). One of these, a RCT in 498 infants, compared antibody responses to HBsAg in seven-month-old infants who received either combined intradermal BCG and HBV or intradermal BCG and intramuscular HBV at birth (133). The investigators found no difference in antibody responses, reported as frequency rates in titre response categories, between the groups. In the second study, Mazzola et al. (134) investigated BCG-specific cytokine responses in a cohort of 85 of these children at 7 months of age. They reported no difference between the groups. The authors also reported no differences in cytokine responses to hepatitis B, but the data, type of stimulant and cytokines measured were not shown. These two studies suggest that route of administration of HBV does not influence cytokine or antibody responses to HBsAg or cytokine responses to mycobacterial antigens.

2.4.1 The influence of BCG and HBV on the immune responses to hepatitis B

Antibody responses

Seven studies have reported on the influence of BCG on antibody responses to HBsAg (Table 2.12). Four studies showed that BCG modifies infant antibody responses to HBsAg in children vaccinated with HBV at various time points with both a lowering (91) and an enhancing effect (88, 93) that persisted over time (135). Three RCTs showed no effect (87, 136, 137).

A RCT in the Gambia (88), which investigated the influence of BCG on antibody and cytokine responses to vaccine antigens, including HBsAg, in three groups of children showed that BCG's effects on immune responses to HBsAg were more pronounced with age. Compared to babies vaccinated with OPV and HBV at birth, babies given BCG and HBV at birth showed statistically significant increases in median IgG HBsAb responses among those vaccinated with BCG at 2 months ($p = 0.03$) and 4.5 months of age ($p = 0.004$) (88). Hesseling and colleagues (93) investigated the effect of delaying BCG vaccination at birth to 14 weeks of age on infant antibody responses to various vaccine antigens, including HBV. They found a trend toward enhanced antibody responses to HBV at 14 weeks of age in the group given BCG at birth ($p = 0.09$), but not at later time points. By contrast, an Australian study including 108 infants (91), showed that BCG at birth reduced antibody responses to HBsAg in 7-month-old infants, compared with BCG-unvaccinated infants ($p = 0.03$).

Coursaget et al. (136) found no difference in antibody responses to HBsAg in a RCT comparing concurrent BCG and HBV at birth with the individual birth vaccine groups ($p = 0.65$) (136). A Turkish RCT investigated antibody responses to HBsAg in infants given concurrent vaccines with HBV compared with HBV given in isolation and found no difference in antibody responses (137). In a more recent RCT in 131 Gambian infants, Darboe and colleagues (87) reported no difference in antibody levels to HBsAg measured at 7 weeks or 18 weeks of age in those vaccinated with BCG at 6 weeks compared with unvaccinated controls.

In two of these studies (87, 93), the first doses of BCG and HBV were separated by 6 weeks. As shown in Table 2.12, differences in geographic location, the age at BCG administration, the BCG strain used, the age when outcome was measured and the other vaccinations the infants received, preclude general conclusions to be made about BCG influences on antibody responses to HBsAg. The clinical relevance of the reported findings from these studies are also unknown as the effects on antibody levels, whether enhanced, unchanged or depressed, did not influence seroprotection from hepatitis B.

Cytokine responses

At the time of writing, there were only two studies that had investigated the influence of BCG on cytokine responses to HBsAg (Table 2.13). Neither of these studies were in neonates. Ota et al. (88) investigated the influence of BCG given at birth or 2 months of age on cytokine responses to vaccine antigens, including HBsAg. They found statistically significant increases in median IFN-γ ($p = 0.008$), IL-5 ($p = 0.04$) and IL-13 ($p = 0.006$) responses to HBsAg at 2 months of age in babies vaccinated with BCG at birth. For IFN-γ, BCG at birth also enhanced responses to HBsAg at 4.5 months of age ($p = 0.05$). By contrast, Libraty et al. (85) in the Philippines, reported no difference in frequency of IFN-γ spot-forming colonies measured by ELISpot in 2- to 3-month old infants vaccinated with BCG within 2 weeks of birth or at age 6 weeks ($p = 0.4$).

As shown in Table 2.12, these two apparently conflicting studies differed in design. The laboratory measurement of cytokine responses also differed, with Ota et al. using an enzyme-linked immunosorbent assay (ELISA) method to measure cytokine responses. The researchers (88) highlighted that timing of BCG in relation to other vaccines influenced immune responses to HBsAg at 4.5 months of age particularly for IFN-γ. BCG at the time of priming with HBV (i.e., at birth) enhanced IFN-γ responses to HBsAg ($p = 0.05$) compared with BCG at the time of boosting (i.e., at 2 months of age [$p = 0.35$]).

At the time of writing, the study by Ota and colleagues (88) was the only study investigating BCG-induced NSEs that included both antibody and cytokine responses to HBV. The researchers showed that enhanced cytokine responses to HBsAg correlated with increased IgG HBsAb levels. The relationship between the responses of different immune cells to unrelated infectious stimulants should be a focus of future studies.

Table 2.12: Studies reporting the influence of BCG and HBV on antibody responses to hepatitis B surface antigen

Author, year, location (reference)	**Study design**	**Age BCG administered**	**Age at outcome**	**Outcome (IgG antibody responses to HBsAg)**	**Comment**
Coursaget, 1992, Senegal (136)	RCT in 108 infants	Group A: Birth: BCG + HBV 2, 3, 9 months: HBV Group B: Birth: HBV 2, 3, 9 months: HBV 4 months: BCG Group C: Birth: BCG 2, 3, 9 months: HBV	9 months	No difference in protective levels of antibody (>10 mIU/L) in Group A compared with Group B (p = 0.65). Antibody responses to HBsAg were similar in babies vaccinated concurrently or separately with BCG and/ HBV.	Babies received other vaccines according to then Senegalese vaccine schedule: DTP, OPV at 2, 3, and 4 months: Comparisons for group A and Group C not reported for antibody levels.
Ota, 2002, The Gambia (88)	RCT in 151 babies	Group 1: 35 babies Birth: BCG + HBV Group 2: 34 babies Birth: HBV 2 months: BCG + HBV Group 3: 34 babies Birth, 2, 4 months: HBV 4.5 months: BCG (controls)	2 months 4.5 months	Babies vaccinated with BCG at birth had enhanced antibody responses at age 2 months and 4.5 months of age. Babies vaccinated with BCG at second dose HBV (2 months) had non-significant difference in antibody responses compared with controls.	All babies revived other vaccines as per recommended The Gambian schedule: OPV at birth, 1 and 2 months HBV at birth, 2 and 4 months DTP at 2,3 and 4 months.
Ota, 2004, The Gambia (135)	Nested cohort study in the above RCT	Group 1: 35 babies Birth: BCG + HBV Group 2: 34 babies Birth: HBV 2 months: BCG + HBV Group 3: 34 babies Birth, 2, 4 months: HBV 4.5 months: BCG (controls)	12 months	Babies vaccinated with BCG at birth had enhanced antibody responses at 12 months of age compared with controls.	All babies revived other vaccines as then recommended The Gambian schedule OPV at birth, 1 and 2 months HBV at birth, 2 and 4 months DTP at 2, 3 and 4 months.

Author, year, location (reference)	Study design	Age BCG administered	Age at outcome	Outcome (IgG antibody responses o HBsAg)	Comment
Artan, 2004, Turkey (137)	RCT in 60 infants	Group 1: 20 babies 2 months BCG + HBV 3, 9 months: HBV Group 2: 20 babies 2 months: BCG 3, 4, 9 months: HBV Group 3: 20 babies Birth, 1 month: HBV 2 months: BCG 6 months: HBV	4-6 weeks after the 9-month vaccines 13 months after 9-month vaccines	No difference in protective levels of antibodies to HBsAg between the 3 groups.	Babies received other vaccines according to then Turkish vaccine schedule: DTwP, OPV at 2 months, 3 and 4 months MV at 9 months.
Ritz, 2013, Australia, (91)	Non-randomised study in 108 infants	Group 1: 56 babies Birth: BCG + HBV 2, 4, 6 months: HBV Group 2: 52 babies Birth, 2, 4, 6 months: HBV (BCG-unvaccinated controls)	7 months	BCG reduced antibody responses to HBsAg.	All babies received other vaccines as then routine Australian schedule: PCV-7 vaccine, DTaP, IPV, Hib and oral rotavirus vaccine at 2, 4 and 6 months.
Hesseling, 2016, South Africa, (93)	RCT in 180 infants	Group 1: 106 babies Birth: BCG 6, 10 and 14 weeks: HBV Group 2: 74 babies 6, 10 weeks: HBV 14 weeks: BCG + HBV	14 weeks 24 weeks 52 weeks	Trend toward higher antibody responses to HBsAg at 14 weeks for babies vaccinated with BCG at birth.	All babies received other vaccines as then routine South African schedule: OPV at birth DTwP, Hib, and OPV at 6, 10 and 14 weeks MV at 9 months.
Darboe, 2017, The Gambia (87)	RCT in 131 infants	Group 1: 67 babies Birth: HBV 6 weeks: BCG 8,12 and 16 weeks: HBV Group 2: 64 babies Birth, 8,12,16 weeks: HBV 18 weeks: BCG (controls)	7 weeks 18 weeks	BCG at 6 weeks of age had no effect on antibody responses at age 7 or 18 weeks compared with controls.	All babies revived other vaccines as then recommended The Gambian schedule: OPV, HBV at birth Hib, HBV, DTwP, PCV-13, OPV at 8,12 and 16 weeks

Abbreviations: DTP = diphtheria, tetanus, pertussis vaccine; DTaP = diphtheria, tetanus, acellular pertussis vaccine; DTwP = diphtheria, tetanus, whole cell pertussis vaccine; HBV = hepatitis B vaccine; HBsAg = hepatitis B surface antigen; Hib = *haemophilus influenzae* type b vaccine; IgG = immunoglobulin G; IPV = inactivated poliovirus vaccine; L = litre; mIU = milli-international unit; MV = measles vaccine; OPV = oral polio vaccine; PCV-7 = 7-valent pneumococcal conjugate vaccine; PCV-13 = 13-valent pneumococcal conjugate vaccine; RCT = randomised control trial

Table 2.13: Studies reporting the influence of BCG and HBV on cytokine responses to hepatitis B surface antigen

Author, year, location (reference)	**Study design**	**Age BCG administered**	**Age at outcome**	**Outcome**	**Comment**
Ota, 2002, The Gambia (88)	RCT in 151 babies	Group 1: 35 babies Birth: BCG + HBV Group 2: 34 babies Birth: HBV 2 months: BCG + HBV Group 3: 34 babies Birth, 2, 4 months: HBV 4.5 months: BCG (controls)	2 months 4.5 months	Babies vaccinated with BCG at birth had enhanced IFN-γ, IL-5 and IL-13 cytokine responses to HBsAg at both time points. Babies vaccinated with BCG at HBV second dose (2 months) had enhanced IL-5 and IL-13 responses to HBsAg at 4.5 months of age.	All babies received other vaccines as per recommended The Gambian schedule: OPV at birth, 1 and 2 months DTP at 2, 3 and 4 months. BCG Pasteur strain.
Libraty, 2014, Philippines (85)	Nested case control in 51 babies	Group 1: 38 babies Within 2 weeks: BCG Group 2: 13 babies 6 weeks: BCG	8-12 weeks	No difference in IFN-γ response to HBsAg between the groups.	All babies presumably received vaccines as per Philippines immunisation schedule 2006-2008: OPV and DTP at approximately 6 weeks of age Authors did not comment on HBV administration introduced in 2007. BCG strain unknown.

Abbreviations: DTP = DTP = diphtheria, tetanus, pertussis vaccine; HBsAg = hepatitis B surface antigen; HBV = hepatitis B vaccine; IFN = interferon; IL = interleukin; OPV = oral polio vaccine; RCT = randomised control trial

2.4.2 The influence of concurrent BCG and HBV vaccination on immune responses to mycobacterial antigens

The three RCTs reporting infant immune responses to mycobacterial antigens in children vaccinated with HBV, lacked unvaccinated control groups for ethical reasons. These studies were also not designed to primarily investigate HBV effects on responses to mycobacterial antigens. The studies were directly or indirectly investigating concurrent BCG and HBV or the timing of these vaccines on immune responses to mycobacterial antigens.

In a RCT in 108 Senegalese infants, Coursaget et al. (136) reported reactivity to tuberculin skin testing in 6-month-old infants. They found no difference in positive tuberculin skin tests between infants receiving concurrent BCG and HBV at birth compared with isolated birth doses of these vaccines (Table 2.14). In The Gambia, Darboe et al. (87) investigated the influence of BCG on cytokine responses to stimulants unrelated to BCG in 7-week and 18-week old infants. Concurrent HPV and OPV were administered at birth and intradermal BCG was administered at 6 weeks of age, after which cytokine responses to purified protein derivative of *M. tuberculosis* (PPD) were compared with infants who were BCG-unvaccinated. There was no difference in cytokine responses to PPD between the infants at either time point (Table 2.14). In the only study with neonatal data, Australian researchers in our laboratory, investigating NSEs of BCG, reported cytokine responses to BCG and *M. tuberculosis* in babies who received intradermal BCG and intramuscular HBV compared at birth with babies receiving only intramuscular HBV (21). They found no difference in cytokine responses between the groups (Table 2.14).

All three trials showed that HBV given concurrently or after BCG vaccine does not change immune responses to mycobacterial antigens in children despite variation in location, other concurrent vaccines administered, BCG strain, age of BCG vaccination, age at measured outcome and in the measure of mycobacterial responses (Table 2.14).

Table 2.14: Studies reporting the influence concurrent BCG and hepatitis B vaccination on immune responses to mycobacterial antigens

Author, year, location (reference)	**Study design**	**Age BCG and HBV administered**	**Age at outcome**	**Outcome**	**Comment**
Coursaget, 1992, Senegal (136)	RCT in 108 infants	Group A: Birth: BCG + HBV 2, 3, 9 months: HBV Group B: Birth: HBV 2, 3, 9 months: HBV 4 months: BCG Group C: Birth: BCG 2, 3, 9 months: HBV	6 months for TST	Positive TST in Group A compared with Groups B and C (p = 0.36). Immune responses to PPD and HBsAg were similar in babies vaccinated concurrently or separately with BCG and/ HBV.	Babies received other vaccines according to then Senegalese vaccine schedule: DTP, OPV at 2, 3, and 4 months: TST results reported at 6 months did not differentiate between the 3 groups. BCG Pasteur strain.
Darboe, 2017, The Gambia (87)	RCT in 131 infants	Group 1: 67 babies Birth: HBV 6 weeks: BCG 8, 12 and 16 weeks: HBV Group 2: 64 babies Birth, 8, 12, 16 weeks: HBV 18 weeks: BCG (controls)	7 weeks 18 weeks	No effect of BCG vaccination on any cytokine responses to PPD at any time point.	All babies revived other vaccines as then recommended The Gambian schedule: OPV, HBV at birth Hib, HBV, DTwP, PCV-13, OPV at 8, 12 and 16 weeks. BCG Russia strain.
Freyne, 2018, Australia (21)	RCT in 212 babies	Group 1: Within the first week of life: BCG + HBV Group 2: Within the first week of life: HBV (controls)	3-21 days	No difference between the groups in cytokine responses to BCG and *M. tuberculosis.*	All babies received HBV at birth. BCG Denmark strain.

Abbreviations: DTP = diphtheria, tetanus, pertussis vaccine; DTwP = diphtheria, tetanus, whole cell pertussis vaccine; HBsAg = hepatitis B surface antigen; HBV = hepatitis B vaccine; Hib = *Haemophilus influenzae* type b vaccine; OPV = oral polio vaccine; PCV-13 = 13-valent pneumococcal conjugate vaccine; PPD = purified protein derivative; RCT = randomised control trial; TST = tuberculin skin test

2.5 Summary

This review of current literature has highlighted the predominance of research in BCG-induced NSE compared with NSEs of HBV. RCTs are under-represented as are studies in the neonatal period, especially for those reporting immunological effects. HBV is currently one of the most commonly administered neonatal vaccines, yet at the time of writing, there were few studies in children and no neonatal studies investigating HBV-induced NSEs. Order and timing of vaccinations has been identified as an important modifier of BCG-induced NSEs. The WHO currently strongly recommends concurrent BCG and HBV when both vaccines are recommended at birth (138). However, neonatal studies investigating NSEs in relation to both BCG and HBV compared with an unvaccinated control group are absent.

An understanding of the non-specific immunological effects of isolated HBV and concurrent BCG vaccination, two of the few vaccines licensed for neonates, from a well-designed RCT, is needed. The information would add to the evidence of the influence of vaccines on neonatal immunity to unrelated stimulants, for consideration by scientists when designing new neonatal vaccine schedules or additional adjuvants to enhance the neonatal defences against infections.

Chapter 3: Materials and Methods

3.1 Introduction

This research project investigated the influence of two common birth vaccines - Bacille Calmette-Guérin vaccine (BCG) and hepatitis B vaccine (HBV) - on neonatal cytokine responses to nine stimulants in the Early Life Vaccines and Immunity Study (ELVIS) (139). This was a collaborative study between the Obstetric and Neonatology Departments at the Mercy Hospital for Women (MHW) in Heidelberg, Melbourne, The Murdoch Children's Research Institute (MCRI) and The Royal Children's Hospital (RCH), Melbourne. This chapter details the experimental design, ethics approvals, procedures and clinical data collection for the trial.

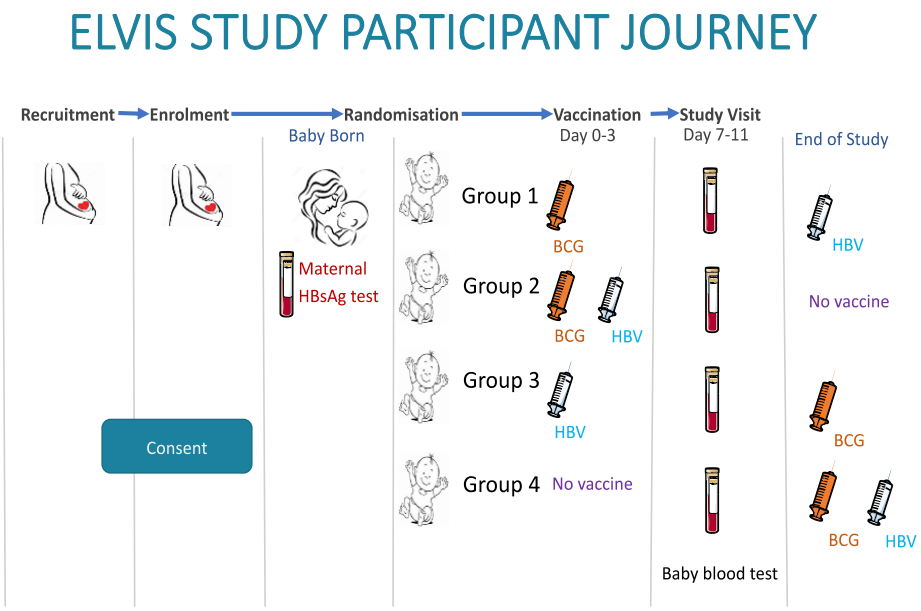
3.2 Clinical trial design

ELVIS was a randomised control trial to investigate the influence of BCG and HBV on neonatal cytokine responses to the different stimulants. Expectant mothers were screened for their likelihood of travel with their babies to areas of high tuberculosis incidence within the following 5 years. Those likely to travel were asked to consider participation in the study. All babies in the study were offered BCG as recommended for protection against tuberculosis while travelling (23). The details of study procedures and interventions are detailed below. Participants progressed through the study with a time commitment of up to 11 days after the birth of the baby (Figure 3.1). Maternal blood was collected as soon as possible after delivery to confirm negative hepatitis B surface antigen (HBsAg) status. Eligible babies were randomised within 3 days of birth and study vaccines were administered at the time of randomisation. Babies returned after 7 ± 4days when a blood sample was taken. At the end of the study, babies received any vaccinations not previously administered, so that all babies received both BCG and HBV.

3.3 Data entry and storage

Study data were collected and managed using Research Electronic Data Capture (REDCap) hosted at RCH (140, 141). REDCap is a secure, web-based software platform designed to support data capture for research studies, providing 1) an intuitive interface for validated data capture; 2) audit trails for tracking data manipulation; 3) automated export procedures for seamless data downloads to common statistical packages, and 4) procedures for data integration and interoperability with external sources. Questionnaires and layout of the electronic database were designed especially for the ELVIS trial and all records were maintained throughout the project. All researchers were trained to access and enter data appropriately into the database, which could only be accessed by authorised researchers.

Figure 3.1: Participant journey through the study



Abbreviations: HBsAg = hepatitis B antigen, HBV = hepatitis B vaccine

3.4 Clinical data

Clinical information from the participants was gathered by researchers at various points of contact: at enrolment, clinical antenatal visits, randomisation and study visits. Data collected and entered into REDCap included demographics, exclusion and inclusion criteria, adverse events and information related to factors known to influence neonatal immunity, e.g., feeding, medication use and medical conditions.

Researchers also performed a general health check before phlebotomy to identify any potential concerns. These assessment details were included in the database. Babies were referred for formal assessment outside of the study if any concerns were identified.

3.5 Ethics approval

Ethics approval was obtained from the Mercy Health Human Research Ethics Committee (HREC) and the RCH HREC. The Mercy Health HREC originally approved the study in August 2014 (Ref. no. R14/02) (Appendix A). Minor amendments to the protocol were submitted and approved in October 2014 (Appendix B), June 2015 (Appendix C) and May 2016 (Appendix D). The HREC at the RCH approved the original study in May 2014 (Ref. no. 34032 A) (Appendix E), and minor amendments to the protocol in February 2015 (Ref. no. 34032B) (Appendix F).

3.6 Participant recruitment

3.6.1 Recruitment site

Potential participants were approached at the MHW in Heidelberg, Melbourne. This hospital provides obstetric care to mothers with low risk pregnancies in their catchment area and provides specialist obstetric and neonatal care for state-wide high-risk pregnancies. Approximately 1,000 babies are born there each year, with caesarean sections accounting for roughly 40% of all deliveries.

3.6.2 Recruitment procedure

Pregnant women attending antenatal clinics or women who had delivered an infant vaginally who had not yet received HBV were approached by researchers to consider participation in the study. Researchers screened parents by asking if they were likely to travel outside of Australia with their babies to a country of high tuberculosis (TB) incidence in the next 5 years. An online resource, the BCG world atlas (142) was used to determine the TB incidences for each country. If the parents were interested in the study and indicated they were likely to travel to countries with a TB incidence of >40 per 100,000 population, researchers sent them the parent/guardian information and consent form (PGICF) (Appendix G) via email. This form described the purpose of the study, the procedures to be followed and the risks and benefits of participation in the study. The contact details for the study co-ordinator were also made available at that time. With a parent's consent, an alert was placed in the maternal or infant record, advising staff that the family were considering involvement in the study. This was to ensure that study vaccines were given by research staff, should the infant be enrolled in the study.

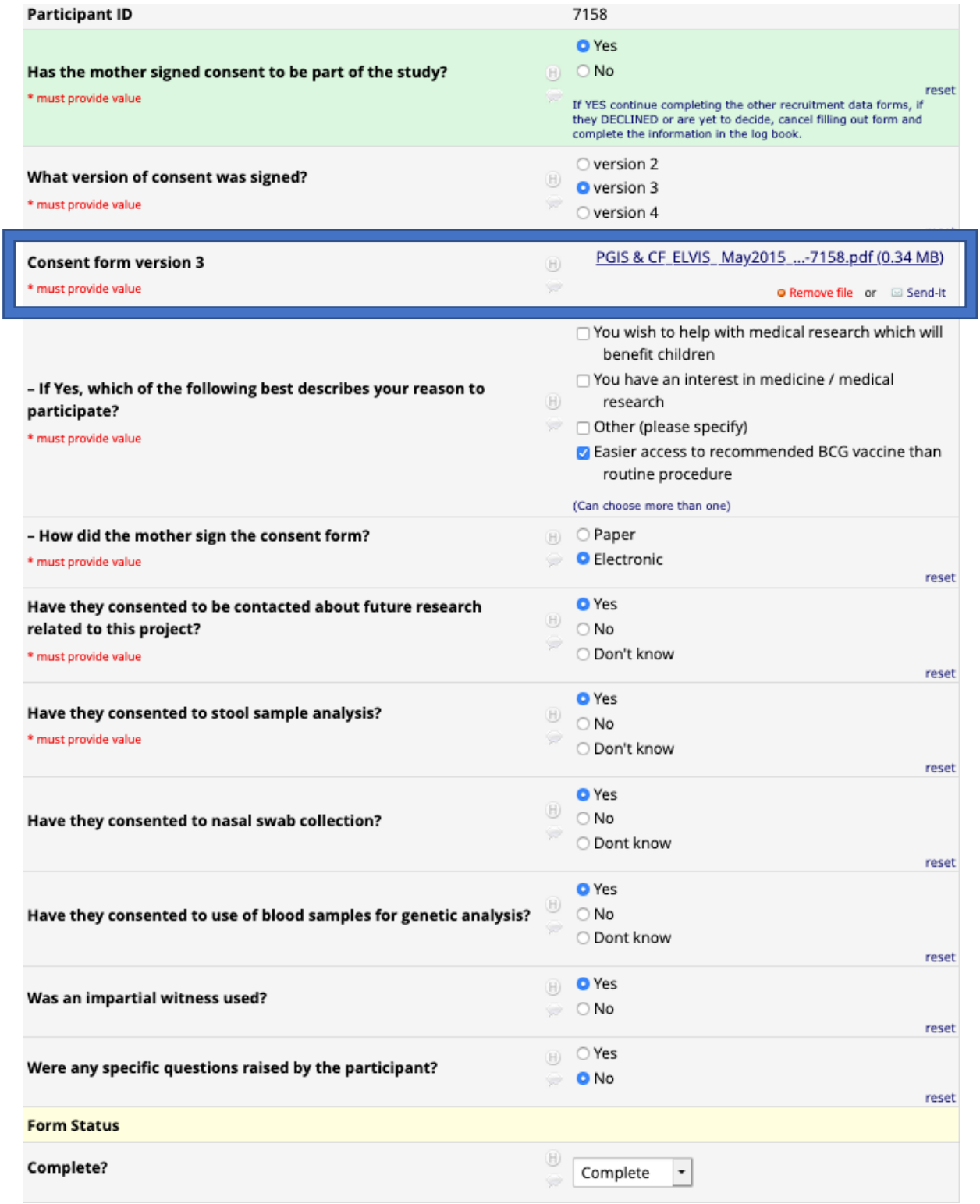
Researchers re-approached these parents either at the next antenatal visit or later the same day if the infant was already born, to establish if the infant would be enrolled in the study.

3.7 Consent procedure

A member of the research team ensured that each infant's parents understood the information presented in the PGICF and provided an opportunity for the parents and family to ask questions or clarify any aspect of the study. Electronic consent was obtained for every participant enrolled in the study before commencing any study procedures or clinical data collection.

Mothers were given access to study iPads to view the PGICF as a PDF document in the presence of a researcher. They signed the PDF file in the presence of a witness using the iPad application iAnnotate (143). A new file was created for each participant and saved with the participant's identification number. One signed copy was then emailed to the participant's parent and another copy was sent to the secure study email address. A signed copy was directly saved into the participant's electronic study record. Figure 3.2 shows the layout in the participants' electronic records where the consent form was stored.

Figure 3.2: Participant electronic consent record entry



Participant ID	7158
Has the mother signed consent to be part of the study? * must provide value	◉ Yes ○ No reset If YES continue completing the other recruitment data forms, if they DECLINED or are yet to decide, cancel filling out form and complete the information in the log book.
What version of consent was signed? * must provide value	○ version 2 ◉ version 3 ○ version 4
Consent form version 3 * must provide value	PGIS & CF_ELVIS_May2015_...-7158.pdf (0.34 MB) Remove file or Send-It
– If Yes, which of the following best describes your reason to participate? * must provide value	☐ You wish to help with medical research which will benefit children ☐ You have an interest in medicine / medical research ☐ Other (please specify) ☑ Easier access to recommended BCG vaccine than routine procedure (Can choose more than one)
– How did the mother sign the consent form? * must provide value	○ Paper ◉ Electronic reset
Have they consented to be contacted about future research related to this project? * must provide value	◉ Yes ○ No ○ Don't know reset
Have they consented to stool sample analysis? * must provide value	◉ Yes ○ No ○ Don't know reset
Have they consented to nasal swab collection?	◉ Yes ○ No ○ Dont know reset
Have they consented to use of blood samples for genetic analysis?	◉ Yes ○ No ○ Dont know reset
Was an impartial witness used?	◉ Yes ○ No reset
Were any specific questions raised by the participant?	○ Yes ◉ No reset
Form Status	
Complete?	Complete

3.8 Eligibility criteria

3.8.1 Inclusion criteria

Infants meeting all the following criteria were eligible for inclusion in the study:

- Singleton pregnancy
- Delivered vaginally
- Gestational age ≥32 weeks
- Birth weight >1,500 g
- Aged <3 days
- Likely to travel to an area with high TB prevalence in the first 5 years of life
- English-speaking mother
- Maternal negative antenatal human immunodeficiency virus (HIV) screen in the current pregnancy
- Maternal negative antenatal hepatitis B infection screen in the current pregnancy
- Not exposed to household contacts infected with hepatitis B.

3.8.2 Exclusion criteria

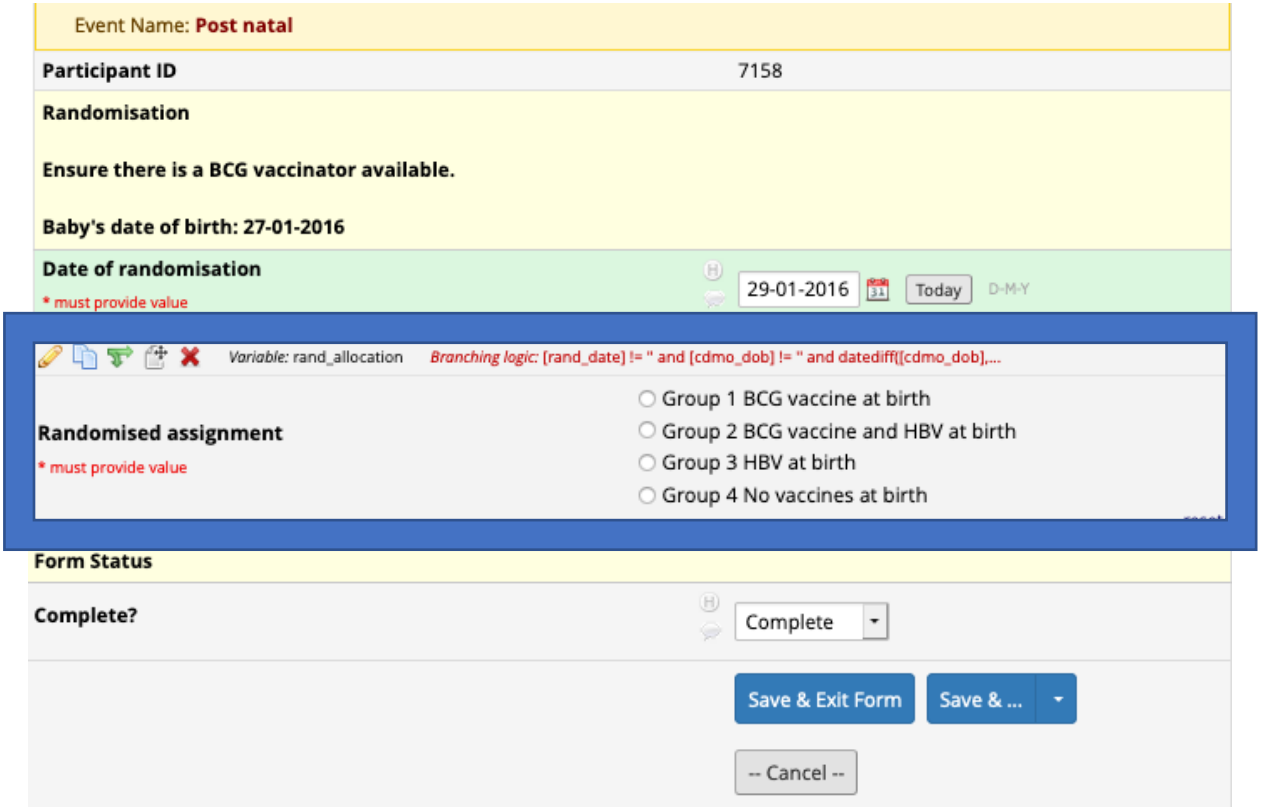
Babies meeting any of the following criteria were excluded from the study:

- Received study vaccinations (HBV or BCG) before randomisation
- Maternal immunosuppression
- Contraindication to receive BCG vaccine:
    - Treatment with immunosuppressive therapy
    - Known malignancy involving bone marrow or lymphatic systems
    - Known cellular immunodeficiency
    - Serious underlying illness, including severe malnutrition
    - Generalised skin disease
    - Significant febrile illness
    - Medically unstable i.e., requiring respiratory or cardiac support
    - Third trimester antenatal exposure to biological disease-modifying anti-rheumatic drugs (bDMARDS)
    - Known family history of immunodeficiency
    - Parental consanguinity

3.9 Randomisation

The randomisation schedule was prepared by an independent biostatistician. Babies were randomly assigned using REDCap to one of the three vaccine groups or the control group within 3 days of birth (Figure 3.3). It was not possible to randomise a baby more than once or to cancel or delete a previous randomised allocation. The randomised allocation was then stored in the participant's study record

Figure 3.3: Participant electronic randomisation record entry



The randomisation allocation in the database is written as vaccine "at birth", however, it was understood by all researchers that the vaccines could be delivered within 72 hours of birth

3.10 Study groups

The effect of giving BCG and HBV at birth on cytokine responses in neonates was evaluated in three different vaccine groups, and a control group who received no birth vaccine (Table 3.1). No placebo vaccine was used in this trial.

Table 3.1: Study groups

Group	Vaccines administered within three days of birth
1	Bacille Calmette-Guérin vaccine
2	Bacille Calmette-Guérin vaccine and hepatitis B vaccine
3	hepatitis B vaccine
4	No vaccine/control

3.11 Vaccines used in the study

3.11.1 BCG Vaccine

Description, dispensing and accountability

BCG Vaccine SSI, Danish strain 1331 is a freeze-dried powder containing live attenuated *M. bovis* strain, BCG. It was supplied in multi-dose vials and reconstituted according to instructions before use.

BCG vaccine was delivered to the MCRI from the supplier and transported to the recruitment site as un-reconstituted vaccine. Strict compliance with cold storage (+ 2°C to +8°C) was maintained throughout the study, as stipulated by the manufacturer. All BCG vials were protected from light, used before the expiry date and any unused reconstituted BCG was discarded after 4 hours.

BCG vaccine for the study was stored at the recruitment study pharmacy, according to standard protocols. It was dispensed according to the recruiting site procedures, with a valid medication order on in-patient drug charts or out-patient scripts to designated members of the research team. A record of BCG vaccine dispensed, reconstituted and administered was kept in a separate REDCap database. The records included details of batch numbers, vial numbers and expiry dates of vaccine.

Dose and route of administration

Study participants received a single 0.05 mL intradermal injection of BCG Vaccine SSI over the distal insertion of the deltoid muscle approximately one third down the upper left arm according to administration instructions by trained members of the research team. A 1 mL syringe, marked in divisions of 0.1 mL, fitted with a short bevel needle of 25G/0.50 mm or 26G/0.45 mm was used for administration of the vaccine.

A record of BCG vaccination was placed in the infant's in-patient medical record as a signed order on the medication chart and as a note in the progress notes of each participant. The

details of vaccination were also included in the participant's state-wide infant record book alongside information about the study for future health care providers.

3.11.2 Hepatitis B vaccine (HBV)

Description, dispensing and accountability

The HBV used in this study was H-B-VAX® II pre-filled paediatric formulation HBV (Merck Sharp & Dohme). This vaccine comprised 5 µg per mL HBsAg, produced by yeast using recombinant DNA technology, adsorbed onto 0.25 mg per mL aluminium hydroxide. This formulation is preservative-free. The vaccine was kept in the postnatal ward and at the recruitment site pharmacy. Vaccine batch numbers and expiry dates were entered into the participants' study records and included in the participant's state-wide infant record book. Records of hepatitis B vaccination were entered in the infant's in-patient medical record as a signed order on the medication chart and as a note in the progress notes of each participant.

Dose and route of administration

Study participants received an intramuscular dose of 0.5 mL of HBV in the outer lateral thigh.

3.12 Laboratory assessments

3.12.1 Examination of maternal blood samples for hepatitis B surface antigen and antibodies

Maternal blood was tested close to delivery to ensure their infants were not at risk of intrapartum transmission of hepatitis B. Maternal immunity to hepatitis B infection was also assessed as this may impact on their infant immune response to HBV.

Maternal blood was collected by phlebotomists at the recruitment hospital site, most often when other blood tests were scheduled. Blood was collected into serum tubes using an evacuation closed system. Samples were transported at room temperature to the diagnostic microbiology laboratory at the Austin Hospital, Melbourne. This laboratory is nationally accredited to perform tests for HBsAg and antibodies. HBsAg was measured using an immunoassay (Elecsys® HBsAg II, Roche Diagnostics). Results were reported as positive (reactive) or negative (non-reactive). Hepatitis B surface antibody (HBsAb) titres were also analysed using an immunoassay (Elecsys® Anti-HBs II, Roche Diagnostics), which is accurate for titres between 2 and 1000 IU/L.

Maternal HBsAg and antibody results for each participant were reviewed from the maternal electronic health records at the recruitment site and entered into the REDCap database. The data, along with other clinical variables, were exported as a .csv file into Excel, and then imported into Stata v.13.1.

HBsAb results were categorised according to titre ranges. Cut-off values were chosen for each category to show mothers who were not seroprotected against hepatitis B infection (HBsAb titres <10 IU/L), and those who were seroprotected against hepatitis B infection with either low (10-100 IU/L) or high (>100 IU/L) titres of HBsAb. The results were reported as percentage frequencies in the four groups.

3.12.2 Neonatal blood samples

Babies were brought back to the recruitment site for blood collection 7 ± 4 days after randomisation. Phlebotomists, experienced in neonatal phlebotomy, collected blood samples using 24-gauge peripheral intravenous cannulas into 7.5-mL sodium-heparin, endotoxin-free tubes (*S-monovette*). Blood samples, labelled with the participant study identity number, date and time of collection, were kept at room temperature and couriered to the MCRI laboratory within 4 hours of collection. Laboratory staff who received the samples were blinded as to the treatment group of each participant.

The whole blood samples were stimulated with nine different stimulants and RPMI (nil control) 4 hours (± 10%) after collection (Table 3.2).

Table 3.2: List of stimulants

Stimulant	Final Concentration
Negative control	
Nil (RPMI medium only)	
Mycobacterial stimulants	
BCG Denmark	75 ug/mL
M. tuberculosis	Equivalent to 10_6 colony-forming units (CFU)/mL
Viral component	
Hepatitis B surface antigen	10 ug/mL
Heat-killed pathogens	
Candida albicans	Equivalent to 10_6 CFU/mL
Escherichia coli	Equivalent to 10_6 CFU/mL
Listeria monocytogenes	Equivalent to 10_6 CFU/mL
Staphylococcus aureus	Equivalent to 10_6 CFU/mL
Toll-like receptor (TLR) agonists	
Peptidoglycan (PEPG) (TLR 2 agonist)	10 ug/mL
Resiquimod (R848) (TLR 7/8 agonist)	3.5 ug/mL

Abbreviations: CFU = colony forming units; mL = millilitres; ug = micrograms

The stimulants listed in Table 3.2 were chosen as they represent a broad range of neonatal pathogenic stimuli including bacteria, viruses and fungi and allow comparisons with other similar studies. The Toll-like receptors (TLR) are a group of ten different pathogen recognition receptors that function as innate immune cell sensors for infections (144). Peptidoglycan (PEPG) was chosen as it forms part of the bacterial cell wall of both Gram-positive and Gram-negative bacteria and is recognised by TLR2 that is present on the immune cell membrane (145). It induces production of inflammatory cytokines from monocytes and T cells (146). Resiquimod (R848) drives production of TH1 cytokines through a range of mechanisms including stimulating dendritic (innate immune) cells (147). It is recognised by TLR7/8 that is present within the cell.

The stimulants were sourced as follows:
RPMI 1640 medium (Gibco); BCG Denmark strain 1331 (Statens Serum Institut, Copenhagen, Denmark); HBsAg (Alpha Diagnostics); PEPG and R848 (Invivogen).

Bacterial and fungal pathogens were clinical isolates obtained from children with invasive infections at the RCH in Melbourne. Pathogens were grown on solid media and re-

suspended in phosphate buffered saline. The viability of isolates was determined by serial dilutions on solid media. A colony count was made on this suspension before heat-killing at 70°C for 2 hours and then tested for sterility by culturing in relevant growth media.

Stimulation assays were performed in MiCROLON® high binding U-bottom 12 by 8-well strip plates (Greiner), which had been prepared in the laboratory. Assay strips for most stimulants were prepared using a robotic system to deliver 20 µL of stimulant to each well and were then stored at -80°C until required. BCG, HBsAg and *M. tuberculosis* were added separately, immediately before performing the assay.
For the assay, pre-prepared assay strips were thawed at 37°C for 10 minutes and centrifuged at 250 x *g* in an Eppendorf centrifuge (Model 5810R) for 5 minutes. 20 µL of BCG, HBsAg and *M. tuberculosis* preparations were then added to separate wells of each test strip. Neonatal whole blood samples were diluted in an equal volume of RPMI-1640, after which 180 µL of diluted blood were added to each well.
Blood samples from each participant were stimulated in order of the following priority:

1. Nil (RPMI only)
2. Bacille Calmette-Guérin (Denmark), (BCG)
3. Hepatitis B surface antigen, (Hep B)
4. *Escherichia coli, (E. coli*)
5. Resiquimod, (R848)
6. Peptidoglycan, (PEPG)
7. *Staphylococcus aureus, (S. aureus)*
8. *Candida albicans, (C. albicans)*
9. *Mycobacterium tuberculosis*, (*M. tuberculosis*)
10. *Listeria monocytogenes, (L. monocytogenes)*

The strips were then covered with a sterile lid and placed into a humidified incubator (37°C; 5% CO_2 in air) for 20 ± 2 hours. Stimulated whole blood samples were centrifuged at 600 x *g* for 5 minutes and the supernatants were transferred to labelled 1.5 mL microfuge tubes. The supernatants were then stored at -80°C until the cytokine concentrations were measured.

In addition to the clinical criteria for exclusion from the study (Chapter 3.8.2), I excluded samples for cytokine measurements if they met any of the following criteria.

Clinical criteria for exclusion were:

- Participant received antibiotics at any time before blood collection
- Participant diagnosed with a congenital infection
- Participant received immunomodulatory treatment e.g., intravenous immunoglobulin or steroids.

Laboratory criteria for exclusion were:

- Insufficient blood volume to run the test
- Presence of blood clots in the sample
- Blood samples showed haemolysis
- Laboratory protocols were breached while performing the assay.

Three cytokines, tumour necrosis factor-alpha (TNF-α), interferon-gamma (IFN-γ) and monocyte chemoattractant protein-1 (MCP-1), were measured in the supernatants using the following commercial enzyme-linked immunosorbent assay (ELISA) kits:
Mabtech 3512-1H-20 ELISA development kit for TNF-α; Mabtech 3420-1H-20 ELISA development kit for IFN-γ and MyBioScource, MBS2088398 96Tx5 ELISA kit for MCP-1.

Test samples were thawed and diluted 1 in 6 before measuring TNF-α and IFN-γ, and 1 in 100 before measuring MCP-1. All assays were done according to the manufacturer's instructions. An extra dilution was made to the standard dilution instructions for MCP-1 resulting in lower measurable values.

ELISA Analysis software (148) using Four Parameter Logistic (4PL) was used to fit standard curves and calculate cytokine concentrations in the test samples. All samples were run in duplicate in the same experimental run. Results were exported as .csv files to Excel and corrected for their dilution factor. The data were merged with the clinical metadata and analysed using Stata v 13.1 (StatCorp) and Prism 8, version 8.0 (GraphPad Software).

3.12.3 'Outliers' and 'non-responders' definitions

In the cytokine-stimulant pair datasets from all participants, 'outliers' were defined as babies with a cytokine measurement that exceeded two standard deviations above or below the mean cytokine measurement for each cytokine-stimulant pair dataset.

Babies were called 'non-responders' if their cytokine response to stimulation, measured by ELISA, was recorded as zero pg/mL. An arbitrary value of half the lowest threshold of detection for that assay was allocated to the zero readings.

3.13 Statistical methods

3.13.1 Sample size calculation

From similar cytokine studies in young infants published before this study was designed, biostatistician, Susan Donath, from the Clinical Epidemiology & Biostatistics Unit (CEBU) at the MCRI estimated that a sample size of 50 neonates in each group would have 80% power to detect an effect size of 0.634 using a two-group t-test log-transformed data with a 0.05 two-sided significance level. The number needed to recruit for this study was closer to 300 babies to allow for attrition of 33% in the trial. The attrition rate takes into consideration that consent was taken antenatally in a hospital where the rate for caesarean section deliveries was approximately 40% at the time of the trial. We estimated the likely caesarean rate to be lower in our study population as we excluded multiple pregnancies or women planning elective caesarean deliveries. Further considerations for attrition were the strict inclusion criteria and technical difficulty in neonatal phlebotomy to achieve adequate blood volumes for analysis. It was calculated this would take 18 months at a recruitment rate of 30%, taking into consideration the demographics of the patients receiving care at the recruitment hospital and likelihood of meeting eligibility criteria.

3.13.2 Choice of parametric vs non-parametric statistical tests

Non-parametric statistical tests were used to analyse the data as the distribution of cytokine measurements were skewed. Therefore, the cytokine measurements were not log-transformed before analyses were performed.

13.3.3 Statistical tests performed to determine the primary outcome

A Kruskal-Wallis (K-Wallis) test was used to determine whether there was a statistically significant difference in the median cytokine-stimulant response for each cytokine-stimulant pair between the four groups (four-group comparison).

For every cytokine-stimulant pair with a statistically significant result from the K-Wallis test, a Mann-Whitney U (MWU) test was used to determine if any vaccine group (Group 1, Group 2 or Group 3) was statistically different from the control group (Group 4) (two-group comparisons).

A p-value was considered significant if the two-sided value was ≤0.05.

13.3.4 Statistical tests performed for secondary analyses

Sex

The cytokine data were analysed for any influence of sex on cytokine-stimulant pair responses using the MWU test.

For every cytokine-stimulant pair with a statistically significant MWU test, a further MWU test was performed to determine whether responses in babies in the birth vaccine groups (Group 1, Group 2 and Group 3) were statistically different for girls or boys compared with unvaccinated babies (Group 4).

13.4 Reporting

The study has been reported in line with the Standards for Reporting Diagnostic Accuracy Studies (STARD) Guidelines (149). This is a checklist that was developed to improve completeness and transparency of reporting of studies of diagnostic accuracy.

13.5 Study Protocol

The detailed study protocol is shown in Appendix H.

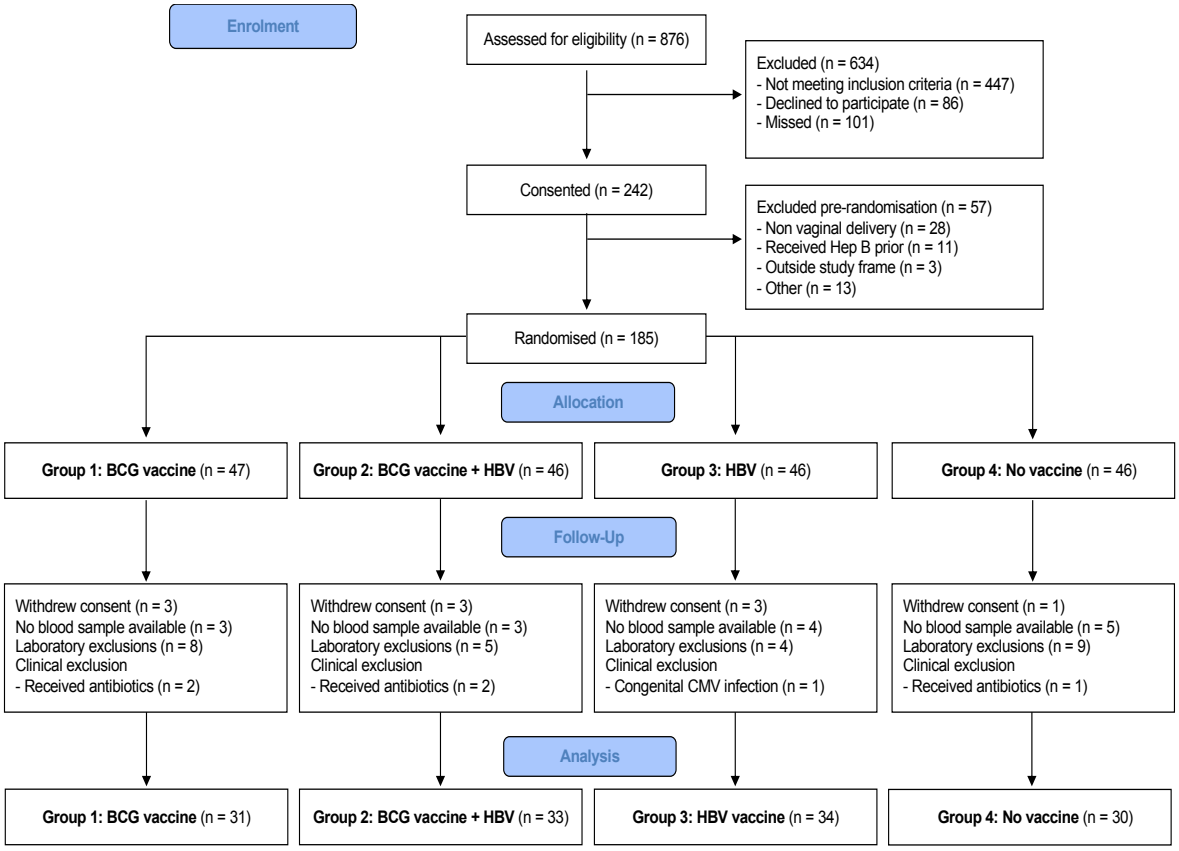
Chapter 4: Results

4.1 Participant recruitment

A total of 876 pregnant mothers were approached for the study (Figure 4.1). Of these, 242 (28%) consented to participate, and 185 (76% of those consented) were randomised to the four study groups.

Recruitment to the study was stopped prematurely due to a global Bacille Calmette-Guérin (BCG)-Denmark vaccine shortage resulting in lower number of participants recruited than planned. After exclusions, a total of 128 participants (69% of those randomised) were included in the final laboratory analyses (Figure 4.1). There were similar numbers in each of the four groups.

Figure 4.1: ELVIS CONSORT flow diagram



4.2 Participant characteristics

Table 4.1 shows the baseline characteristics of the babies and mothers for whom samples were analysed. The groups were well balanced for the majority of characteristics. However, there was a slightly higher proportion of males in the No vaccine group (group 4) and females in the BCG vaccine group (Group 1). However, the proportions between the four groups was not statistically different (analysis of variance (ANOVA) test, p = 0.8).

Babies were appropriate weights for their gestational ages. Birth weights were similar across the four groups (mean 3,272 g ± 442 g) and approached the national average of 3,327 g for that year (150). Nine babies weighed above the mean + standard deviation (SD) and 23 babies weighed below the mean less the SD. Most babies were exclusively breastfed.

Almost 90% of the mothers reported that they received BCG vaccination in childhood, having been born in countries where BCG vaccination is routinely offered. Just over half of mothers (53%) were seroprotected against hepatitis B infection. Babies receiving hepatitis B vaccine (HBV) at birth (Group 3) had the highest proportion of maternal seroprotection to hepatitis B (68%).The difference was not statistically significant between the groups (ANOVA, p = 0.2) Approximately half the babies were born to parents who migrated from India and other South Asian countries (Nepal, Sri Lanka, Afghanistan and Pakistan); one-fifth were born to parents of Chinese ancestry. The rest were born to parents with ancestral backgrounds from many different countries, predominantly South East Asian countries, but also Europe, South America, South Pacific and Africa.

4.3 Trial vaccines

All babies received the trial vaccinations according to the protocol. In each of the vaccine groups, 46% of the babies received the vaccine within 24 hours of birth.

4.4 Adverse events

No adverse events related to vaccination with BCG or HBV were reported by caregivers or health care professionals within 3 months of vaccination for any baby or any time during the study thereafter.

Table 4.1: Baseline characteristics of study participants

	Group 1 **BCG vaccine**	Group 2 **BCG vaccine + HBV**	Group 3 **HBV**	Group 4 **No vaccine**	Total
	No. (%)	No. (%)	No. (%)	No. (%)	No. (%)
All participants	31 (24)	33 (26)	34 (27)	30 (24)	128 (100)
Infant factors					
Sex					
Male	11 (36)	16 (52)	17 (50)	18 (60)	62 (48)
Female	20 (65)	17 (49)	17 (50)	12 (40)	66 (52)
Birth weight, mean ± SD (g)	3221 ± 313	3106 ± 389	3179 ± 280	3300 ± 461	3272 ± 442
Gestational age, mean ± SD (w)	39.4 ± 1.0	39.2 ± 1.2	39.3 ± 1.0	39.5 ± 1.2	39.3 ± 1.0
Feeding					
Exclusive breast	24 (78)	23 (70)	27 (79)	21 (70)	95 (74)
Exclusive formula	0 (0)	1 (3)	0 (0)	0 (0)	1 (1)
Mixed	7 (23)	9 (28)	7 (21)	9 (30)	32 (25)
Time to first vaccine (h)					
<24	14 (45)	16 (49)	15 (44)	-	-
24-72	16 (52)	17 (52)	18 (53)	-	-
Not recorded	1 (3)	0 (0)	1 (3)	-	-
Maternal factors					
Maternal BCG					
Yes	28 (90)	30 (91)	31 (91)	23 (77)	112 (88)
No	3 (10)	3 (9)	2 (6)	3 (10)	11 (9)
Unknown	0 (0)	0 (0)	1 (3)	4 (13)	5 (4)
Maternal hepatitis B surface antibody (HBsAb) titre (IU/L)					
0-10*	17 (55)	15 (46)	11 (32)	14 (47)	57 (45)
10-100	3 (10)	9 (27)	6 (18)	6 (20)	24 (19)
>100	11 (36)	6 (18)	17 (50)	10 (33)	44 (34)
Unrecorded	0 (0)	3 (9)	0 (0)	0 (0)	3 (2)
Maternal vaccination in pregnancy[a]					
Yes	23 (74)	27 (82)	26 (77)	21 (70)	97 (76)
No	5 (16)	5 (15)	7 (21)	8 (27)	25 (20)
Unknown	3 (10)	1 (3)	1 (3)	1 (3)	6 (5)
Maternal region of birth					
South East Asia[b]	1 (3)	2 (6)	3 (9)	7 (23)	13 (10)
South Asia[c]	20 (65)	21 (64)	12 (35)	11 (37)	64 (50)
East Asia[d]	6 (19)	4 (12)	12 (35)	3 (10)	25 (20)
Other	4 (13)	6 (18)	6 (18)	8 (27)	24 (19)
Unknown	0 (0)	0 (0)	1 (3)	1 (3)	2 (2)

* HBsAb titre <10 IU/L considered non protected
a *Boostrix* (Diphtheria-Tetanus-acellular Pertussis) in third trimester ± Influenza vaccine
b South East Asia includes Vietnam, Indonesia, Philippines, Singapore, Malaysia
c South Asia includes India, Nepal, Sri Lanka, Afghanistan, Pakistan
d East Asia includes China and Hong Kong
Abbreviations: g = grams; h = hours; SD = standard deviation; w = weeks

4.5 Samples for cytokine analysis

Of the babies for whom there was sufficient blood for analysis, the mean blood volume obtained at collection was 3.75 mL (range 0.27 mL-7.55 mL; median 3.47mL).

The number of cytokine-stimulant pair values available for analyses differed between babies as a result of the differing blood volumes collected. For monocyte chemoattractant protein-1 (MCP-1), the loss of two samples during laboratory processing meant that the final cytokine-stimulant pair numbers were less than those assayed for tumour necrosis factor-alpha (TNF-α) and interferon-gamma (IFN-γ). In addition, a pre-determined priority list for stimulations resulted in a different number of samples that were tested for each stimulant (chapter 3.12.2). The final number of samples for each stimulant is the number obtained after sample exclusion for laboratory reasons post the stimulation assays and therefore did not match the pre-determined priority list. However, similar number of samples for each stimulant across the trial groups were assayed for all three cytokines (Table 4.2).

***Table 4.2*: Sample numbers for all cytokine-stimulant pairs**

Stimulants	Group 1		Group 2		Group 3		Group 4		Total	
	BCG vaccine		**BCG vaccine + HBV**		**HBV**		**No vaccine**			
	No.		No.		No.		No.		No.	
	TNF-α IFN-γ	**MCP-1**	**TNF-α IFN-γ**	**MCP-1**	**TNF-α IFN-γ**	**MCP-1**	**TNF-α IFN-γ**	**MCP-1**	**TNF-α IFN-γ**	**MCP-1**
Negative control										
Nil	31	30	33	33	34	34	30	29	128	126
Mycobacterial stimulants										
BCG	28	27	31	31	31	31	29	28	119	117
M. tuberculosis	21	20	27	27	27	27	22	23	97	97
Viral component										
Hep B	21	21	28	28	25	25	24	23	98	97
Heat-killed pathogens										
C. albicans	22	21	24	25	27	27	22	22	95	95
E. coli	30	29	32	32	32	32	28	27	122	120
L. monocytogenes	19	18	23	23	26	26	20	20	88	87
S. aureus	19	18	24	24	26	26	22	22	91	90
Toll-like receptor agonists										
PEPG	29	28	31	31	34	34	25	25	119	118
R848	30	29	32	32	34	34	26	25	122	120

Abbreviations: IFN-γ = interferon-gamma; IQR = interquartile range; MCP-1 = monocyte chemoattractant protein-1; TNF-α = tumour necrosis factor-alpha
Stimulants: Nil, RPMI medium; Hep B, hepatitis B surface antigen; PEPG, peptidoglycan; R848, resiquimod

4.6 Cytokine measurement

4.6.1 Data distribution

The following figures and text describe the general data distribution for each cytokine-stimulant pair for all samples. Outliers and non-responders will be discussed separately (see Chapters 4.6.2 and 4.6.3).

Tumour necrosis factor-alpha

In the negative control (RPMI) samples, TNF-α responses were minimal (Figure 4.2) and significantly lower compared with responses to all stimulants (Mann-Whitney U (MWU) test, $p < 0.05$ for all stimulants). There was a large variation (generally more than 2-log-fold) in responses between individual babies for all stimulants. The median TNF-α response also varied more than 2-log-fold amongst stimulants. The response was highest following stimulation with resiquimod (R848) (for which the variation between babies was lowest).

Visualisation of the values and the frequency of distribution curves of all TNF-α-stimulant pairs showed their distribution to be non-normal (Figure 4.3).

Interferon-gamma

The lowest responses were seen in the negative control (RPMI) samples and were significantly less than responses for all stimulants (MWU test, $p < 0.05$ for all stimulants). There was a large variation in individual responses for all stimulants (generally 2-3-log-fold). Responses to R848 stimulation were highest overall and showed the greatest variation, approaching a 5-log-fold difference between babies.

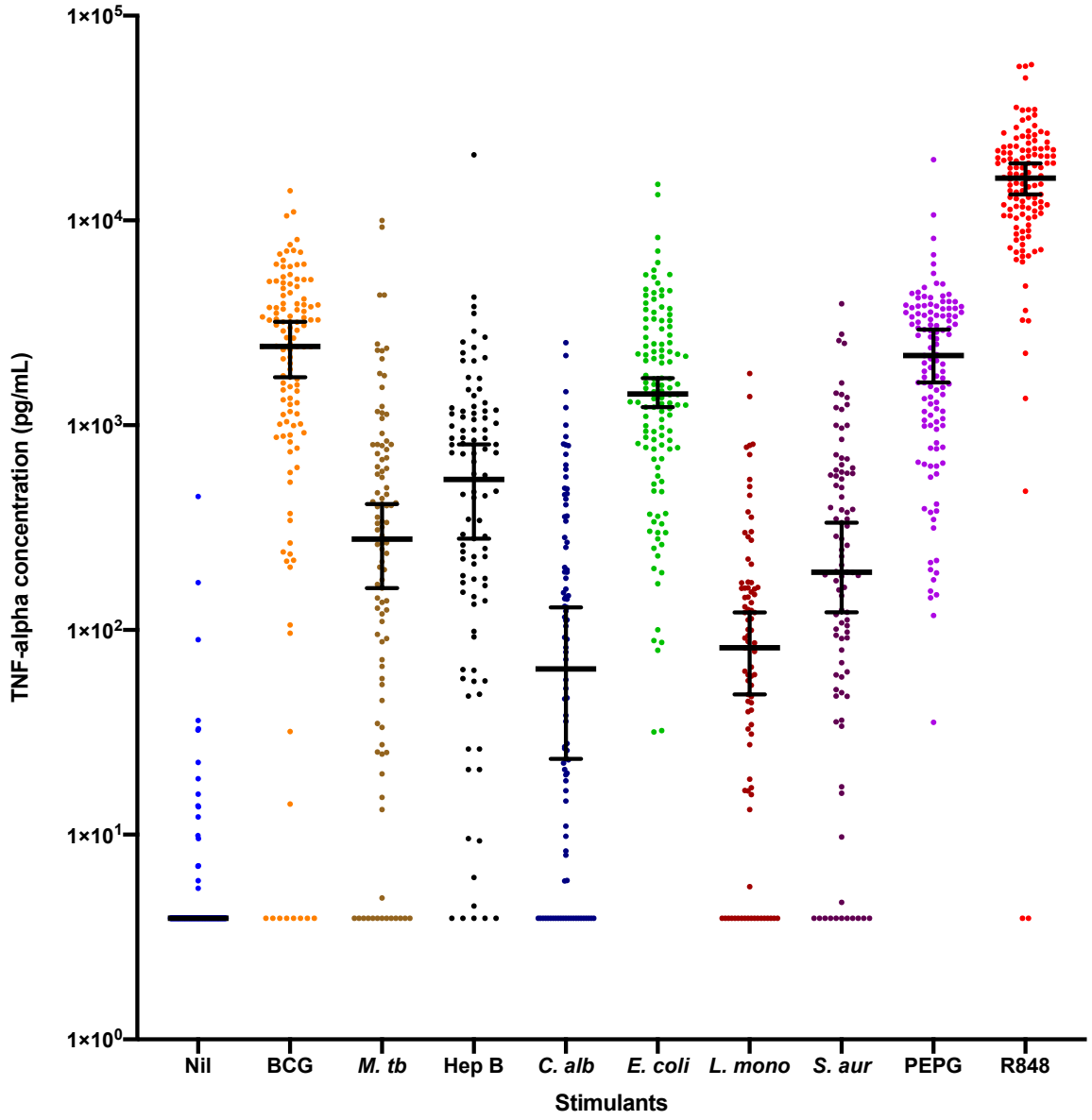
Visualisation of the values and the frequency of distribution curves of all IFN-γ-stimulant pairs showed their distribution to be non-normal (Figure 4.5)

Monocyte chemoattractant protein-1

There were statistically lower, but measurable, MCP-1 responses in most of the negative control (RPMI) samples compared with other stimulants (MWU test $p < 0.05$; Figure 4.6). The inter-individual variation in responses for all stimulant showed a different pattern compared with the other cytokines. Most babies either produced a robust response or failed to produce a measurable response to each stimulant. For the babies who responded, the variation between responses was less than the other cytokines but still encompassed a 1-2-log-fold difference. The median responses were similar for all stimulants (MWU $p > 0.05$ between all stimulants).

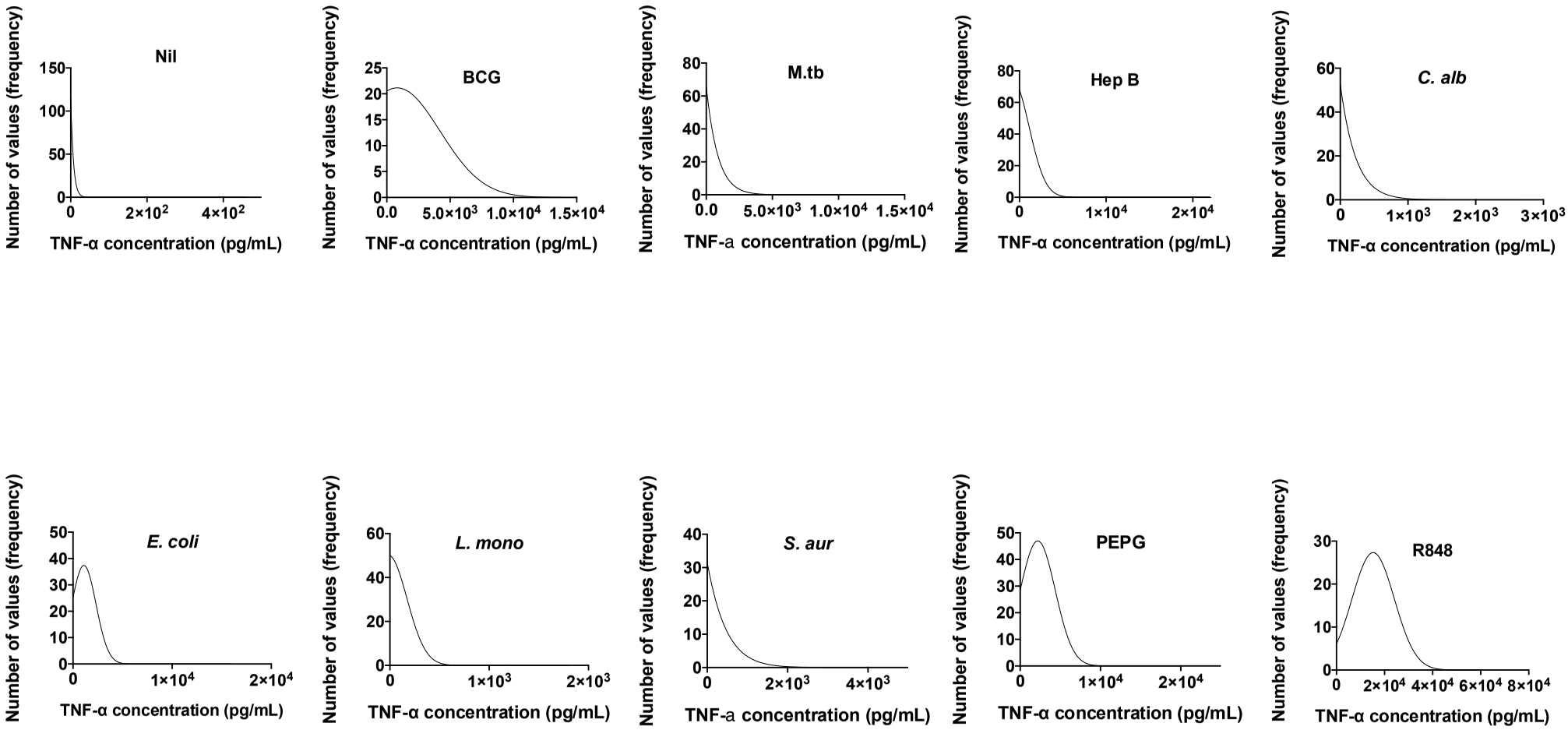
Visualisation of the values and the frequency of distribution curves for all MCP-1-stimulant pairs showed their distribution to be non-normal (Figure 4.7).

Figure 4.2: Distribution of TNF-α responses following in vitro stimulation with the nil control, killed pathogens and two Toll-like receptor agonists. Error bars depict the median and 95% upper and lower confidence intervals.



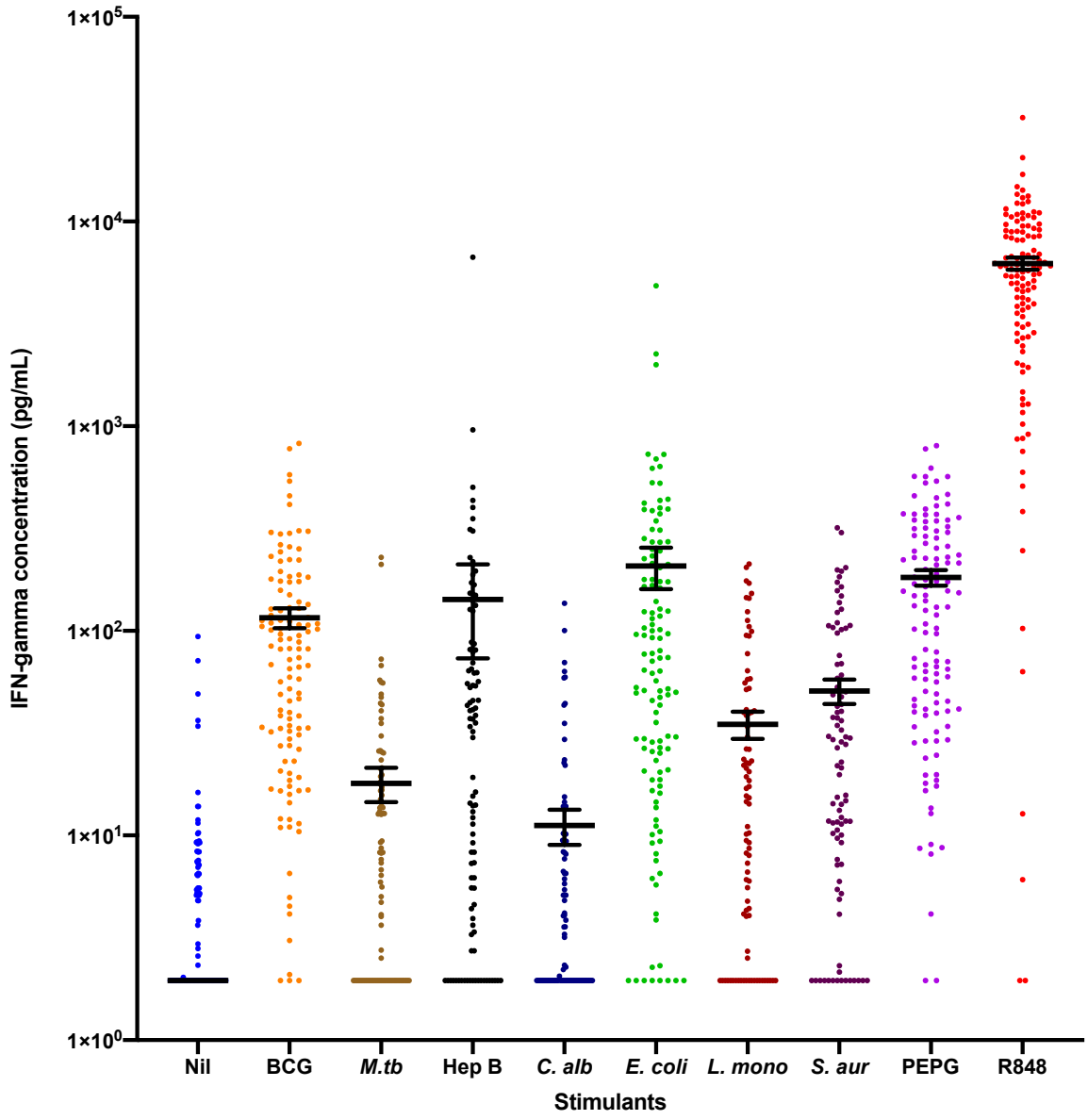
Abbreviations: pg/mL = picogram/ millilitre; TNF-α = tumour necrosis factor-alpha
Stimulants: Nil, RPMI medium; BCG; *M. tb, killed Mycobacterium tuberculosis*; Hep B, hepatitis B surface antigen; *C. alb*, killed *Candida albicans*; *E. coli*, killed *Escherichia coli*; *L. mono*, killed *Listeria monocytogenes*; *S. aur*, killed *Staphylococcus aureus;* PEPG, peptidoglycan; R848, resiquimod

Figure 4.3: Data frequency histograms of TNF-α responses for each stimulant. Normal distribution curves superimposed.



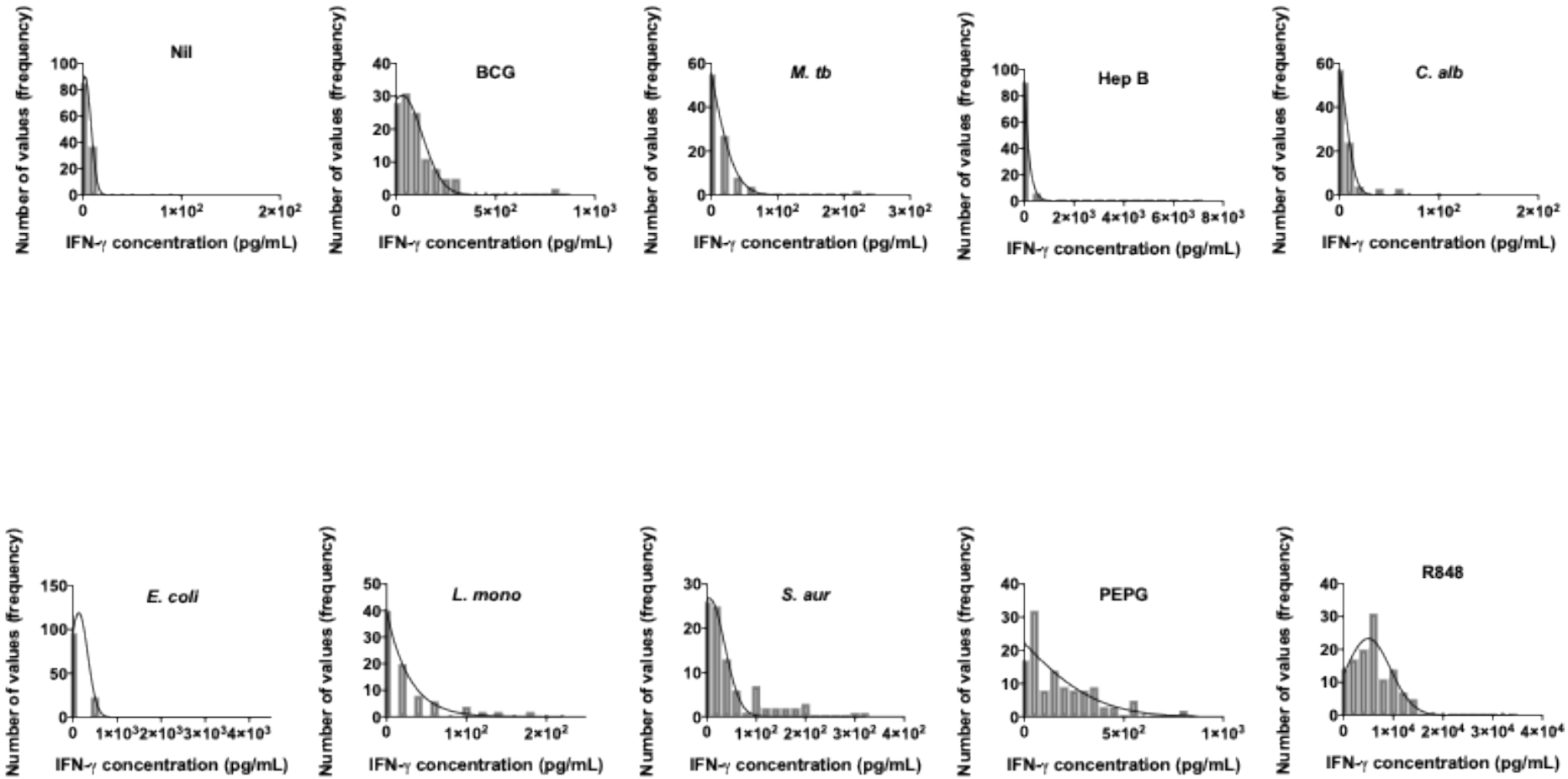
Abbreviations: pg/mL = picograms/millilitre; TNF-α = tumour necrosis factor-alpha
Stimulants: Nil, RPMI; Hep B, Hepatitis B surface antigen; PEPG, peptidoglycan; R848, resiquimod

Figure 4.4: Distribution of IFN-γ responses following in vitro stimulation with the nil control, killed pathogens and two Toll-like receptor agonists. Error bars depict the median and 95% upper and lower confidence intervals.



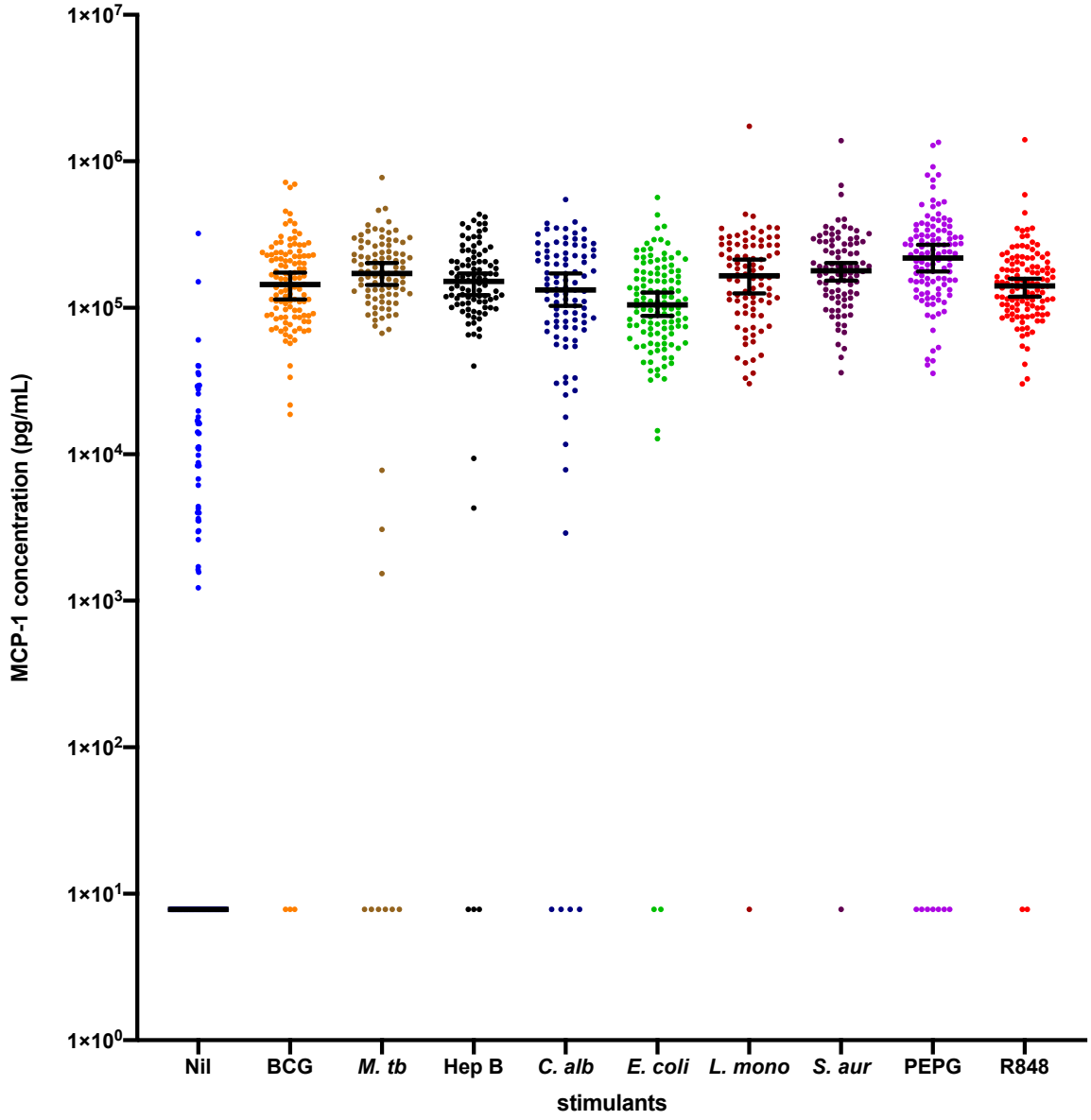
Abbreviations: IFN-γ = interferon-gamma; pg/mL = picogram/ millilitre
Stimulants: Nil, RPMI medium; BCG; *M. tb, killed Mycobacterium tuberculosis*; Hep B, hepatitis B surface antigen; *C. alb*, killed *Candida albicans*; *E. coli*, killed *Escherichia coli*; *L. mono*, killed *Listeria monocytogenes*; *S. aur*, killed *Staphylococcus aureus;* PEPG, peptidoglycan; R848, resiquimod

Figure 4.5: Data frequency histograms of IFN-γ responses for each stimulant. Normal distribution curves superimposed.



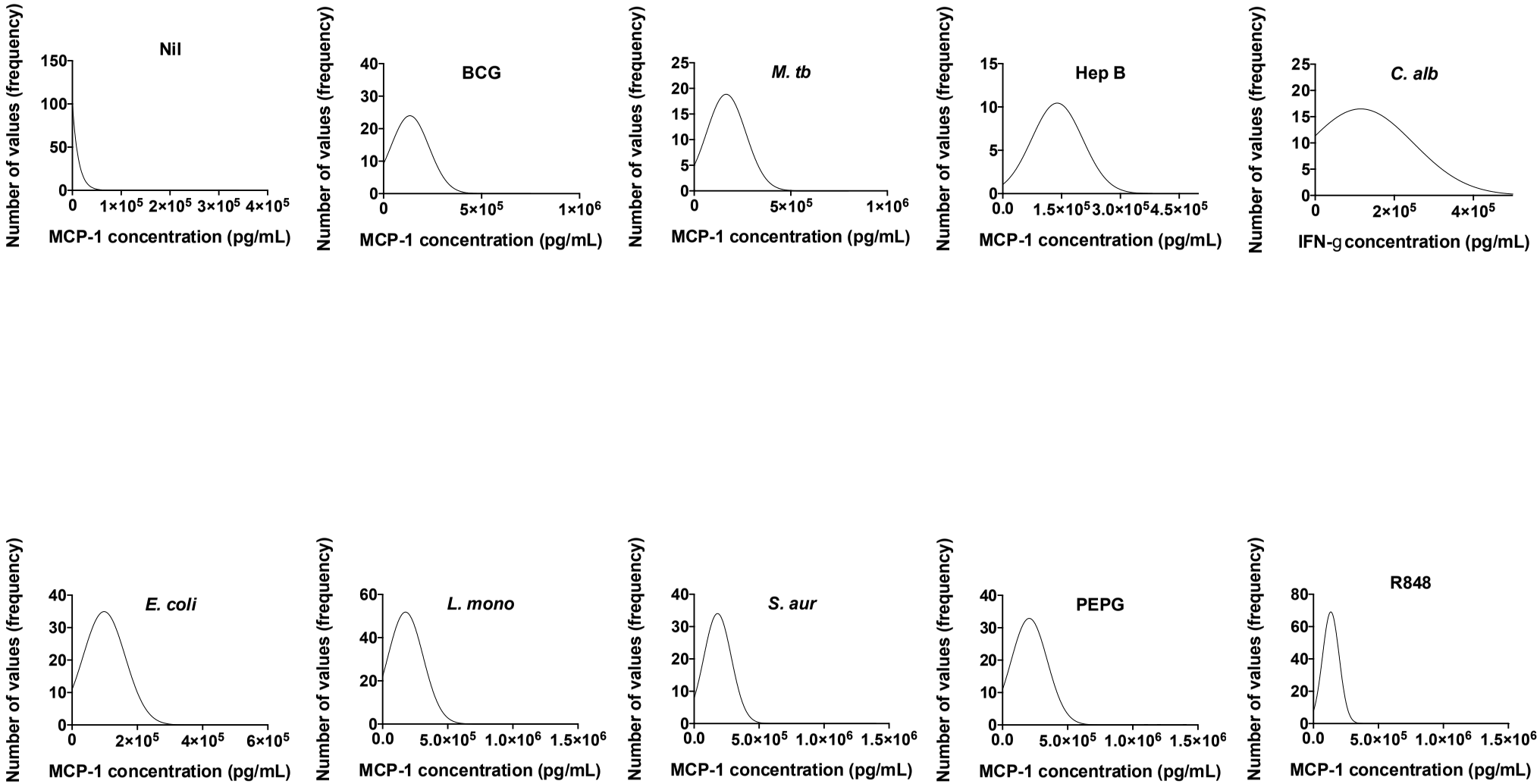
Abbreviations: IFN-γ = interferon-gamma; pg/mL = picograms/millilitre
Stimulants: Nil, RPMI; Hep B, Hepatitis B surface antigen; PEPG, peptidoglycan; R848, resiquimod

Figure 4.6: Distribution of MCP-1 responses following in vitro stimulation with the nil control, killed pathogens and two Toll-like receptor agonists. Error bars depict the median and 95% upper and lower confidence intervals.



Abbreviations: MCP-1 = monocyte chemoattractant protein-1; pg/mL = picogram/ millilitre
Stimulants: Nil, RPMI medium; BCG; *M. tb, killed Mycobacterium tuberculosis*; Hep B, hepatitis B surface antigen; *C. alb*, killed *Candida albicans*; *E. coli*, killed *Escherichia coli*; *L. mono*, killed *Listeria monocytogenes*; *S. aur*, killed *Staphylococcus aureus;* PEPG, peptidoglycan; R848, resiquimod

Figure 4.7: Data frequency histograms of MCP-1 responses for each stimulant. Normal distribution curves superimposed.



Abbreviations: MCP-1 = monocyte chemoattractant protein-1; pg/mL = picograms/millilitre
Stimulants: Nil, RPMI; Hep B, Hepatitis B surface antigen; PEPG, peptidoglycan; R848, resiquimod

Summary of cytokine-stimulant pair data

Median cytokine responses were statistically lower in the nil (RPMI) samples compared with stimulated samples for each cytokine (MWU p <0.05), as expected. Overall, cytokine responses to stimulants were lowest for IFN-γ and highest for MCP-1 (Table 4.3). There was large variation in cytokine responses between babies for all cytokine-stimulant pairs. The cytokine-stimulant response patterns differed between the three cytokines. Every cytokine-stimulant pair dataset was non-normally distributed.

Table 4.3: Median cytokine responses for all stimulants

Stimulant	Median (IQR) cytokine responses (ng/mL)		
	TNF-α	**IFN-γ**	**MCP-1**
Negative control			
Nil (RPMI)	0.004 (0.004-0.004)	0.002 (0.002-0.006)	0.008 (0.008-0.008)
Mycobacterial stimulants			
BCG	2.43 (0.91-3.98)	0.08 (0.03-0.14)	144 (87-229)
M. tuberculosis	0.28 (0.05-0.73)	0.008 (0.002-0.02)	171 (108-240)
Viral component			
Hep B	0.54 (0.14-1.14)	0.03 (0.003-0.08)	151 (103-210)
Heat-killed pathogens			
C. albicans	0.07 (0.006-0.26)	0.002 (0.002-0.009)	132 (73-227)
E. coli	1.42 (0.77-2.80)	0.07 (0.02-0.21)	104 (66-171)
L. monocytogenes	0.08 (0.02-0.16)	0.01 (0.002-0.04)	165 (97-267)
S. aureus	0.19 (0.06-0.58)	0.02 (0.007-0.07)	178 (118-282)
Toll-like receptor agonists			
PEPG	2.19 (1.00-3,60)	0.13 (0.04-0.29)	219 (125-316)
R848	16 (10-22)	5 (2-9)	141 (98-199)

Abbreviations: IFN-γ = interferon-gamma; IQR = interquartile range; MCP-1 = monocyte chemoattractant protein-1; ng/mL = nanograms/millilitre; TNF-α = tumour necrosis factor-alpha
Stimulants: Nil, RPMI; Hep B, hepatitis B surface antigen; PEPG, peptidoglycan; R848, resiquimod

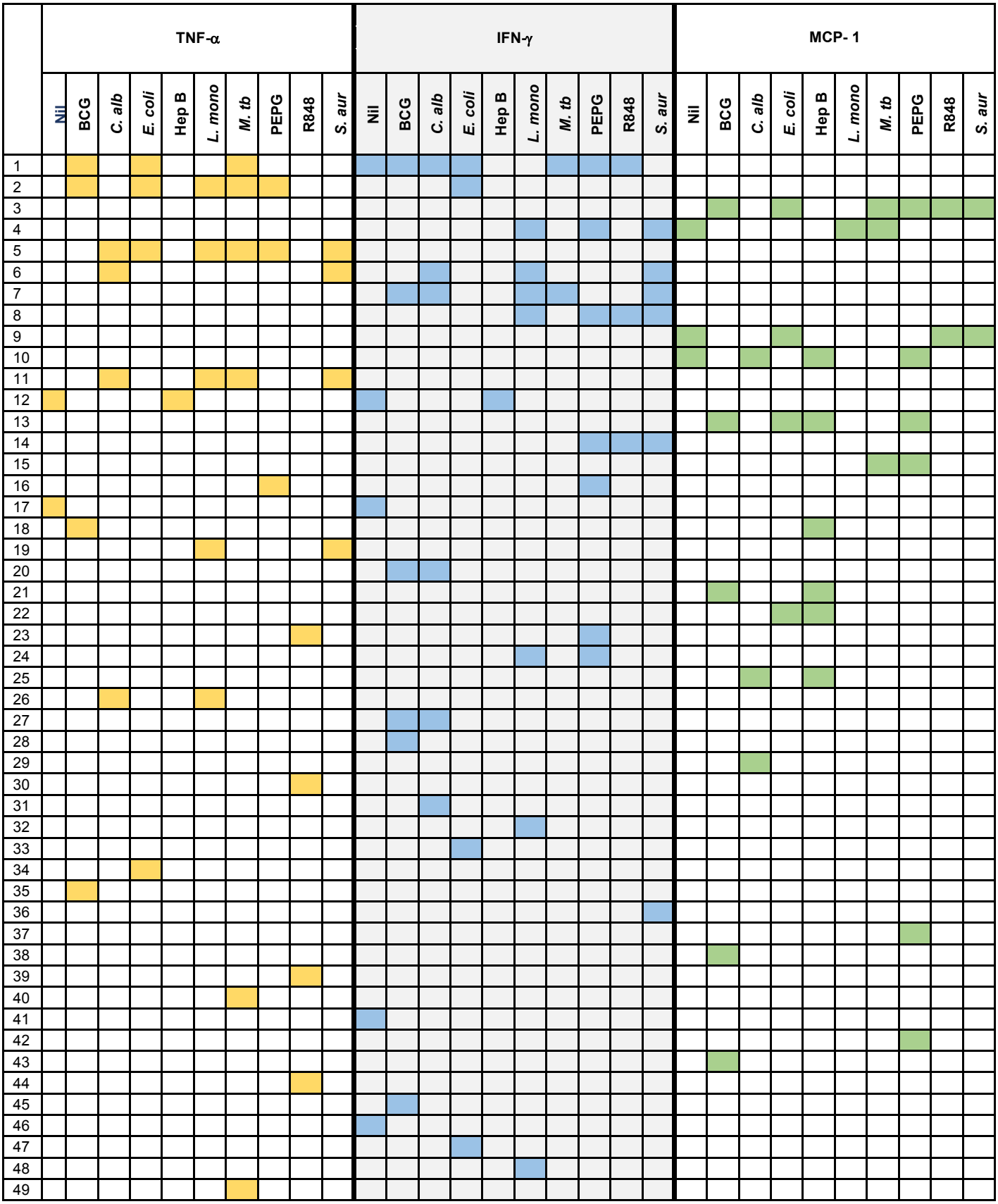
4.6.2 Outliers

'Outliers' had been defined as babies with cytokine responses exceeding two standard deviations above or below the mean value for each cytokine-stimulant pair. No outliers exceeded two standard deviations lower than the mean value for any cytokine-stimulant pair dataset.

Forty-nine babies (38%) were identified as an outlier in at least one cytokine-stimulant pair (Table 4.4). No baby was an outlier for every cytokine-stimulant pair nor were there any babies with outlier values for a specific stimulant in all three cytokines or for all stimulants in one cytokine.

Small numbers of TNF-α outliers were observed for every stimulant (Table 4.5). Outliers were more prevalent for IFN-γ compared with the other cytokines, but overall numbers were small for each stimulant. There were few outliers with hepatitis B surface antigen (HBsAg), *M. tuberculosis* and R848 compared with the other stimulants (Table 4.6). There were very few outliers for all stimulants for MCP-1, being mostly for responses to peptidoglycan (PEPG), HBsAg and BCG (Table 4.7). The number of outliers for each cytokine-stimulant pair across the four study groups were not statistically different (ANOVA $p > 0.05$; Tables 4.5-4.7).

Table 4.4: Summary of all 49 babies with outlier values for each cytokine-stimulant pair



Abbreviations: IFN-γ = interferon-gamma; MCP-1 = monocyte chemoattractant protein-1; TNF-α = tumour necrosis factor-alpha
Stimulants: Nil, RPMI medium; BCG; *M. tb, killed Mycobacterium tuberculosis*; Hep B, hepatitis B surface antigen; *C. alb*, killed *Candida albicans*; *E. coli*, killed *Escherichia coli*; *L. mono*, killed *Listeria monocytogenes*; *S. aur*, killed *Staphylococcus aureus;* PEPG, peptidoglycan; R848, resiquimod
Rows ordered by decreasing frequency of outlier values

Table 4.5: Outliers for TNF-α-stimulant responses

Stimulant	Group 1 **BCG vaccine** No./Total	Group 2 **BCG vaccine + HBV** No./Total	Group 3 **HBV** No./Total	Group 4 **No vaccine** No./Total	Total No./Total
Negative control					
Nil	1/31	0/33	0/34	1/30	**2/128**
Mycobacterial stimulants					
BCG	1/28	2/31	0/31	1/29	**4/119**
M. tuberculosis	2/21	3/27	0/27	1/22	**6/97**
Viral component					
Hep B	1/21	0/28	0/25	0/24	**1/98**
Heat-killed pathogens					
C. albicans	1/22	2/24	0/27	1/22	**4/95**
E. coli	1/30	1/32	1/32	1/28	**4/122**
L. monocytogenes	1/19	2/23	1/26	1/20	**5/88**
S. aureus	0/19	2/24	1/26	1/22	**4/91**
Toll-like receptor agonists					
PEPG	1/29	1/31	0/34	1/25	**3/119**
R848	2/30	0/32	1/34	1/26	**4/122**

Stimulants: Nil, RPMI; Hep B, hepatitis B surface antigen; PEPG, peptidoglycan; R848, resiquimod

Table 4.6: Outliers for IFN-γ responses

Stimulant	Group 1	Group 2	Group 3	Group 4	Total
	BCG vaccine	**BCG vaccine + HBV**	**HBV**	**No vaccine**	
	No./Total	No./Total	No./Total	No./Total	No./Total
Negative control					
Nil	2/31	1/33	1/34	1/30	**5/128**
Mycobacterial stimulants					
BCG	1/28	1/31	3/31	1/29	**6/119**
M. tuberculosis	1/21	0/27	0/27	1/22	**2/97**
Viral component					
Hep B	1/21	0/28	0/25	0/24	**1/98**
Heat-killed pathogens					
C. albicans	1/22	0/24	3/27	2/22	**6/95**
E. coli	1/30	0/32	2/32	1/28	**4/122**
L. monocytogenes	1/19	0/23	1/26	5/20	**7/88**
S. aureus	1/19	0/24	1/26	4/22	**6/91**
Toll-like receptor agonists					
PEPG	3/29	0/31	1/34	3/25	**7/119**
R848	1/30	0/32	1/34	1/26	**3/122**

Stimulants: Nil, RPMI; Hep B, hepatitis B surface antigen; PEPG, peptidoglycan; R848, resiquimod

Table 4.7: Outliers for MCP-1 responses

Stimulant	Group 1 **BCG vaccine** No./ Total	Group 2 **BCG vaccine + HBV** No./Total	Group 3 **HBV** No./Total	Group 4 **No vaccine** No./Total	Total No./Total
Negative Control					
Nil	0/30	1/33	1/34	1/29	**3/126**
Mycobacterial stimulants					
BCG	0/27	0/31	0/31	5/28	**5/117**
M. tuberculosis	0/20	0/27	1/27	2/23	**3/97**
Viral component					
Hep B	0/21	4/28	0/25	2/23	**6/97**
Heat-killed pathogens					
C. albicans	0/21	3/25	0/27	0/22	**3/95**
E. coli	0/29	1/32	1/32	2/27	**4/120**
L. monocytogenes	0/18	0/23	0/26	1/20	**1/87**
S. aureus	0/18	0/24	1/26	1/22	**2/90**
Toll-like receptor agonists					
PEPG	0/28	2/31	2/34	2/25	**6/118**
R848	0/29	0/32	1/34	1/25	**2/120**

Stimulants: Nil, RPMI; Hep B, hepatitis B surface antigen; PEPG, peptidoglycan; R848, resiquimod

4.6.3 Cytokine levels in RPMI samples

As expected, few babies produced measurable cytokine responses to RPMI (negative control) overall (Tables 4.9-4.11). In 34% of the babies, the only stimulant that failed to produce a cytokine response was RPMI (negative control). For TNF-α, 14 babies (11%) produced a cytokine response that was able to be measured by the assay with a general equal distribution among the groups (Table 4.9). Proportionately more babies, 45 babies (35%) for IFN-γand 47 babies (37%) for MCP-1, produced measurable responses in the RPMI samples compared with TNF-α. There was no consistency for each baby between cytokines for the response in RPMI samples i.e., failure to respond to RPMI for one cytokine was not predictive of the response to RPMI for a different cytokine (Table 5.8). For IFN-γ, non-responders in the RPMI samples were proportionately higher in babies who received BCG vaccine (Groups 1 and 2; Table 4.10). In contrast MCP-1 non-responders to RPMI were proportionately higher in babies who did not receive BCG (Groups 3 and 4; Table 4.11). These differences were not significant (ANOVA p >0.05).

4.6.4 Non-responders

Non-responders were previously defined as babies with unrecordable (zero pg/mL) cytokine responses to stimulation.

Non-responders for cytokine-stimulant pairs in all samples

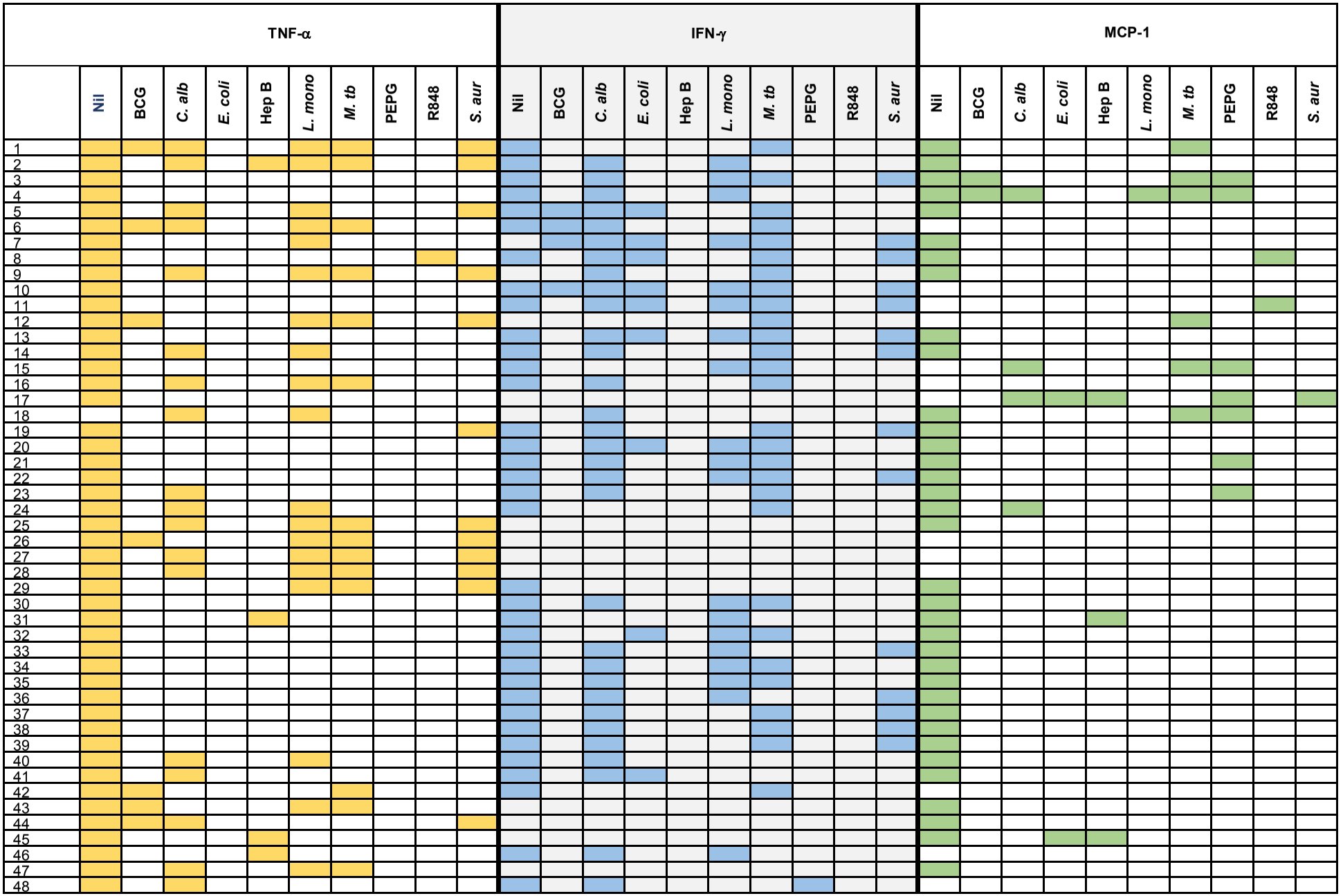
Overall, there was no identifiable pattern for non-responders across the cytokines or stimulants. Ninety four of 128 babies (73%) failed to produce a measurable cytokine response in at least (and most frequently) only one cytokine-stimulant pair. Only one baby failed to produce a measurable cytokine response to the same stimulant, namely *M. tuberculosis*, for all three cytokines (Table 4.8).

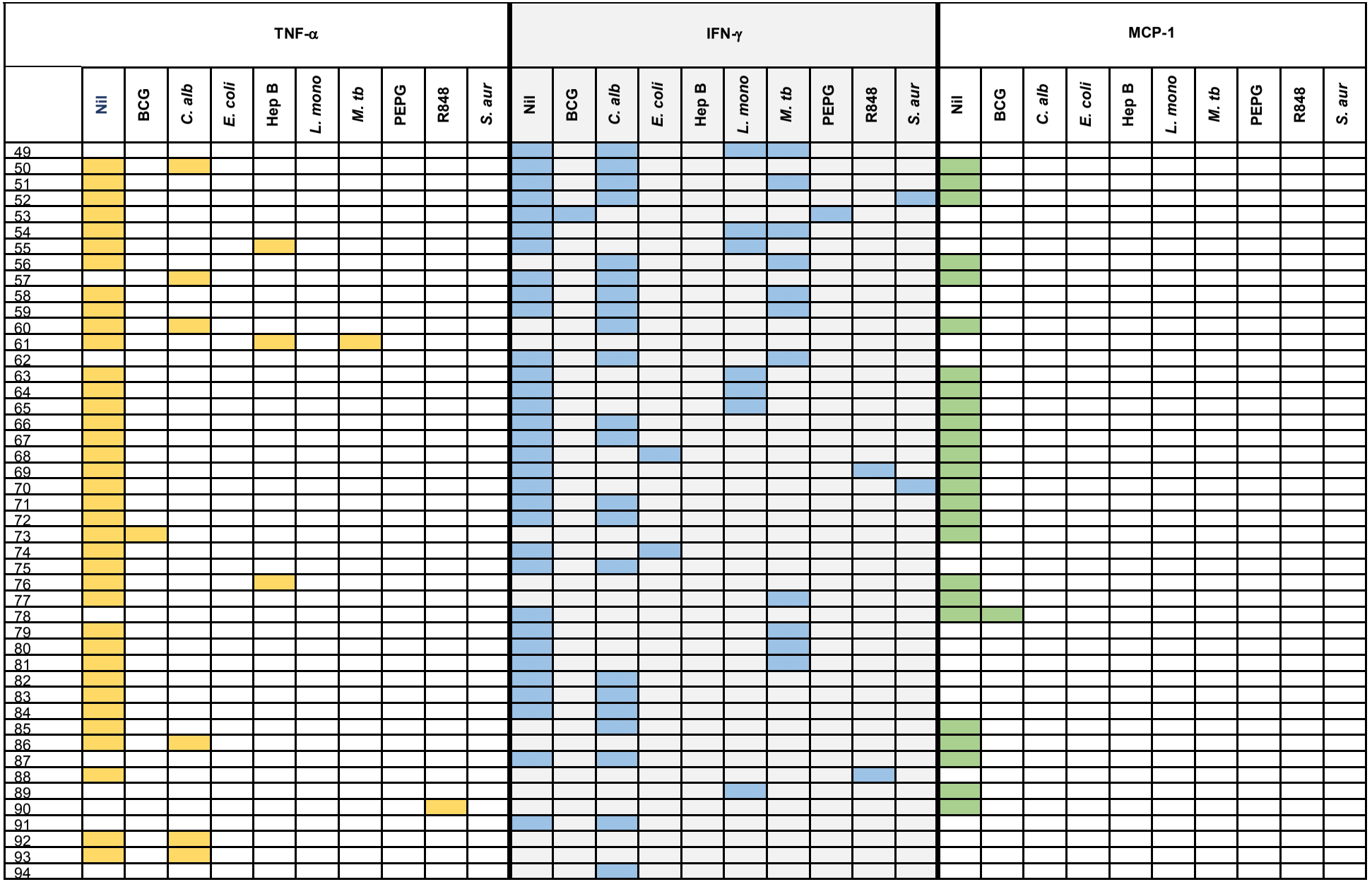
TNF-α non-responders were highest for the heat-killed pathogens, C. *albicans* (25%) and *L. monocytogenes* (22%). In contrast, all babies produced measurable TNF-α in response to *E. coli* and PEPG and most (>90%) responded to R848, HBsAg and BCG (Table 4.9).

IFN-γ-stimulant pairs had more non-responders compared with the other cytokines. Non-responder values varied by stimulant, being most abundant for the stimulants yielding lowest overall responses, i.e., *C. albicans* and *M. tuberculosis* with 56% and 40% non-responders respectively. BCG, PEPG, R848 and *E. coli* stimulation, in contrast, resulted in measurable IFN-γ responses in >90% of babies (Table 4.10).

MCP-1 responses were unrecordable for the assay in more than half (56%) of babies in response to *C. albicans* stimulation. Non-responders were generally high in response to *M. tuberculosis* (40%), HBsAg (27%), *L. monocytogenes* (27%) and *S. aureus* (18%). However, few babies failed to respond to R848, PEPG, BCG and *E. coli* (Table 4.11).

Table 4.8: Non-responders for cytokine-stimulant pairs





Stimulants: Nil, RPMI medium; BCG; *M. tb, killed Mycobacterium tuberculosis*; Hep B, hepatitis B surface antigen; *C. alb*, killed *Candida albicans*; *E. coli*, killed *Escherichia coli*; *L. mono*, killed *Listeria monocytogenes*; *S. aur*, killed *Staphylococcus aureus;* PEPG, peptidoglycan; R848, resiquimod

Abbreviations: IFN-γ = interferon-gamma; MCP-1 = monocyte chemoattractant protein-1; TNF-α = tumour necrosis factor-alpha

Rows ordered by decreasing frequency of outlier values

Non-responders for cytokine-stimulant pairs by group allocation

For TNF-α-stimulant pairs, absolute numbers of non-responders in each groups were small (Table 4.9). For all stimulants, the difference in non-responders between the groups was not significant (ANOVA p >0.05). HBV-vaccinated babies (Groups 2 and 3) had a higher proportion of non-responders in samples stimulated with *L. monocytogenes* compared with babies not vaccinated with HBV (Group 1 and Group 4). BCG-vaccinated babies (Group 1) had a lower proportion of non-responders for samples stimulated with *S. aureus.*

For IFN-γ-stimulant pairs, there were no statistically different numbers of non-responders between the groups for any stimulant (ANOVA p >0.05). Non-responders in HBsAg-stimulated samples were proportionately lower in HBV-vaccinated babies (Groups 2 and 3), although absolute numbers were similar across the four groups. The Toll-like receptor (TLR) agonists had few overall non-responders (Table 4.10).

Similarly, for MCP-1-stimulant pairs, the absolute numbers were very low for non-responders with a non-significant difference between groups for all stimulants (ANOVA p >0.05; Table 4.11).

Table 4.9: Non-responders for TNF-α

Stimulants	Group 1	Group 2	Group 3	Group 4	Total
	BCG vaccine	**BCG vaccine + HBV**	**HBV**	**No vaccine**	
	No./ Total (%)	No./ Total (%)	No./ Total (%)	No./ Total (%)	No./ Total (%)
Negative control					
Nil	28/31 (90%)	30/33 (91%)	30/34 (88%)	26/30 (87%)	**114/128 (89%)**
Mycobacterial stimulants					
BCG	3/28 (11%)	1/31 (3%)	2/31 (6%)	2/29 (7%)	**8/119 (7%)**
M. tuberculosis	4/21 (19%)	4/27 (15%)	4/27 (15%)	3/22 (14%)	**15/97 (15%)**
Viral component					
Hep B	1/21 (5%)	2/28 (7%)	1/25 (4%)	3/24 (13%)	**7/98 (7%)**
Heat-killed pathogens					
C. albicans	5/22 (23%)	6/24 (25%)	7/27 (26%)	6/22 (27%)	**24/95 (25%)**
E. coli	0/30 (0%)	0/32 (0%)	0/32 (0%)	0/28 (0%)	**0/122 (0%)**
L. monocytogenes	3/19 (16%)	6/23 (26%)	7/26 (27%)	3/20 (15%)	**19/88 (22%)**
S. aureus	1/19 (5%)	3/24 (13%)	5/26 (19%)	3/22 (14%)	**12/91 (13%)**
Toll-like receptor agonists					
PEPG	0/29 (0%)	0/31 (0%)	0/34 (0%)	0/25 (0%)	**0/119 (0%)**
R848	1/30 (3%)	0/32 (0%)	1/34 (3%)	0/26 (0%)	**2/122 (2%)**

Stimulants: Nil, RPMI; Hep B, hepatitis B surface antigen; PEPG, peptidoglycan; R848, resiquimod

Table 4.10: Non-responders for IFN-γ

Stimulants	Group 1	Group 2	Group 3	Group 4	Total
	BCG vaccine	**BCG vaccine + HBV**	**HBV**	**No vaccine**	
	No./ Total (%)	No./ Total (%)	No./ Total (%)	No./ Total (%)	No./ Total (%)
Negative control					
Nil	23/31 (74%)	22/33 (67%)	21/34 (62%)	17/30 (57%)	**83/128 (65%)**
Mycobacterial stimulants					
BCG	1/28 (4%)	2/31 (6%)	2/31 (6%)	0/29 (0%)	**5/119 (4%)**
M. tuberculosis	8/21 (38%)	11/27 (41%)	13/27 (48%)	7/22 (32%)	**39/97 (40%)**
Viral component					
Hep B	7/21 (33%)	6/28 (21%)	6/25 (24%)	7/24 (29%)	**26/98 (27%)**
Heat-killed pathogens					
C. albicans	14/22 (64%)	13/24 (54%)	15/27 (56%)	11/22 (50%)	**53/95 (56%)**
E. coli	3/30 (10%)	4/32 (13%)	3/32 (9%)	1/28 (4%)	**11/122 (9%)**
L. monocytogenes	3/19 (16%)	8/23 (35%)	9/26 (35%)	4/20 (20%)	**24/88 (27%)**
S. aureus	1/19 (5%)	5/24 (21%)	5/26 (19%)	5/22 (23%)	**16/91 (18%)**
Toll-like receptor agonists					
PEPG	1/29 (3%)	0/31 (0%)	1/34 (3%)	0/25 (0%)	**2/119 (2%)**
R848	0/30 (0%)	1/32 (3%)	1/34 (3%)	0/26 (0%)	**2/122 (2%)**

Stimulants: Nil, RPMI; Hep B, hepatitis B surface antigen; PEPG, peptidoglycan; R848, resiquimod

Table 4.11: Non-responders for MCP-1

Stimulants	Group 1	Group 2	Group 3	Group 4	Total
	BCG vaccine	**BCG vaccine + HBV**	**HBV**	**No vaccine**	
	No./ Total (%)	No./ Total (%)	No./ Total (%)	No./ Total (%)	No./ Total (%)
Negative control					
Nil	17/30 (57%)	19/33 (58%)	24/34 (71%)	19/29 (66%)	**79/126 (63%)**
Mycobacterial stimulants					
BCG	0/27 (0%)	0/31 (0%)	2/31 (6%)	1/28 (4%)	**3/117 (3%)**
M. tuberculosis	0/20 (0%)	1/27 (4%)	5/27 (19%)	0/23 (0%)	**6/97 (6%)**
Viral component					
Hep B	1/21 (5%)	1/28 (4%)	0/25 (0%)	1/23 (4%)	**3/97 (3%)**
Heat-killed pathogens					
C. albicans	1/21 (5%)	0/25 (0%)	3/27 (11%)	0/22 (0%)	**4/95 (4%)**
E. coli	1/29 (3%)	1/32 (3%)	0/32 (0%)	0/27 (0%)	**2/120 (2%)**
L. monocytogenes	0/18 (0%)	0/23 (0%)	1/26 (4%)	0/20 (0%)	**1/87 (1%)**
S. aureus	1/18 (6%)	0/24 (0%)	0/26 (0%)	0/22 (0%)	**1/90 (1%)**
Toll-like receptor agonists					
PEPG	1/28 (4%)	0/31 (0%)	4/34 (12%)	2/25 (8%)	**7/118 (6%)**
R848	0/29 (0%)	0/32 (0%)	1/34 (3%)	1/25 (4%)	**2/120 (2%)**

Stimulants: Nil, RPMI; Hep B, hepatitis B surface antigen; PEPG, peptidoglycan; R848, resiquimod

4.7 Primary statistical analyses of cytokine-stimulant responses

4.7.1 Tumour necrosis factor-alpha

There were no statistically significant differences in the median TNF-α responses to any of the stimulants between the four groups (p >0.05; Table 4.12).

For the BCG vaccine-related antigens, the median TNF-α response to *M. tuberculosis* was highest in babies vaccinated with BCG (Groups 1 and 2) compared with controls (Group 4). The addition of HBV reduced the response, with a lower median TNF-α response to *M. tuberculosis* in Group 2 compared with Group 1. However, the differences between median responses between any of the two-groups compared were not statistically significant for stimulation with *M. tuberculosis* (MWU p >0.05). The median TNF-α response to stimulation with BCG, however, was not enhanced by BCG vaccination (Group 1) compared with controls (Group 4; MWU p = 0.6). Babies who received HBV (Groups 2 and 3) had higher responses to HBsAg compared with controls (Group 4; Table 4.12), however, these differences were not statistically significant (MWU p = 0.7 for Group 3 compared with Group 4 and p = 0.6 for Group 2 compared with Group 4).

For the heat-killed pathogens and TLR agonists, there was no consistent pattern. The median TNF-α response to *C. albicans* was similar between all groups. Compared with controls (Group 4), the median response to *E. coli* was lower in babies who received any vaccine, but lowest in babies given BCG vaccine (Group 1; MWU p = 0.1), the median response to *S. aureus* was highest in babies given BCG vaccine (Group 1; MWU p = 0.7), the median responses to PEPG was highest in babies given both vaccines (Group 2; MWU p = 0.5) and the median responses to R848 was lowest in babies given both vaccines (Group 2; MWU p = 0.4; Table 4.12).

Table 4.12: Four-group differences in median (IQR) TNF-α-stimulant responses (ng/mL)

Stimulants	Group 1 **BCG vaccine**	Group 2 **BCG vaccine + HBV**	Group 3 **HBV**	Group 4 **No vaccine**	**p-value ***
Negative control					
Nil (RPMI)	0.004 (0.004-0.004)	0.004 (0.004-0.004)	0.004 (0.004-0.004)	0.004 (0.004-0.004)	0.89
Mycobacterial stimulants					
BCG	2.42 (0.62-4.25)	2.02 (1.03-4.00)	3.00 (0.92-4.30)	3.07 (0.92-4.51)	0.96
M. tuberculosis	0.44 (0.07-0.86)	0.29 (0.03-0.69)	0.17 (0.02-0.74)	0.26 (0.11-0.67)	0.76
Viral component					
Hep B	0.54 (0.10-1.13)	0.73 (0.15-1.14)	0.73 (0.20-1.32)	0.47 (0.07-1.09)	0.91
Heat-killed pathogens					
C. albicans	0.09 (0.007-0.17)	0.04 (0.005-0.34)	0.04 (0.004-0.30)	0.09 (0.005-0.23)	0.99
E. coli	1.17 (0.53-1.85)	1.52 (0.66-2.52)	1.49 (0.89-2.81)	1.90 (0.77-3.58)	0.39
L. monocytogenes	0.09 (0.02-0.17)	0.06 (0.006-0.16)	0.10 (0.004-0.19)	0.08 (0.02-0.16)	0.94
S. aureus	0.28 (0.09-0.60)	0.18 (0.07-0.58)	0.19 (0.02-0.57)	0.17 (0.05-0.69)	0.92
Toll-like receptor agonists					
PEPG	2.19 (1.19-3.70)	3.09 (1.08-3.71)	1.87 (0.63-3.20)	2.06 (0.86-3.82)	0.57
R848	17.14 (10.00-57.75)	13.56 (9.00-20.66)	17.45 (9.15-24.43)	15.47 (11.65-22.04)	0.78

Abbreviations: IQR = interquartile range; ng/mL = nanograms/millilitre; TNF-α = tumour necrosis factor-alpha
Stimulants: Nil, RPMI; Hep B, hepatitis B surface antigen; PEPG, peptidoglycan; R848, resiquimod

* Kruskal-Wallis

4.7.2 Interferon-gamma

Compared to controls, BCG and HBV influenced responses for some cytokine-simulant pairs. There was a statistically significant difference in the median IFN-γ response to R848 between the four groups (H 8.707, p = 0.03; Table 4.13; Figure 4.8).

In two-group analyses, the difference in the median R848-induced IFN-γ response was significant between BCG-vaccinated babies (Group 1) and unvaccinated babies (Group 4; 4101 vs 8209 pg/mL, difference -4106 pg/mL; 95% CI -4993 to -954, p = 0.006) and between HBV (Group 2) and unvaccinated babies (Group 4; 5709 vs 8209 pg/mL, difference -2501 pg/mL; 95% CI -4109 to -188, p = 0.04). The IFN-γ response was lower in HBV-vaccinated babies (Group 3) compared with unvaccinated babies (Group 4; median 6142 vs 8209 pg/mL difference -2067 pg/mL), but this difference was not statistically significant (p = 0.4; Table 4.14; Figure 4.9).

For vaccine-related antigens, BCG and *M. tuberculosis* failed to induce higher IFN-γ responses in babies vaccinated with BCG (Group 1) compared with babies not vaccinated with BCG (Groups 3 and 4). HBsAg, likewise, failed to induce higher responses in those vaccinated with HBV (Groups 2 and 3) compared with controls (Group 4; Table 4.13).

None of the heat-killed pathogens or PEPG induced statistically significant different median responses between the four groups (p >0.05; Table 4.13). However, compared with unvaccinated controls (Group 4), BCG and HBV did appear to influence IFN-γ responses to some stimuli, in that the median IFN-γ response to most stimulants was lower in babies given BCG alone (Group 1). The median response to *E. coli* was lower in babies given BCG alone (Group 1) compared with other groups and this effect was not seen in the babies given concurrent BCG and HBV (Group 2). In contrast, the median response to *S. aureus* was higher in babies given BCG (Group 1) compared with other groups. Two-group analyses showed no statistical differences between the median responses to *E. coli or S. aureus* between the vaccinated group (Groups 1, 2 and 3) and the control group (Group 4; MWU p >0.05). However, median responses were significantly higher in response to *S. aureus* in babies vaccinated with BCG alone (Group 1) compared with babies given both vaccines (Group 2; MWU p = 0.005).

For the TLR agonists, the vaccinated groups (Groups 1, 2 and 3) had lower median responses compared with the control group (Group 4). The lowest responses to PEPG were seen in babies vaccinated with HBV (Group 3), an effect that was lower with concurrent vaccination (Group 2) compared with controls (Table 4.13). Two-group analyses showed no statistical differences between the median PEPG responses between any two groups compared (MWU p > 0.05).

Table 4.13: Four-group differences in median (IQR) IFN-γ-stimulant responses (pg/mL)

Stimulants	Group 1 **BCG vaccine**	Group 2 **BCG vaccine + HBV**	Group 3 **HBV**	Group 4 **No vaccine**	**p-value ***
Negative control					
Nil	2 (2-2)	2 (2-2)	2 (2-2)	2 (2-2)	0.52
Mycobacterial stimulants					
BCG	43 (24-116)	95 (30-174)	88 (19-179)	74 (29-136)	0.84
M. tuberculosis	8 (2-36)	8 (2-21)	4 (2-14)	13 (2-17)	0.69
Viral component					
Hep B	11 (3-52)	15 (4-60)	41 (5-161)	50 (2-116)	0.59
Heat-killed pathogens					
C. albicans	2 (2-9)	2 (2-7)	4 (2-9)	4 (2-14)	0.80
E. coli	49 (10-137)	82 (16-203)	85 (21-196)	73 (29-272)	0.45
L. monocytogenes	15 (4-77)	8 (2-39)	16 (2-36)	22 (6-135)	0.23
S. aureus	40 (15-110)	11 (5-35)	23 (8-63)	24 (4-146)	0.07
Toll-like receptor agonists					
PEPG	131 (40-373)	120 (29-253)	114 (39-328)	175 (67-317)	0.37
R848	4104 (1671-6277)	5709 (2620-6730)	6142 (3000-9843)	8209 (4643-9767)	0.03

Abbreviations: IFN-γ = interferon-gamma; IQR = interquartile range; pg/mL = picograms/millilitre
Stimulants: Nil, RPMI; Hep B, hepatitis B surface antigen; PEPG, peptidoglycan; R848, resiquimod

* Kruskal-Wallis

Significant result in red

Table 4.14: Two-group differences in median IFN-γ-R848 responses

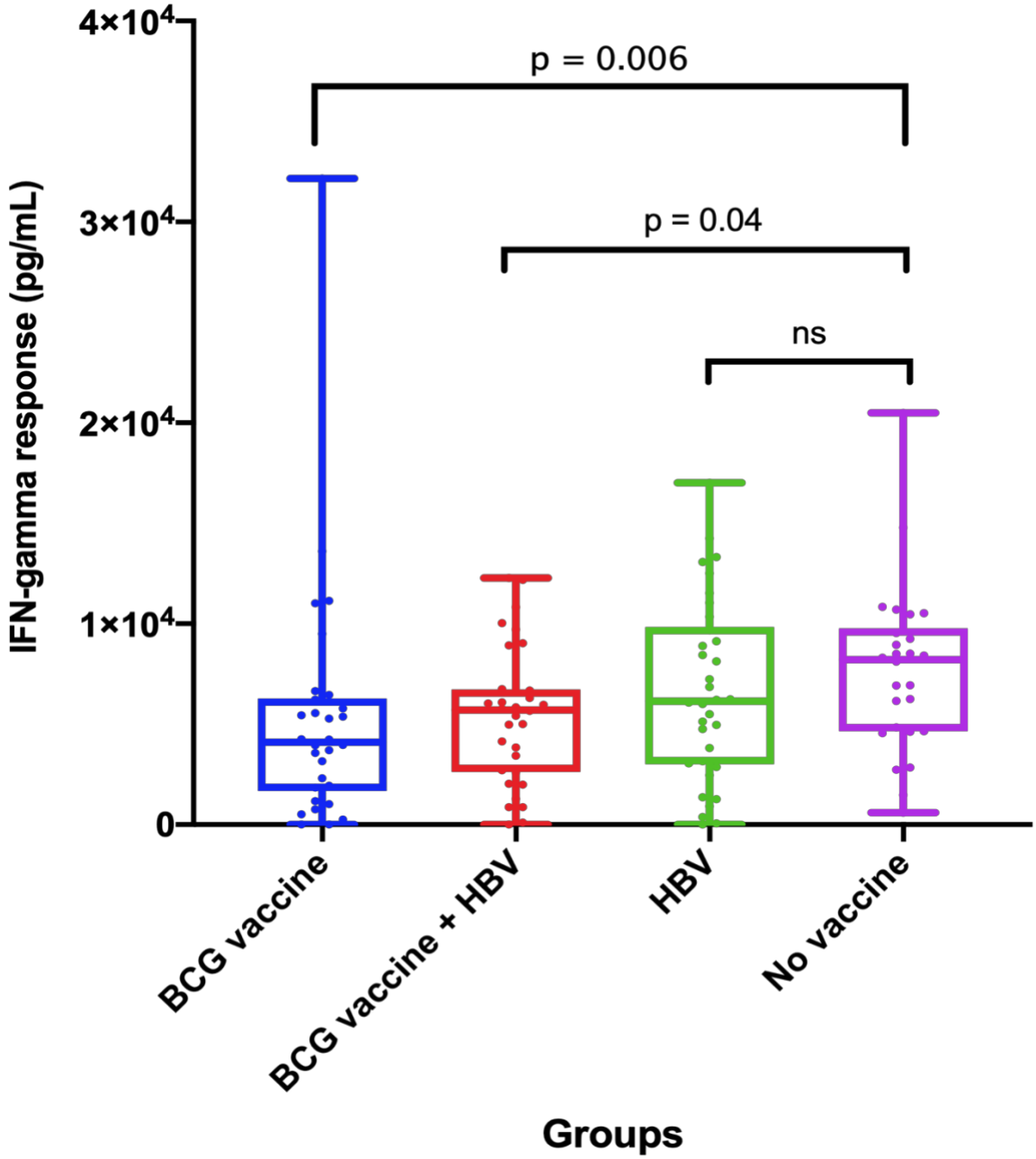
Group compared	Median response (pg/mL)	p-value*	Difference in median response (pg/mL)	95% CI of median difference	
				LCI	UCI
BCG vaccine (Group 1)	4,104	0.006	-4,106	-4,993	-954
No vaccine (Group 4)	8,209				
BCG vaccine + HBV (Group 2)	5,709	0.04	-2,501	-4,109	-188
No vaccine (Group 4)	8,209				
HBV (Group 3)	6,142	0.4	-2,067	-3,345	1,257
No vaccine (Group 4)	8,209				

Abbreviations: CI = confidence interval; HBV = hepatitis B vaccine; IFN-γ = interferon-gamma; LCI = lower confidence interval; UCI = upper confidence interval; pg/mL = picograms/millilitre; R848 = resiquimod

Significant results in red

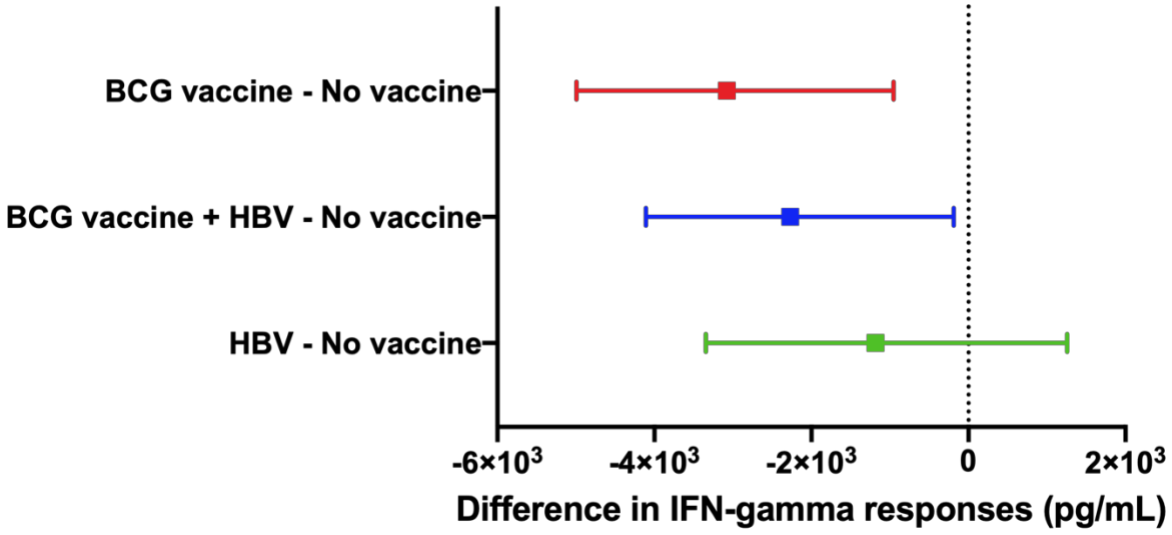
* Mann-Whitney-U

Figure 4.8: Median IFN-γ responses (pg/mL) to R848. Error bars depict median responses, boxes show 25th to 75th interquartile ranges and whiskers show total range. Significant results (p <0.05) and non-significant (ns) results analysed by Mann-Whitney U test are shown.



Abbreviation: HBV = hepatitis B vaccine; IFN-γ = interferon-gamma; pg/mL = picograms/millilitre; R848 = resiquimod

Figure 4.9: Difference in median IFN-γ responses (pg/mL) to R848 in the vaccine groups compared with the unvaccinated group (Group 4). The vertical line at zero represents no difference. Points depict the difference in median responses and the lines depict 95% upper and lower confidence intervals.



Abbreviations: HBV = hepatitis B vaccine; IFN-γ = interferon-gamma; pg/mL = picograms/millilitre; R848 = resiquimod

4.7.3 MCP-1

Compared to controls, BCG and HBV influenced MCP-1 responses to certain stimulants. Specifically, there was a statistically significant difference in the median MCP-1 response to *E. coli* between the four groups (H [8.886]; p = 0.03; Table 4.15; Figure 4.10).

In two-group analyses, the difference in median *E. coli*-induced MCP-1 responses was statistically significant between BCG-vaccinated babies (Group 1) and unvaccinated babies (Group 4; 75 ng/mL vs 123 ng/mL; difference -48; 95% CI -84 to -17; p = 0.006; Table 4.16). The difference in median MCP-1 responses to *E. coli* was also significantly lower in BCG-vaccinated babies (Group 1) compared with those vaccinated with HBV (Group 3; 75 ng/mL vs 115 ng/mL; difference -40; 95% CI -73 to -3; p = 0.03). Median MCP-1 responses to *E. coli* were also low in the other vaccine groups (Groups 2 and 3) compared with the unvaccinated group (Group 4), but these differences were not significant (Table 4.16; Figure 4.11).

Median responses to all other stimulants were also not significantly different between the four groups (p >0.05; Table 4.15).

For vaccine-related antigens, the median MCP-1 response to *M. tuberculosis* and BCG stimulation in BCG-vaccinated babies (Groups 1 and 2) was not appreciably enhanced compared with unvaccinated controls (Table 4.15). The median response to HBsAg was higher in babies vaccinated with HBV (Group 3) compared with controls (Group 4), but this difference was not statistically significant (MWU p = 0.4). When BCG and HBV were given concurrently (Group 2) the enhancing effect on responses to HBsAg was not significantly weakened compared to babies vaccinated with HBV alone (Group 3; MWU p = 0.2) and was also not significantly lower than responses in unvaccinated controls (Group 4; MWU p = 0.6; Table 4.15).

There was no consistent pattern of responses for the heat-killed pathogens and TLR agonists. BCG-vaccinated babies (Groups 1 and 2) had lower responses to *C. albicans* and *S. aureus* than unvaccinated babies (Group 4) that were not statistically different (MWU p >0.05 for all two group analyses). For *C. albicans,* the lowered response was mitigated by HBV. All vaccine groups (i.e., Groups 1, 2 and 3) showed lower responses to *L. monocytogenes* than the unvaccinated group (Group 4). The two-group analyses showed trends toward significant differences for those vaccinated with HBV alone (Group 3; MWU p = 0.07) and for those given both vaccines (Group 2; MWU p = 0.06) compared with controls. Responses to both PEPG and R848 in BCG-vaccinated babies (Group 1 and 2) were lower than in unvaccinated babies (Group 4;Table 4.15).The two-group analyses showed responses to PEPG in babies vaccinated with BCG alone (Group 1) was statistically lower than responses in unvaccinated babies (Group 4; MWU p = 0.03). All other two-group analyses for PEPG and R848 responses found no statistical differences for any two groups compared (MWU p >0.05). The response patterns of BCG-vaccinated babies (Group 1), and BCG plus HBV-vaccinated babies (Group 2) were similar to each other

Sub-analysis

For MCP1-stimulant pair responses, babies either clearly responded or failed to respond within the assay's level of detection (i.e., designated non-responder). A further analysis with non-responders excluded did not significantly alter results, i.e., there remained a statistically significant difference in the median MCP-1 responses to *E. coli* between the four groups (Kruskal-Wallis p = 0.048), with a statistically significant lower median MCP-1 response in babies vaccinated with BCG (Group 1) compared with unvaccinated babies (Group 4; MWU p = 0.01).

Table 4.15: Four-group differences in median (IQR) MCP-1-stimulant responses (ng/mL)

Stimulants	Group 1 **BCG vaccine**	Group 2 **BCG vaccine + HBV**	Group 3 **HBV**	Group 4 **No vaccine**	**p-value ***
Negative Control					
Nil	0.008 (0.008-0.008)	0.008 (0.008-0.008)	0.008 (0.008-0.008)	0.008 (0.008-0.008)	0.58
Mycobacterial stimulants					
BCG	114 (89-224)	159 (79-215)	138 (88-225)	165 (86-268)	0.62
M. tuberculosis	206 (112-262)	164 (119-223)	153 (88-220)	189 (124-279)	0.69
Viral component					
Hep B	105 (81-206)	135 (110-192)	185 (115-256)	162 (110-241)	0.59
Heat-killed pathogens					
C. albicans	103 (55-246)	124 (77-220)	163 (70-225)	146 (87-244)	0.78
E. coli	75 (40-147)	100 (72-155)	115 (64-197)	123 (96-178)	0.03
L. monocytogenes	191 (113-268)	122 (73-236)	159 (74-227)	236 (120-305)	0.18
S. aureus	154 (122-305)	143 (98-282)	190 (126-273)	191 (155-293)	0.65
Toll-like receptor agonists					
PEPG	177 (107-271)	230 (144-368)	206 (102-293)	271 (183-354)	0.11
R848	115 (82-185)	152 (106-218)	138 (102-183)	154 (93-211)	0.45

Abbreviations; IQR = interquartile range; MCP-1 = monocyte chemoattractant protein-1; ng/mL = nanograms/millilitre

Stimulants: Nil, RPMI; Hep B, hepatitis B surface antigen; PEPG, peptidoglycan; R848, resiquimod

* Kruskal-Wallis

Significant result in red

Table 4.16: Two-group differences in median MCP-1- *E. coli* responses

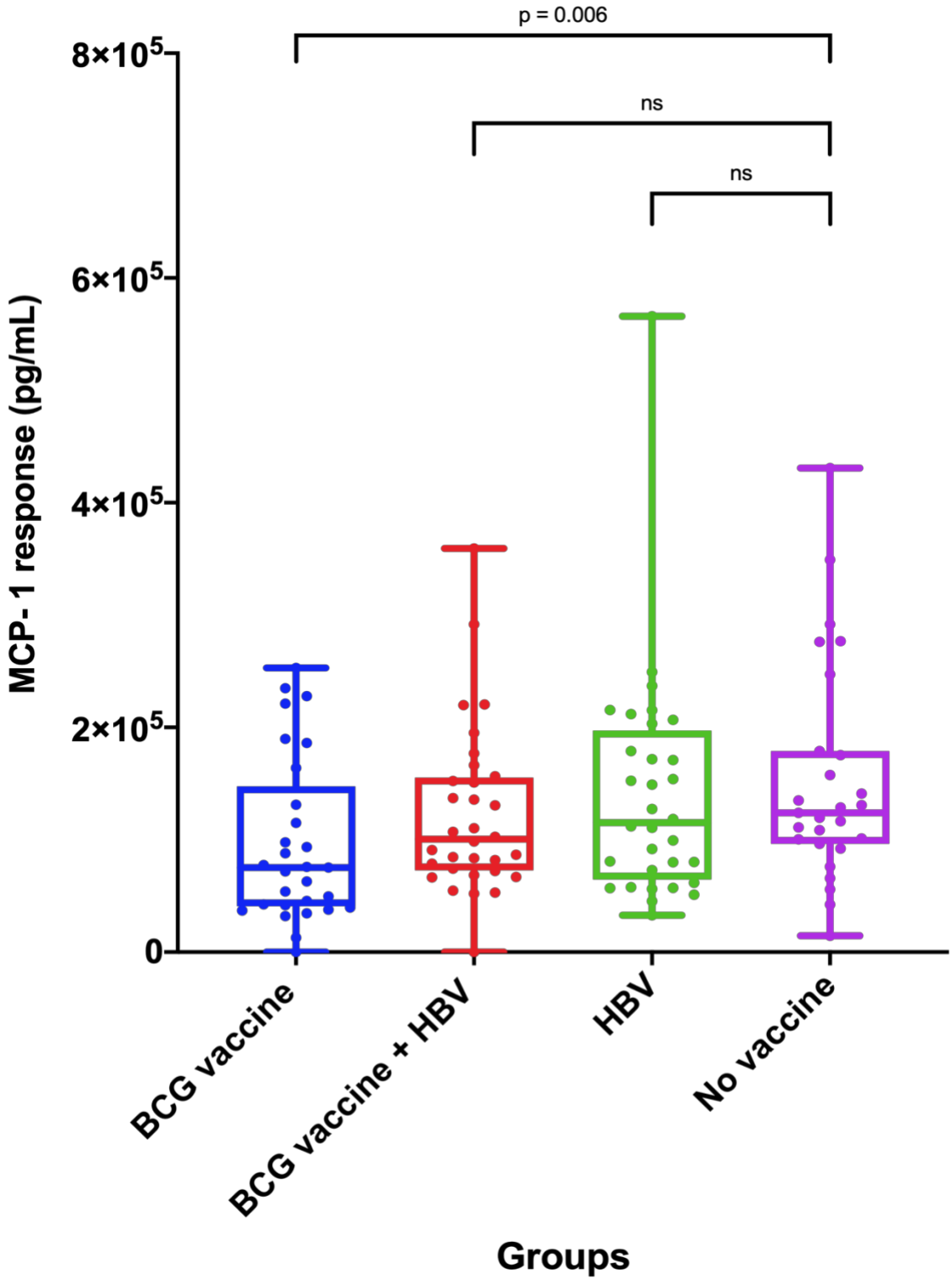
Group compared	Median response (ng/mL)	p-value*	Difference in median responses (ng/mL)	95% CI of median difference	
				LCI	UCI
BCG vaccine (Group 1)	75	0.006	-48	-84	-17
No vaccine (Group 4)	123				
BCG vaccine + HBV (Group 2)	100	0.2	-23	-55	13
No vaccine (Group 4)	123				
HBV (Group 3)	115	0.5	-8	-53	30
No vaccine (Group 4)	123				

Abbreviations: CI = confidence interval; HBV = hepatitis B vaccine; LCI = lower confidence interval; MCP-1 = monocyte chemoattractant protein-1; UCI = upper confidence interval; ng/mL = nanograms/millilitre

Significant results in red

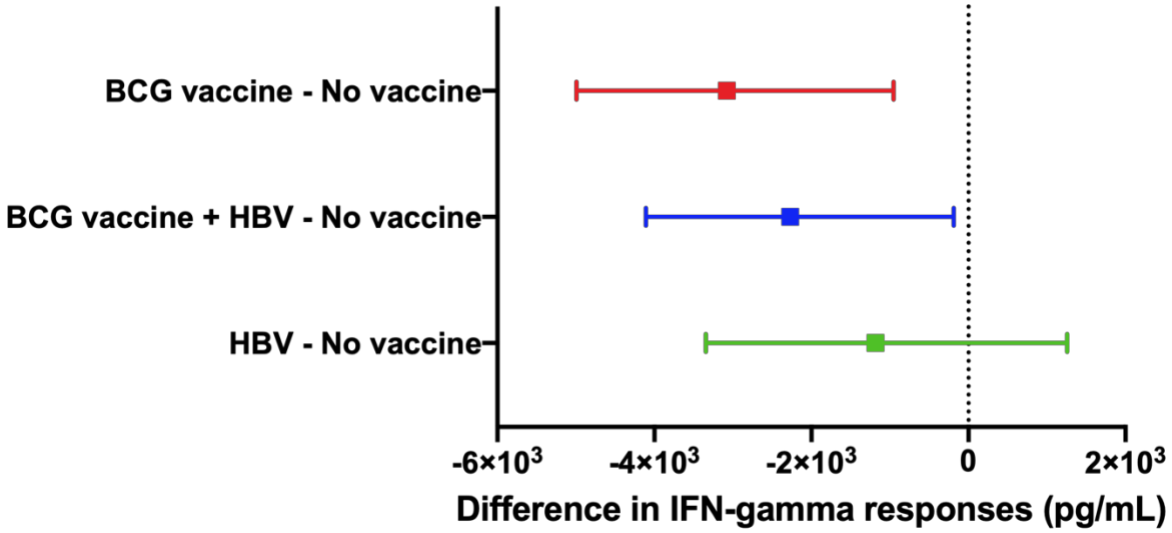
* Mann-Whitney-U

Figure 4.10: Median MCP-1 responses (pg/mL) to *E. coli*. Error bars depict median responses, boxes show 25th to 75th interquartile ranges and whiskers show total range. Significant results ($p < 0.05$) and non-significant (ns) results analysed by Mann-Whitney U test are shown.



Abbreviations: HBV = hepatitis B vaccine; MCP-1 = monocyte chemoattractant protein-1; pg/mL = picograms/millilitre

Figure 4.11: Difference in median MCP-1 responses (pg/mL) to *E. coli* in the vaccine groups compared with the unvaccinated group (Group 4). The vertical line at zero represents no difference. Points depict the difference in median responses and the lines depict 95% upper and lower confidence intervals.



Abbreviations: HBV = hepatitis B vaccine; MCP-1 = monocyte chemoattractant protein-1; pg/mL = picograms/millilitre

4.7.4 Summary of results of statistical analyses

Overall, there were few statistically significant differences in cytokine-stimulant responses between the four groups for all three cytokines.

Significant differences were seen only for IFN-γ in response to R848, and for MCP-1 in response to *E. coli.* In general, babies receiving vaccines at birth (Groups 1, 2 and 3) had lower cytokine responses compared with controls (Group 4). Specifically, babies vaccinated with BCG (Group 1) had significantly lower cytokine responses than the unvaccinated group. The addition of HBV (concurrent vaccine administration- Group 2) mitigated the differences for both these cytokine-stimulant pairs.

Although the differences in median responses to other stimulants between the four groups were not statistically different, we observed influences on the responses from the birth vaccines for certain cytokine-stimulant pairs. Both BCG and HBV independently influenced cytokine-stimulant responses. For some cytokine-stimulant pairs, the changes induced by BCG were mitigated by HBV and vice versa.

4.8 Secondary sex analyses of cytokine-stimulant responses

The sex of the baby influenced several cytokine-stimulant responses overall (Tables 4.17, 4.18 and 4.19). There was a significant difference in median responses between males and females for TNF-α ($p = 0.04$) and IFN-γ in response to *L. monocytogenes* ($p = 0.005$) and for IFN-γ in response to the TLR agonist, PEPG ($p = 0.03$).

For TNF-α-stimulant responses, there was no consistent sex-related pattern. The median responses to the mycobacterial antigens and HBsAg between boys and girls were similar. For the heat-killed pathogens and TLR agonists, the median responses in males were higher compared with females but these differences were not significant ($p > 0.05$; Table 4.17).

For IFN-γ-stimulant responses, the median responses were higher in males compared with females for all stimulants, approaching statistical significance for *S. aureus* ($p = 0.05$) and BCG ($p = 0.07$; Table 4.18). The differences in median responses between males and females for all of the stimulants, were not significant ($p > 0.05$).

For MCP-1-stimulant responses, the median responses were higher in males compared with females for all stimulants, but these differences were not significant ($p > 0.05$; Table 4.19).

For the cytokine-stimulant pairs with significantly different median responses between males and females, data were further analysed by group allocation. This revealed a significantly lower median IFN-γ response to *L. monocytogenes* in males receiving both vaccines (Group 2) compared with unvaccinated males (Group 4; MWU $p = 0.03$). However, there were no statistically significant differences in median cytokine responses between the girls and boys in the vaccine groups compared with unvaccinated babies for all of the other cytokine-stimulant pairs (MWU $p > 0.05$).

Table 4.17: Sex influence on TNF-α-stimulant responses

Stimulant Group compared	Median response (pg/mL)	p-value*	Difference in median response (pg/mL)	95% CI of median difference	
				LCI	UCI
Negative Control					
Nil					
Females	4	0.9	0	0	0
Males	4				
Mycobacterial stimulants					
BCG					
Females	2424	0.9	-36	-859	752
Males	2460				
M. tuberculosis					
Females	229	0.3	-37	-213	32
Males	267				
Viral component					
Hep B					
Females	556	0.5	30	-275	127
Males	527				
Heat-killed pathogens					
C. albicans					
Females	36	0.08	-78	-108	0.
Males	114				
E. coli					
Females	1288	0.1	-263	-915	78
Males	1552				
L. monocytogenes					
Females	57	0.04	-44	-81	0.
Males	100				
S. aureus					
Females	146	0.06	-113	-218	0.
Males	259				
Toll-like receptor agonists					
PEPG					
Females	1988	0.404	-577	-842	343
Males	2565				
R848					
Females	17342	0.6	1790	-2244	4290
Males	15552				

Abbreviations: CI = confidence interval; LCI = lower confidence interval; pg/mL = picograms/millilitre; TNF-α = tumour necrosis factor-alpha; UCI = upper confidence interval

Significantly different results between females and males are shown in red

*Mann-Whitney-U

Table 4.18: Sex influence on IFN-γ-stimulant responses

Stimulant Group compared	Median response (pg/mL)	p-value*	Difference in median response (pg/mL)	95% CI of median difference	
				LCI	UCI
Negative Control					
Nil					
Females	2	0.6	0	0	0
Males	2				
Mycobacterial stimulants					
BCG					
Females	50	0.07	43	-2	57
Males	93				
M. tuberculosis					
Females	4	0.2	-4	-6	0
Males	8				
Viral component					
Hep B					
Females	13	0.08	-11	-40	0
Males	38				
Heat-killed pathogens					
C. albicans					
Females	2	0.1	-2	-2	0
Males	4				
E. coli					
Females	63	0.1	-13	-65	5
Males	76				
L. monocytogenes					
Females	7	0.005	-17	-25	-2
Males	23				
S. aureus					
Females	13	0.05	-17	-28	0
Males	31				
Toll-like receptor agonists					
PEPG					
Females	102	0.03	-63	-104	-5
Males	165				
R848					
Females	5463	0.2	-589	-2529	551
Males	6052				

Abbreviations: CI = confidence interval; IFN-γ = interferon-gamma; LCI = lower confidence interval; pg/mL = picograms/millilitre; UCI = upper confidence interval

Significantly different results between females and males are shown in red

*Mann-Whitney-U

Table 4.19: Sex influence on MCP-1-stimulant responses

Stimulant Group compared	Median response (ng/mL)	p-value*	Difference in median response (ng/mL)	95% CI of median difference	
				LCI	UCI
Negative Control					
Nil					
Females	0.008	0.4	0	0	0
Males	0.008				
Mycobacterial stimulants					
BCG					
Females	131	0.3	-32	-47	19
Males	164				
M. tuberculosis					
Females	163	0.9	-13	-38	49
Males	176				
Viral component					
Hep B					
Females	126	0.3	-43	-48	20
Males	169				
Heat-killed pathogens					
C. albicans					
Females	129	0.4	-6	-60	24
Males	136				
E. coli					
Females	95	0.3	-21	-36	13
Males	117				
L. monocytogenes					
Females	156	0.4	-16	-64	27
Males	172				
S. aureus					
Females	171	0.6	-17	-53	30
Males	189				
Toll-like receptor agonists					
PEPG					
Females	189	0.9	-40	-50	60
Males	230				
R848					
Females	129	0.5	-20	-35	17
Males	150				

Abbreviations: CI = confidence interval; LCI = lower confidence interval; MCP-1 = monocyte chemoattractant protein-1; ng/mL = nanograms/millilitre; UCI = upper confidence interval

* Mann-Whitney-U

Chapter 5: Discussion and summary

For this study, I investigated whether isolated Bacille Calmette-Guérin vaccine (BCG), hepatitis B vaccine (HBV) or concurrent HBV and BCG, given at birth, influenced cytokine responses to unrelated stimulants at 1 week of age. In this discussion, each cytokine response induced by a unique stimulant will be referred to as a 'cytokine-stimulant signature'.

We measured cytokine responses by enzyme-linked immunosorbent assays (ELISAs) that cannot reliably detect concentrations below a threshold lower limit set by the manufacturer. Measurements of responses marginally below the threshold are therefore extrapolated values. There is no standard across studies on how extrapolated values should be reported or analysed. We assigned these a value of half the lowest threshold of detection for that assay, a method with proven validity for similar analyses (21).

In this study, at the chosen statistical significance level of 0.05, there were few statistically significant differences in the median cytokine responses to any of the stimulants between the groups. This may have been due to type 2 error with the relatively small sample size and the large variation in responses between individuals. However, despite these limitations, we found an influence from these birth vaccines for some cytokine-stimulant signatures compared with unvaccinated controls.

The influence on cytokine responses was strongest for babies receiving BCG vaccine, alone or concurrently with HBV. Isolated HBV vaccination also induced a unique increase and decrease in cytokine responses to unrelated stimulants, but unlike for BCG vaccination, the differences in median cytokine responses compared with controls were not statistically significant. Interestingly, the statistically significant effects from BCG, when found, were mitigated by concurrent HBV. Specifically, when stimulated with resiquimod (R848), a Toll-like receptor (TLR) 7/8 agonist, the interferon-gamma (IFN-γ) response was significantly decreased in babies who were vaccinated with BCG, at birth. This dampening effect on IFN-γ production was mitigated by the concurrent administration of HBV. Similarly, when stimulated with *E.* coli, monocyte chemoattractant protein 1 (MCP-1) concentrations were decreased in babies vaccinated with BCG and/or HBV at birth. The difference reached statistical significance for BCG-vaccinated babies only, the lowering effect again being mitigated by concurrent HBV administration.

Although differences in median cytokine responses did not reach statistical significance, this pattern was replicated for MCP-1 responses to hepatitis B surface antigen (HBsAg), *C. albicans*, peptidoglycan (PEPG) and R848, where BCG lowered the effect on MCP-1 responses that were not apparent following co-administration with HBV. In contrast, BCG increased MCP-1 responses to *M. tuberculosis,* a BCG vaccine-related stimulant*,* an effect that was also weakened by the co-administration of HBV.

5.1 Comparisons with other studies

There are few other published cytokine studies in this research area in paediatric and neonatal participants. These studies are heterogeneous in design with respect to the method of cytokine measurement and the cytokine panel investigated, the stimulant panel and duration of stimulation, the age of the child and other factors now known to influence heterologous immunity. Because of this, comparisons with results from other studies need to be interpreted with caution.

5.1.1 Similarities with other studies

We demonstrated measurable cytokine production from neonatal cells - as previously reported (21, 87, 89, 151, 152). Specifically, BCG vaccination induced a decrease or an increase in cytokine responses, depending on the stimulant, as shown in similar studies (21, 86, 89). Concurrent vaccination with BCG and HBV tended to induce the same cytokine response pattern to unrelated stimulants as BCG alone. This pattern was also reported by researchers who measured cytokine responses in cord blood stimulated with these vaccines (153), suggesting that BCG drives these cytokine response patterns.

In this study, we found considerable differences in cytokine responses between individuals, consistent with previous reports (21, 86, 154, 155). The magnitude of each of the three cytokine responses for all stimulants was highest for MCP-1 and lowest for IFN-γ, as found in other paediatric studies that included these three cytokines (21, 156). However, MCP-1 responses have not been extensively investigated in the context of pathogen-associated infectious stimulant exposure in neonates.

In this study, stimulation with R848, the TLR 7/8 agonist, induced the highest tumour necrosis factor-alpha (TNF-α) and IFN-γ responses compared with other stimulants. This finding is consistent with an earlier report that R848 stimulation results in the highest cytokine production of all TLR agonists in newborns (157).

In keeping with other studies, minimal cytokine concentrations were detected in most RPMI samples (21, 89). However, 35% and 37% of participants had measurable background IFN-γ and MCP-1 detected. This is consistent with other studies where cytokines were detected in baseline sample from infants (21, 90, 156, 158), and is unlikely to reflect contamination of RPMI with immune stimulants. In further support of non-contamination, measurable cytokine responses in the RPMI samples were not detected for all three cytokines from any individual sample. For this reason, we did not exclude the “positive” RPMI samples from our analyses.

The cytokine-stimulant signatures described in this study are comparable to those reported in similar paediatric studies (21, 86, 87, 89). For example, no differences were found in TNF-α or IFN-γ responses in neonates 4 days after birth vaccination with BCG, compared with unvaccinated babies, in response to stimulation with *E. coli* or *C. albicans* (89). MCP-1 was not included in that cytokine panel. Stimulation with BCG, *M. tuberculosis* and *S. aureus* also resulted in no significant differences in neonatal TNF-α, IFN-γ or MCP-1 responses 1 week after vaccination with BCG compared to those given BCG and HBV vaccines at birth (21). Blood from infants given BCG at 6 weeks of age showed no difference in TNF-α or IFN-γ

responses to heat-killed *C. albicans*, *E. coli*, *L. monocytogenes* or purified protein derivative (PPD) 1 week after vaccination compared with unvaccinated subjects (87). MCP-1 responses were not measured in this study.

In a case-control study of 21 infants in the UK, Smith and co-workers (86) reported no differences in cytokine responses to vaccine-unrelated antigens in unvaccinated and BCG-vaccinated children at 6 months of age. They found neither TNF-α, IFN-γ or MCP-1 levels were significantly altered in response to *C. albicans*, *E. coli* or *S. aureus* in the BCG-vaccinated infants, consistent with findings in this current study. In another study, Libraty et al. (85) reported no difference in IFN-γ enzyme-linked immunosorbent spot (ELISPOT)-forming cell colonies to HBsAg in BCG-vaccinated neonates compared with controls, consistent with our study.

When stimulated with R848, cells from babies vaccinated at birth with BCG and/or HBV, produced significantly less IFN-γ than those from unvaccinated controls. The results were consistent with findings in a study comparing babies vaccinated with both vaccines compared to HBV alone (21). This study shares many similarities with the Early Life Vaccines and Immunity study (ELVIS), including the same protocols for blood collection, laboratory procedures for stimulation and storage of samples, research laboratory and staff. R848 has been shown to induce dendritic cell production of cytokines (147). In our study, vaccination with BCG and/or HBV appeared to have modified this response, with HBV dampening the effect of BCG. The clinical significance of this finding for humans is unknown. IFN-γ has several immune effects and is produced by various immune cells that is regulated through complex interactions involving other cytokines (159). From an infection perspective, IFN-γ supports RSV clearance (160), protects against Group B streptococcal infection (161) and low levels are associated with greater severity of *B. pertussis* (162) in neonatal mice. However, exaggerated pro-inflammatory responses may result in harmful tissue damage. BCG may be attenuating potentially harmful proinflammatory states in very early life; potentially providing a biological mechanism for the observations for BCG-induced protective effects on infant mortality seen in West African infants.

Several stimulants including BCG, HBsAg, *C. albicans*, *E. coli, L. monocytogenes, S aureus*, PEPG and R848 induced lower MCP-1 responses in BCG-vaccinated babies compared with controls. These results were consistent with findings by other researchers in our group for the stimulants *S. aureus, E. coli, L. monocytogenes, C. albicans*, PEPG and R848 (21). Again, the dampening effect on cytokine responses from BCG in this study was mostly ameliorated by concurrent HBV. These differences only reached statistical significance for MCP-1 responses to *E. coli*. MCP-1 is primarily produced by monocytes or macrophages (163) and is a chemokine that attracts additional monocytes, memory T cells and NK cells to the sites of inflammation (164). In relation to infectious diseases, Flores-Villanueva et al. (165) have shown that people with certain MCP-1 genotypes produce more MCP-1 and are more likely to develop TB, suggesting that their higher levels of MCP-1 inhibit the production of other cytokines in response to *M. tuberculosis*. High plasma levels of MCP-1 are also found in patients with human immunodeficiency virus (HIV)-associated dementia, and in the CSF of patients with HIV encephalitis (166) and HIV-positive patients with cytomegalovirus (CMV) encephalitis (167). The high level of MCP-1 may be responsible for local tissue damage and higher morbidity (168). The decreased production of IFN-γ and MCP-1 in BCG-vaccinated

babies that we and others have reported may result in a reduction in host inflammatory responses during infections and a consequent reduction in local tissue damage. Our finding that concurrent HBV and BCG vaccination at birth weakened the effect of BCG is in keeping with observations that non-live vaccines negate non-specific effects (NSEs) of live vaccines (118, 169).

5.1.2 Discrepancies with other studies

Differences in the stimulants and cytokines investigated in studies similar to the ELVIS trial, allowed few comparisons with the cytokine-stimulant signatures investigated in the current study. Previous data from our group showed statistically significant differences in MCP-1 responses to several common stimulants in a study comparing BCG-vaccinated babies with BCG-unvaccinated controls, and a non-significant decrease in IFN-γ in response to R848 between the groups (21). This may be explained simply as being due to the larger sample size in that study. However, there are also notable differences in the study design that could have influenced the results. First, the previous study compared babies receiving BCG alone vs babies receiving HBV, whereas this study compared babies receiving BCG, BCG together with HBV and babies who received HBV alone with unvaccinated babies and involved analyses of four study groups as opposed to the two-group analyses of the earlier study. Second, the timing of vaccination was different between the studies. Babies were vaccinated in the first 10 days of life in the previous study, but within the first 3 days in our study. Evidence shows that the timing of vaccination, even in the early days and weeks of life, can influence cytokine responses (89). Third, the participant groups in the two studies differed, in that the earlier study included babies from a predominantly Caucasian Australian background, whereas our study recruited families likely to travel to areas of high tuberculosis (TB) prevalence within the next 12 months, resulting in a study population with predominantly migrant Indian and Chinese ethnic backgrounds. Variations in neonatal innate immune responses to BCG in different populations are well documented (170). Fourth, babies in the previous study included some born via caesarean section and those born vaginally, whereas our study included babies born vaginally only. The mode of delivery does influence the immune response to BCG (170), and the combination of these factors may explain some of the different findings between the two studies.

We found no significant differences in the cytokine responses to *M. tuberculosis* in BCG-vaccinated babies. This finding may have been expected as an indicator of the specific effect of BCG, or a 'positive control' as reported in other studies (86, 88, 90). Researchers who investigated cytokine responses induced by BCG in older infants in the United Kingdom reported that stimulation with *M. tuberculosis* (i.e., the specific response) resulted in increased levels of IFN-γ and TNF-α in BCG-vaccinated infants compared with controls (86). Similarly, researchers in West Africa also described higher IFN-γ and TNF-α responses to *M tuberculosis* or tuberculin in BCG-vaccinated infants (88, 90). However, there are several differences in the design of these studies and ours. These include location, older age of participants, longer duration between vaccination and cytokine measurement, use of other concurrent vaccinations, administration of other vaccines, and the duration of stimulation of blood samples before cytokine measurement. Along with the population differences (171) and the age of participants, other vaccinations also influence immune responses (18, 88, 131). The combination of all these factors explain the differences observed between the studies. However, the more likely factors that may have been responsible for the

differences between the studies are a combination of timing of investigation after vaccination and the stimulation time before cytokine measurement. In one of the studies, blood samples were stimulated with tuberculin for 6 days compared with 20 hours in our study, which may have been too short to induce maximal T cell-derived IFN-γ in neonates (80). The IFN-γ measured in this study, likely originated from innate immune natural killer (NK) cells as the stimulation time would not have allowed recall memory T cell responses to be detected.

Ota el. (88) investigated IFN-γ responses to HBsAg in 2-month-old Gambian infants receiving BCG, HBV and OPV at birth compared with babies receiving HBV and OPV at birth. They showed that concomitant BCG and HBV significantly enhanced production of IFN-γ in response to HBsAg at 2 months and 4.5 month of age. By contrast, we did not find any statistically significant differences in median IFN-γ responses to HBsAg between our groups. The concentration of HBsAg used by Ota et al. for stimulation was lower than in our study, but the stimulation period was much longer (6 days vs 20 hours), allowing time for IFN-γ, produced by TH1 CD4 T cells be detected by the assay. Participants in the Gambian study also received concomitant OPV at birth. Along with older patients from a different population group in whom cytokine responses were measured months after vaccination, the longer interval and stimulation time, again, may explain the discrepancy in production of the IFN-γ between the two studies.

5.2 Limitations and recommendations for future studies

This study has several limitations. From a study design perspective, comparison with other studies was limited due to the small number of cytokines investigated. We included only three cytokines: TNF-α and IFN-γ, being the most common of all cytokines investigated in this context and MCP-1. This allowed comparison of at least two cytokines in all similar studies in neonates and infants. Potentially, more differences between the four groups would have been noted in the cytokine-stimulant responses, had more cytokines been investigated.

The only study investigating the mechanism responsible for BCG NSEs at the time of this study design, was an adult study that showed BCG vaccination induced epigenetic reprogramming of monocytes, altering their function in response to unrelated antigen exposure (99). In our research group, BCG was shown to influence MCP-1 responses to unrelated stimulants in neonates (21). Therefore, MCP-1 was chosen for the cytokine panel. However, it was later shown that monocytes from adult humans differ in their response to cytokines compared with fetal cells (172). Subsequently, researchers studied the effect of BCG vaccination on unrelated antigens in infants and found no difference in monocyte function or monocyte-derived cytokine responses (86). Potentially, monocyte-derived cytokines, like MCP-1, are not the ideal cytokines to be investigated in neonatal studies on immune responses to the antigens we used here.

With the short stimulation time, the cytokines chosen were presumed to be primarily secreted by innate immune cells TNF-α and IFN-γ by NK cells and innate lymphoid cells (173) and MCP-1 predominantly by innate monocytes (159, 163, 174). However, we did not collect enough blood for additional intracellular staining and flow cytometry to document

the origin of the cytokine we measured. In this regard, Watkins et al. (175) showed that IFN-γ production in response to BCG stimulation in cord blood originates from innate natural killer (NK) cells, whereas T cells are responsible for IFN-γ production in older infants following BCG vaccination at birth. As cord blood is functionally different from neonatal blood (83), describing the source of cytokines produced by neonatal cells in studies of heterologous immunity would inform whether adaptive or innate immune cells are predominantly involved in these responses.

Further limitations involve the stimulant panel. The stimulants were chosen to represent a variety of infectious antigens but did not include some of the most common infecting agents in neonates around the world. We would ideally have included group B Streptococcus, HSV, RSV, CMV, enteroviruses and *Bordetella pertussis* in addition to the stimulants investigated in this study. However, none of the stimulants were live organisms and therefore responses measured in our study may not mirror those seen in vivo. Live attenuated organisms with a shorter incubation time may better replicate the complex antigen presenting process and cytokine production pathways in vivo.

The current available evidence suggests that different stimulants influence cytokine responses through various pathogen recognition receptor pathways (86, 99, 100, 102). We included two stimulants of the TLR group, PEPG and R848. It may add more understanding of the cytokine-stimulant signatures in future studies to investigate stimulants based on their pathogen recognition receptor pathway. In addition, the optimal concentration of any given stimulant for ex vivo cytokine measurement is uncertain. Nissen et al. (89) have described differences in concentration of BCG stimulant that resulted in higher IFN-γ responses. Although we used the same concentrations of stimulants that were standardised in our laboratory for an earlier study (21), these may not have been the ideal concentrations for our study population. With several cytokine concentrations in this study falling below the level of detection, it is possible that higher concentration of stimulants would have induced higher responses, allowing a more precise evaluation of differences between groups. Future studies may warrant additional preliminary optimisation studies for different stimulants.

We were also limited by the relatively small neonatal blood volumes we collected to assay. The amount of blood we collected from each participant was often too low to measure the full panel of cytokine-stimulant signatures. Although the blood volumes we collected were similar to those in other neonatal studies (21, 89), our cytokine measurements by ELISA required more blood than techniques such as multiplex cytokine analysis that require smaller volumes to analyse many more cytokines in a sample. Now, newer laboratory methods, using only 100 µl of blood can detect over 50 immune cell populations and more than 250 plasma proteins (83). These novel methods could overcome the difficulty involved with collecting large blood volumes from neonates, while providing a more comprehensive analysis of immune responses to different stimulants.

One of the biggest limitations of my study was the sample size, as we did not analyse the number of samples required by our power calculation. The large inter-individual variation in cytokine responses requires large numbers for such studies, and the feasibility and cost should be considered. However, it should be noted that studies with as few as 10-15

participants per group have been published in this area, with effects from BCG reported (85, 86, 101). The larger published studies investigating the influence of BCG on heterologous immunity are mostly two group comparisons, generally disregarding other vaccinations for primary outcome analyses (21, 85, 87, 91, 93). Despite our intentions and available resources to include more participants and strengthen the conclusions from analyses, we were unable to do so, for reasons beyond our control. Towards the end of the study period, there was a worldwide BCG shortage and a supply of BCG Denmark strain could no longer be provided. In addition, a new BCG strain could not be guaranteed for the entire duration of a new study. There was also no time to repeat the study with a different BCG strain within my candidature. Knowing that BCG strain influences BCG-related adverse events (176), cytokine responses in neonates and infants (177, 178) and responses to unrelated antigens in children (179), we opted to end the study to minimise this potential confounding effect. Future studies should aim to include more participants and consider investigating the influence of BCG strain and different HBV preparations on neonatal heterologous immunity.

The lack of a full panel of stimulants for each cytokine decreased the power for some cytokine-stimulant signatures even further and impacted our results. The findings of our study should therefore be interpreted with caution, owing to possible spurious statistical non-significance due to low sample size.

We also did not have adequate power to draw conclusions from secondary subgroup analyses in this population. Current evidence suggests that maternal BCG (21, 89), mode of delivery (21), sex (21, 89) and timing of vaccine within the first 2 days of life (21, 89) influence early neonatal cytokine responses to unrelated stimulants. We analysed our data for any sex effect on cytokine responses. Few cytokine-stimulant pair responses were significantly different for boys compared with girls, with a trend toward higher responses in boys compared with girls for the three cytokines. A meta-analysis of three randomised control trials (RCTs) in low-birth-weight (LBW) boys by Biering-Sorensen et al. found an influence of sex on the BCG effect on neonatal mortality (110), reporting that BCG at birth was statistically more protective for boys compared with girls in the first week of life. This analysis from clinical trials align with the timing of outcome measure in our study. With larger sample size, these subgroup analyses should be performed to allow comparisons with current published immunological and clinical studies.

It is also important to acknowledge that cytokine responses to stimulants are of uncertain clinical significance. The ELVIS trial, however, was not designed to investigate clinical outcomes. A much larger study would need to be done to investigate a correlation in cytokine responses with neonatal clinical outcomes. To date there are no studies where the primary outcome investigated the influence of BCG or HBV immunisation of neonates on clinical outcomes. There is also no evidence for a correlation with cytokine responses to unrelated antigens with clinical outcomes. Andersen et al. (106) found no correlation between cytokine-stimulant response signatures with unrelated antigens at 6 weeks of age and mortality by age one year in a study in Guinea-Bissau. Similarly for BCG protection against tuberculosis (TB), South African researchers showed no clinical correlate of protection for cytokine signatures induced by BCG (180); and in another South African study, no differences in cytokine expression were detected in 10-week old infants who ultimately developed TB in childhood compared to those who did not (181). To date, in

developed countries, BCG vaccination at birth has not been shown to influence morbidity in childhood (59, 60), despite several studies in developing countries showing a protective effect of BCG on infant and neonatal morbidity and mortality (9, 13, 18, 54). Future studies should focus on the correlation of cytokine-stimulant signature with clinical outcomes in various settings. This would be a significant undertaking, requiring considerable resources and the need to follow up patients closely over time.

5.3 Strengths

The ELVIS trial was a novel study that randomised newborn infants to three vaccine groups and a control group receiving no vaccine. The trial considered vaccine policies regarding two neonatal vaccines that has not been previously explored. To our knowledge, this is only the second trial in this research area in neonates where the control group received no vaccine. The randomised allocation was only possible in this setting as BCG is not routinely offered in Australia and there was confidence in excluding babies born to hepatitis B-infected mothers from the study. The extensive inclusion and exclusion criteria controlled for many factors affecting the immune response, including gestational age, birthweight and mode of delivery. Baseline characteristics were similar across all four groups including infant feeding method, ethnicity, maternal BCG status and time from birth to vaccination. There was strict compliance with standardised protocols for sample collection, transport, storage and processing (182). Few researchers were involved in laboratory procedures ensuring standardisation in technical aspects of the procedures. The study design, resources and patient commitment to the study allowed early blood collection within the neonatal period. This trial allowed comparisons with other published studies investigating neonatal immune responses to unrelated stimulants.

5.4 General recommendations for future studies

Ideally, researchers could pool funds and expertise to allow coordinated, large scale, multi-site, collaborative, randomised control trials that use advanced methods in centralised laboratories, with stringent protocols, to interrogate a comprehensive range of stimulants and cytokines. Several soluble plasma factors influence cytokine immune responses in neonates including sex hormones (183), adenosine, prostaglandins (184-186) and micronutrients (187). Future studies that explore these factors in relation to heterologous vaccine effects would add to our current understanding.

Although several clinical factors influence neonatal responses to infections and vaccines including maternal, infant and environmental factors (83, 188), there are still factors we don't understand. I believe it would be challenging to design a study at this time with results that could be generalisable to all populations. Accordingly, I believe that studies should be focused on clinically relevant questions, while controlling for as many variables as possible. With relevance to this study, neonatal HBV at birth should be considered an environmental factor that may influence neonatal responses to unrelated infections.

Our understanding of the development and evolution of the immune system in very early life is limited. Large studies that investigate the development of the neonatal and infant immune systems over time are lacking (83). Nevertheless, we do know that marked changes in cell composition, plasma proteins and cell phenotypes occur during the first week of life

(152) and that these changes show a set pattern, ordered by the day of life (83), and converge by 3 months (84). In support of these findings, studies with larger sample sizes have detected differences in immune responses in babies vaccinated in the first 24 hours of life compared with babies vaccinated from days 2-7 (89, 94). These insightful studies suggest that the timing of vaccination and measurements of immune outcomes in the first week of life are important considerations in the design and comparison of studies in neonates. Future studies should consider vaccine timing and duration from vaccination to investigation in the study design or include these in subgroup analyses, particularly for neonates. I would recommend further mechanistic studies of vaccines' heterologous effects in neonates by using novel and emerging technologies to identify the cellular origins of the cytokines produced in response to various stimulants.

As discussed above, the most useful investigations into the NSEs of vaccines are clinical studies that provide meaningful evidence of beneficial effects that clinicians and vaccine policy developers could exploit when designing immunisation schedules.

Recently, results of large randomised trials of BCG on clinical outcomes in children were published from the Danish Calmette Study (58, 59) and the MIS BAIR trial (65). Both these trials are in developed, low TB disease prevalent, countries where randomisation of BCG in neonates and infants is ethical. RCTs in developing countries, with high disease burdens will be more limited due to ethical considerations. The observational studies should therefore be collaborative and more homogeneous in design to allow comparison between studies with subgroup analyses considering as many modifiable factors on immunity as possible.

5.5 Summary and conclusion

This study aimed to determine the influence of BCG and HBV at birth on neonatal immune responses to related and unrelated antigens by measuring cytokine responses at 1 week of age and comparing them across three groups receiving either or both of the vaccines, with an unvaccinated control group.

To this end, I designed a randomised control trial and measured TNF-α, IFN-γ and MCP-1 cytokine responses after 20 hours of whole blood stimulation with RPMI medium, BCG, *M. tuberculosis*, HBsAg, *E. coli, C. albicans, L. monocytogenes, S. aureus*, PEPG or R848.

Cytokine-stimulant signatures were highly variable between neonates. Immunisation with BCG and/or HBV induced different cytokine responses to unrelated stimulants. Concurrent immunisation with both vaccines generally induced the same response pattern as BCG alone.

I found few statistically significant differences in cytokine-stimulant responses between the groups. The TLR agonist R848 significantly decreased IFN-γ responses in BCG-vaccinated babies, whether HBV was administered or not. Several unrelated antigens decreased the MCP-1 responses in BCG-vaccinated babies, with or without concurrent HBV, reaching statistical significance for *E. coli* in isolated BCG-vaccinated babies only. In general, the lowering effect of BCG on cytokine responses to unrelated antigens was weakened by concurrent vaccination with HBV.

This study adds to the evidence for vaccine influence on immunity to unrelated infections, particularly in the neonatal period. It is also the first study to investigate HBV influence on unrelated antigens, and the first study to show that concurrent HBV and BCG vaccination at birth weakens the NSE of BCG for certain cytokine-stimulant pairs in neonates.

This study had several limitations, most notably the small sample size, which may have led to type 2 errors. From a study design perspective, an investigation of more cytokines would have allowed a more comprehensive analysis and comparison with other studies in different settings. In this context, the relatively small blood volumes for cytokine analysis was a limiting factor. In future, more expensive assays could be used to analyse more cytokines in smaller blood volumes and include an analysis of the cells of origin of the cytokines produced. As new evidence on pathogen recognition pathways that influence cytokine responses emerges, the choice of stimulants may be refined for each of those pathways and allow more comprehensive, targeted analyses.

The results of this study suggest that HBV vaccine, given concurrently with BCG at birth weakens the effects of BCG on cytokine responses to unrelated antigens in neonates. This is in keeping with several studies on the influences of BCG on heterologous immunity in infants which found that non-live vaccines mitigated the heterologous beneficial effects of live vaccines, including BCG. Where both HBV and BCG vaccines are recommended, the World Health Organization (WHO) currently advises concurrent HBV with BCG (189). This recommendation, which doesn't take into account the heterologous benefits of BCG, may need to be reconsidered in future. Several factors which influence neonatal immune responses need to be taken into consideration when designing future studies like this one, but clinicians and vaccine policy developers would benefit most from an understanding of the clinical correlation of cytokine responses to unrelated stimulants in neonates in terms of morbidity and mortality. These issues should guide future research into this area.

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Appendices

A. Original Mercy HREC approval letter

Mercy Health
Care first

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19th August 2014

Professor Nigel Curtis
Head of Infectious Diseases
The Royal Children's Hospital
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Dear Professor Curtis

Re: R14/02: A Randomised Controlled Trial Investigating the Influence of BCG and Hepatitis B Immunisation at Birth on Neonatal Immune Responses. (The Early Life Vaccine and Immunity Study – ELVIS)

I am pleased to advise that your amendments comply with the requirements of the Human Research Ethics Committee meeting of 8 April 2014 and as such you may now commence with the study. Specifically, the following documentation is approved:

NEAF	Dated 13 May 2014
Site Specific Assessment Form (SSA)	Dated 13 May 2014
Victorian Specific Module	Dated 27 February 2014
Protocol	Version 1.0, Dated 9 May 2014
Participant Information Sheet/Consent Form	Dated 9th May 2014

Documentation submitted for governance purposes for this clinical trial have been received by the Administrative Officer of Mercy Health HREC and have been signed by hospital management 19 August 2014. Therefore all the requirements for ethical approval have been met.

The Human Research Ethics Committee is constituted and functions in accordance with the National Health and Medical Research Council National Statement on Ethical Conduct in Human Research (2007).

This approval by the Mercy Health Human Research Ethics Committee is valid from 19 August 2014 to 18 August 2017. That is, the project should be completed by the approval expiry date, of **18 August 2017.** Should it become apparent that an extension of the 3-year period is required, the principal researcher should apply, in writing, through the Administrative Officer of the Human Research Ethics Committee. Please note that the research project should be commenced within 12 months from the date of this letter.

Would you kindly advise me the date that you commence your research.

In accordance with the NHMRC Guidelines, approval is subject to:

1. Immediate notification to the Administrative Officer, The Mercy Health Human Research Ethics Committee and sponsor, of any serious adverse effects on participants;
2. Immediate notification of any unforeseen events that may affect the continuing ethical acceptability of the project;
3. Notification and reasons for ceasing the project prior to its expected date of completion;
4. The completion of a progress report at 6 months and then annually for the duration of the project; (progress report form will be emailed to you with a copy of this letter);
5. The Mercy Health Human Research Ethics Committee approval of any proposed modifications to the project;
6. The submission of a final report and papers published on completion of the project.

Please also note:

7. Consent Forms must be available for audit by the Mercy Health Human Research Ethics Committee and retained for the period required by law;
8. The Principal Investigator upon leaving the Institution must inform the Mercy Health Human Research Ethics Committee as to the nominated person to replace him/her.

If you have any queries, please do not hesitate to contact me on 8458 4808.

Yours sincerely,

Carole Branch
Administrative Officer
Mercy Health Human Research Ethics Committee

B. Mercy HREC amendment approval letter version 2

Mercy Health
Care first

Mercy Health
Level 2, 12 Shelley Street
Richmond Vic 3121
Phone: +61 3 8416 7777
Fax: +61 3 8416 7888
mercyhealth.com.au

10 October 2014

Professor Nigel Curtis
C/o Dr Lianne Cox
Department of Infectious Diseases & Microbiology
The Murdoch Childrens Research Institute
The Royal Children's Hospital
Flemington Road
Parkville
VIC 3052

Dear Professor Curtis

Re: R14-02: A randomised, controlled trial investigating the influence of BCG and Hepatitis B Immunisation at Birth on Neonatal Immune Responses.

I am pleased to advise that the Mercy Health Human Research Ethics Committee meeting of 7 October 2014 **approved** the amendments for this research.

Amendment request to approved research	Dated 18 September 2014
Additional researchers	Dated 18 September 2014
Updated Protocol (tracked and clean copy)	Version 2.0, Dated 20 September 2014
Updated Information and Consent Form. (tracked and clean copy)	Version 2, Dated 20 September 2014

The Human Research Ethics Committee is constituted and functions in accordance with the National Health and Medical Research Council's National Statement on Ethical Conduct in Human Research (2007). In accordance with the NHMRC Guidelines, approval is subject to:

1. Immediate notification to the Administrative Officer, Human Research Ethics Committee and sponsor, of any serious adverse effects.
2. Immediate notification of any unforeseen events that may affect the continuing ethical acceptability of the project;
3. Notification and reasons for ceasing the project prior to its expected date of completion;
4. The completion of a progress report annually for the duration of the project;
5. Human Research Ethics Committee approval of any proposed modifications to the project;
6. The submission of a final report and papers published on completion of the project.

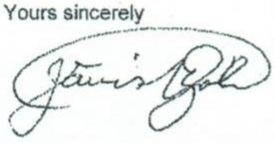
Please also note:

7. Consent Forms must be available for audit by the Human Research Ethics Committee and retained for the period required by law;

8. The Principal Investigator upon leaving the Institution must inform the Human Research Ethics Committee as to the nominated person to replace him/her.

If you have any queries, please do not hesitate to contact Ms Carole Branch, Administrative Officer, Mercy Health Human Research Ethics Committee on (03) 8458 4808.

Yours sincerely



Professor Jānis (John) T. Ozoliņš
Chair, Mercy Health Human Research Ethics Committee

C. Mercy HREC amendment approval letter version 3

Mercy Health
Care first

Mercy Health
Level 2, 12 Shelley Street
Richmond Vic 3121
Phone: +61 3 8416 7777
Fax: +61 3 8416 7888
mercyhealth.com.au

29 June 2015

Professor Nigel Curtis
C/o Dr Lianne Cox
The Royal Children's Hospital
Murdoch Childrens Research Institute
Flemington Road
Parkville
VIC 3052

Dear Professor Curtis

Re: R14/02: A Randomised Controlled Trial Investigating the Influence of BCG and Hepatitis B Immunisation at Birth on Neonatal Immune Responses. (The Early Life Vaccine and Immunity Study - ELVIS)

I am pleased to advise that the Mercy Health Human Research Ethics Committee meeting of 16 June 2015 **approved** the amendments for this research.

ELVIS Protocol	Version 3, Dated 15th May 2015
ELVIS Participant Information and Consent Form	Version 3, Dated 15th May 2015

Please note this research has ethics approval to 18 August 2017.

The Human Research Ethics Committee is constituted and functions in accordance with the National Health and Medical Research Council's National Statement on Ethical Conduct in Human Research (2007). In accordance with the NHMRC Guidelines, approval is subject to:

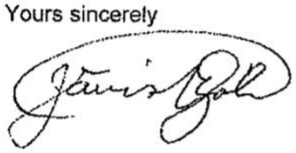
1. Immediate notification to the Administrative Officer, Human Research Ethics Committee and sponsor, of any serious adverse effects.
2. Immediate notification of any unforeseen events that may affect the continuing ethical acceptability of the project;
3. Notification and reasons for ceasing the project prior to its expected date of completion;
4. The completion of a progress report annually for the duration of the project;
5. Human Research Ethics Committee approval of any proposed modifications to the project;
6. The submission of a final report and papers published on completion of the project.

Please also note:

7. Consent Forms must be available for audit by the Human Research Ethics Committee and retained for the period required by law;
8. The Principal Investigator upon leaving the Institution must inform the Human Research Ethics Committee as to the nominated person to replace him/her.

If you have any queries, please do not hesitate to contact Ms Carole Branch, Administrative Officer, Mercy Health Human Research Ethics Committee on (03) 8458 4808.

Yours sincerely



Professor Jānis (John) T. Ozoliņš
Chair, Mercy Health Human Research Ethics Committee

Cc: Dr Lianne Cox, The Royal Childrens Hospital

D. Mercy HREC amendment approval letter version 4

Mercy Health
Care first

Mercy Health
Level 2, 12 Shelley Street
Richmond Vic 3121
Phone: +61 3 8416 7777
Fax: +61 3 8416 7888
mercyhealth.com.au

4 May 2016

Professor Nigel Curtis
C/o Dr Lianne Cox
Murdoch Childrens' Research Institute
The Royal Childrens Hospital
Flemington Road
Parkville
VIC 3052.

Dear Professor Curtis

Re:R14/02: The Early Vaccine & Immunity Study (ELVIS).

I am pleased to advise that your amendment request has been considered by members of the Human Research Ethics Committee Expedited Review Working Party and has been granted approval.

This approval is effective immediately and enables you to continue the study but is subject to the endorsement of the Mercy Health HREC.

In particular, the following is approved:

Extension of ethics approval to March 2017	Dated 15 March 2016
ELVIS Protocol	Version 4.0, Dated 18 November 2015
ELVIS Participant Information & Consent Form	Version 4, Dated 18 November 2015

The full Human Research Ethics Committee will be advised of these amendments at its next meeting to be held on **7 June 2016**. You will receive a formal letter of endorsement from the Mercy Health HREC following this meeting

In accordance with the NHMRC National Statement on Ethical Conduct in Human Research (2007), approval is subject to:

- Immediate notification to the Administrative Officer, Human Research Ethics Committee and sponsor, of any serious adverse effects on participants;
- Immediate notification of any unforeseen events that may affect the continuing ethical acceptability of the project;
- Notification and reasons for ceasing the project prior to its expected date of completion;
- The completion of a progress report annually for the duration of the project;
- Human Research Ethics Committee approval of any proposed modifications to the project;

- The submission of a final report and papers published on completion of the project.

Please also note:

- Consent Forms must be available for audit by the Human Research Ethics Committee and retained for the period required by law;
- The Principal Investigator upon leaving the Institution must inform the Human Research Ethics Committee as to the nominated person to replace him/her.

If you have any queries, please do not hesitate to contact me on 8458 4808.
Yours sincerely,

Carole Branch
Administrative Officer, Mercy Health Human Research Ethics Committee

E. Original RCH HREC approval letter

The Royal Children's Hospital Melbourne
50 Flemington Road
Parkville Victoria 3052 Australia
TELEPHONE +61 3 9345 5522
www.rch.org.au

The Royal Children's Hospital Melbourne

RCH HUMAN RESEARCH ETHICS COMMITTEE APPROVAL

HREC REF. No:	**34032 A**
PROJECT TITLE:	A Randomised, Controlled Trial Investigating the Influence of BCG and Hepatitis B Immunisation at Birth on Neonatal Immune responses.
DOCUMENTS APPROVED:	PGIS & CF v1.0 dated 9 Mar 2014
APPROVED PROTOCOL:	Protocol v1.0 dated 9 May 2014
PRINCIPAL INVESTIGATOR:	Nigel Curtis
DATE OF ORIGINAL APPROVAL:	**20 May 2014**
DURATION:	48 months
DATE OF APPROVAL EXPIRY:	**20 May 2018**

SIGNED: 20 May 2014

COMMITTEE REPRESENTATIVE

CONDITIONS:

APPROVED SUBJECT TO THE FOLLOWING CONDITIONS:

ALL PROJECTS

1. The study must not commence until all Research Agreements have been executed (if applicable)
2. Must comply with the Investigator's Responsibilities in Research Procedure and other Campus Research Policies and Procedures
3. Any proposed change in the protocol or approved documents or the addition of documents must be submitted to the Human Research Ethics Committee (HREC) for approval prior to implementation, including:
   - flyers, brochures, advertising material
   - Increase in recruitment target
4. The Principal Investigator must notify Research Development & Ethics of:
   - Any serious adverse effects of the study on participants and steps taken to deal with them.
   - Any unforeseen events (e.g. protocol violations or complaints).
   - Investigators withdrawing from or joining the project.
5. A progress report must be submitted annually and at the conclusion of the project.
6. RCH HREC approval must remain current for the entire duration of the project. If the project is not completed in the allocated time a renewal request must be submitted to the Research Development & Ethics. Investigators undertaking projects without current HREC approval risk their indemnity, funding and publication rights.

CLINICAL TRIALS

7. Must comply with Good Clinical Practice (GCP)
8. Must report all internal (occurring in RCH participants) Serious Adverse Events (SAE) to the sponsor and the RCH HREC within 72 hours of occurrence.
9. Must report all Suspected Unexpected Serious Adverse Reactions (SUSARS) to the Therapeutic Goods Administration (TGA) (for sponsored studies the sponsor may take this responsibility).

F. RCH HREC amendment approval letter version 2

The Royal Children's Hospital Melbourne
50 Flemington Road
Parkville Victoria 3052 Australia
TELEPHONE +61 3 9345 5522
www.rch.org.au

The Royal Children's Hospital Melbourne

RCH HUMAN RESEARCH ETHICS COMMITTEE APPROVAL

HREC REF. No:	**34032 B**
PROJECT TITLE:	A Randomised, Controlled Trial Investigating the Influence of BCG and Hepatitis B Immunisation at Birth on Neonatal Immune responses.
DOCUMENTS APPROVED:	PGIS & CF v2.0 dated 20 Sep 2014
APPROVED PROTOCOL:	Protocol v2.0 dated 20 Sep 2014
PRINCIPAL INVESTIGATOR:	Nigel Curtis
DATE OF ORIGINAL APPROVAL:	**06 February 2015**
DURATION:	39 months
DATE OF APPROVAL EXPIRY:	**20 May 2018**

SIGNED: _____ Date: 6th February 2015

COMMITTEE REPRESENTATIVE

APPROVED SUBJECT TO THE FOLLOWING CONDITIONS:

ALL PROJECTS

1. The study must not commence until all Research Agreements have been executed (if applicable)
2. Must comply with the Investigator's Responsibilities in Research Procedure and other Campus Research Policies and Procedures
3. Any proposed change in the protocol or approved documents or the addition of documents must be submitted to the Human Research Ethics Committee (HREC) for approval prior to implementation, including:
   - flyers, brochures, advertising material
   - Increase in recruitment target
4. The Principal Investigator must notify Research Development & Ethics of:
   - Any serious adverse effects of the study on participants and steps taken to deal with them.
   - Any unforeseen events (e.g. protocol violations or complaints).
   - Investigators withdrawing from or joining the project.
5. A progress report must be submitted annually and at the conclusion of the project.
6. RCH HREC approval must remain current for the entire duration of the project. If the project is not completed in the allocated time a renewal request must be submitted to the Research Development & Ethics. Investigators undertaking projects without current HREC approval risk their indemnity, funding and publication rights.

CLINICAL TRIALS

7. Must comply with Good Clinical Practice (GCP)
8. Must report all internal (occurring in RCH participants) Serious Adverse Events (SAE) to the sponsor and the RCH HREC within 72 hours of occurrence.
9. Must report all Suspected Unexpected Serious Adverse Reactions (SUSARS) to the Therapeutic Goods Administration (TGA) (for sponsored studies the sponsor may take this responsibility).

G. Parent/Guardian information and consent form

HREC Project Number:	R1402		
Research Project Title:	A randomised, controlled trial investigating the influence of BCG and Hepatitis B immunisation at birth on neonatal immune responses		
Principal Researcher:	Professor Nigel Curtis Leader of Infectious Diseases & Microbiology Group, Murdoch Children's Research Institute, Professor of Paediatric Infectious Diseases, Department of Paediatrics, The University of Melbourne, Head of Infectious Diseases, The Royal Children's Hospital Melbourne		
Version Number:	4	**Version Date:**	18th November 2015

Thank you for taking the time to read this **Parent/Guardian Information Statement and Consent Form**. We would like to invite your child to participate in a research project that is explained below. This document is 8 pages long. Please make sure you have all the pages.

What is an Information Statement?

These pages tell you about the research project. It explains to you clearly and openly all the steps and procedures of the project. The information is to help you decide whether or not you would like your child to take part in the research. Please read this Information Statement carefully.

Before you decide if you want your child to take part or not, you can ask us any questions you have about the project. You may want to talk about the project with your family, friends or health care worker.

If you would like your child to take part in the research project, please sign the consent form at the end of this information statement. By signing the consent form, you are telling us that you:

- Understand what you have read
- Had a chance to ask questions and received satisfactory answers
- Consent to your child taking part in the project.

We will give you a copy of this information and consent form to keep either in hard copy or as an email if you have consented electronically.

1. What is this research project about?

Hepatitis B vaccine is routinely given to all newborn babies in Australia at birth (or up to 7 days of birth) and is used to prevent Hepatitis B infection that can be passed from a mother, who may not be aware of her infection, to her child. The current National Immunisation Program schedule recommends all babies get Hepatitis B vaccine at birth (or up to 7 days of age), and at 2, 4 and 6 months of age. These later doses of the Hepatitis B vaccine are given to protect the baby from infection he/she may get from other ways throughout his/her life.

Tuberculosis (TB) is an infectious disease that usually affects the lungs. The vaccine that protects against TB is called BCG. Because the rates of TB in Australia are so low, BCG vaccination is not part of the Immunisation Program schedule. However, BCG vaccination is recommended for certain groups of people who are at risk of infection. These groups include children under the age of 5 years who will be travelling outside Australia to an area where TB is much more common.

Research has shown that some vaccines given in early life may help a child's immunity and protect against other infections and improve their response to other vaccines. For example, studies in developing countries have shown that children given BCG vaccine were also protected from other infections and their response to other vaccines was improved.

How BCG, and other vaccines, causes this effect on the immune system is not completely understood. This project will study this effect. By allowing us to understand how these vaccines (Hepatitis B Vaccine and BCG) affect children's immunity, the results from this study may help us find new ways to improve immune responses in children living in Australia and overseas.

In addition to vaccines, we also know that the normal, unharmful, germs that live in the gut and in the nose also influences the immune system and we would like to understand this better.

The aim of this project is to find out the effect of Hepatitis B vaccine and BCG vaccine given at birth on children's immunity, germs in the gut and nose, and the relationship between these.

We will include up to 300 mothers and their newborn babies from the Mercy Hospital for Women in this project. We will compare blood samples from the babies in four separate groups, depending on when they receive their BCG and Hepatitis B vaccines.

2. Who is funding this research project?

The Principal Investigator, Professor Nigel Curtis, has started this research study. It is being funded by the Murdoch Childrens Research Institute (MCRI) and conducted by researchers based at the Mercy Hospital for Women (MHW), The Royal Children's Hospital (RCH), The University of Melbourne and MCRI. You will not be paid to take part in this research study

3. Why am I being asked to be in this research project?

We are inviting you to take part because you are giving birth at the Mercy Hospital for Woman and you plan to travel with your baby to a country with high levels of TB in the next five years. Because you are planning on travelling to a country with high levels of TB, it is recommended that your baby be given the BCG vaccine before travelling, to prevent him/her getting TB while outside Australia.

Your baby will also be given the Hepatitis B vaccine, as all babies are in Australia.

Because it is recommended that your baby be given both these vaccines, should you accept these recommendations, we will be able to study the effect of the vaccines on immunity, the germs in the gut and nose, and the relationship between these.

4. What does participation in this research involve?

Study length

Your participation in the study will last approximately 7-11 days and up to 3 months (if you choose to) from when your child is born.

Randomisation

As part of this study your baby will be given Hepatitis B and BCG vaccines. We will randomly (like flipping a coin) put your baby in one of the following groups:

- Group 1: BCG vaccine at birth then Hepatitis B vaccine at approximately 7 days old
- Group 2: BCG and Hepatitis B vaccine at birth
- Group 3: Hepatitis B vaccine at birth then BCG vaccine at 7 days old
- Group 4: Hepatitis B and BCG vaccine at 7 days old

Visits and Procedures

After your baby is born, we will:

- Confirm that you still want your baby to take part in the study
- Make sure you and your baby are still suitable to take part in the study
- Check your baby can be safely given the BCG vaccine

All visits and procedures will be completed at the hospital. The visits will happen within 3 days of your baby being born and again about a week after the initial visit, provided there is a trained staff member available to carry out the procedures.

The following procedures will be completed:

Mother

- We will access your hospital medical record to collect information about any medical conditions you may have and treatment you are currently receiving.
- Ask you some questions to give us information about your general health and details such as your age, country of birth etc.
- Collect a blood sample (about a teaspoon, 5 ml) after you have given birth to check for Hepatitis B infection.
- Ask you some questions about your diet, general health and the well-being of your baby when you visit us about a week after your baby is born.

Baby

- Vaccinations will be given according to the group your baby is placed in
- Collect a blood sample (up to one and a half teaspoons, from a vein) about a week after your baby is born.

<table>
<tr><td rowspan="2">Antenatal Clinics/ Postnatal Ward</td><td>Study point 1</td><td colspan="4">Recruitment</td></tr>
<tr><td>Study point 2</td><td colspan="4">Enrolment</td></tr>
<tr><td colspan="6"></td></tr>
<tr><td rowspan="6">Postnatal ward</td><td>Study point 3</td><td colspan="4">Maternal blood sample (5 ml of blood) (Day 0-5 after delivery)</td></tr>
<tr><td rowspan="2">Study point 4</td><td colspan="4">Randomisation (Day 0-3 after delivery)</td></tr>
<tr><td>Group 1</td><td>Group2</td><td>Group 3</td><td>Group 4</td></tr>
<tr><td rowspan="4">Study point 5</td><td colspan="4">Infant Vaccination (Day 0-3 after delivery)</td></tr>
<tr><td>Group 1</td><td>Group 2</td><td>Group 3</td><td>Group 4</td></tr>
<tr><td rowspan="2">BCG</td><td>BCG</td><td rowspan="2">Hep B</td><td rowspan="2">Nil</td></tr>
<tr><td>Hep B</td></tr>
<tr><td colspan="6"></td></tr>
<tr><td>Post delivery</td><td>Study point 6</td><td colspan="4">Confirm recent maternal negative hepatitis B test (Day 0-7 after delivery)</td></tr>
<tr><td colspan="6"></td></tr>
<tr><td rowspan="5">Mercy Hospital</td><td rowspan="5">Study point 7 (Day 7+/-4 from study point 5)</td><td colspan="4">Researcher completes questionnaire</td></tr>
<tr><td colspan="4">Infant Blood sample (up to 7,5 ml)</td></tr>
<tr><td colspan="4">Infant Vaccination</td></tr>
<tr><td>Group 1</td><td>Group 2</td><td>Group 3</td><td>Group 4</td></tr>
<tr><td>(Hep B)</td><td>Nil</td><td>BCG</td><td>(Hep B) BCG</td></tr>
</table>

Consent for optional sample collection

1. We will ask you to consider collecting a small stool (poo) sample on any day your baby has a dirty nappy from birth until the study point 7 visit.

These will be stored in provided plastic specimen bags in an airtight container that will be kept in the freezer. This should take no more than 5 minutes of your time. If you or your baby is still in hospital when stool samples are collected, these will be stored in a freezer in the hospital, otherwise the samples will be kept in your home freezer. The study team will collect the samples from the hospital or from yourself about 1 week after your baby is born (the study point 7 visit).

2. We will also ask you to consider collection of a nose swab from you and your baby at the times we visit you and again when your baby is between 1 and 3 months old.

A member of the study team will collect this sample from you and your baby within 3 days of birth and again when the baby is about 1 week old. At this visit, a member of the study team will show you how to take this swab, as you will need to take the nose swab from yourself and your baby when he/she is between 1 and 3 months old.

If you decide that you don't want to collect the stool or nose swab samples, you will still be able to continue in the study. You may also choose to collect either the stool or nasal swab samples without being excluded from the study.

Consent for optional genetic analysis in baby's blood sample

Our bodies are made up of different types of cells. Inside these cells you find genes. Genes are passed down in families from parents to children: you get half your genes from your mother and half from your father. Our genes contain all the information that makes us what we are, including our eye colour, blood type, and height and whether we are born as a boy or a girl.

There are about 23,000 genes that make up a human being and genes are arranged along a chemical substance called DNA. We will extract DNA from your baby's blood samples. We will look to see if there are genetic features in your baby's DNA that might be associated with the immune response to BCG vaccination.

The genetic analysis that we are doing is for research purposes only and the significance of the results relating to the immune system is unknown, therefore we will not provide individual results to you.

This part of our study is voluntary, if you are happy for us to undertake genetic analysis, you can let us know by checking the box on the consent form (last page).

We will respect your decision and will only use the samples and data as you have asked for it to be used.

Consent to be contacted about future research related to this project

Researchers may contact you to discuss participation in future studies conducted by this research facility. If you decide that you do not want to be contacted, you will still be able to continue in the study. We will not contact you unless you provide consent for us to do so.

5. What will happen to the test samples?

All samples from you and your baby will be collected and labelled with an allocated study number.

We will analyse various components of the immune response in your baby's blood sample.

The stool and nasal swabs will be transported to the Infectious Diseases and Microbiology research laboratory at the MCRI and stored in freezers until further analysis. We will determine the "germs" in stool and nasal swabs.

Only the members of the research team will be able to access any of these samples and will update reports on their location and processing. The freezers are locked and can only be opened by members of the research team who have access to the key.

6. What will happen to information about my baby or me?

In this study we will collect and use personal and health information about you and your child for research purposes. Any information we collect that can identify you or your child will be treated as confidential and used only in this project unless otherwise specified. We can disclose the information only with your permission, except as required by law.

All written information will be stored securely in the Infectious diseases and Microbiology department at the Murdoch Childrens Research Institute. Electronic information, the information stored on our computer systems, can be accessed only by research staff that has a secure password. We will back up this information and store these files at the Murdoch Childrens Research Institute.

The following people may access information collected as part of this research project:

- The research team involved with this project
- The Royal Children's Hospital Human Research Ethics Committee
- The Mercy Health Human Research Ethics Committee

In accordance with the National Medical Health and Research Council Guidelines, the Mercy Health Human Research Ethics Committee or the Royal Children's Hospital Human Research Ethics Committees are required to conduct audits of research products from time to time. It may therefore be possible that these Committees, which have approved this research, will seek to view a copy of your signed consent form, or to contact you, to ensure that the research is being conducted according to the ethical standards required by these guidelines.

The stored information will be re-identifiable. This means that we will remove identifying information such as names and give the information a special code number. Only the research team can match your names to your code number, if it is necessary to do so.

We are required to keep information collected as part of a research project for a certain length of time. Because the participants in this project are under 18 years old, we must keep information until the youngest participant turns 25 years old and at least 15 years have passed since we have published any results from this study. The research information may be destroyed after this time.

At the end of the study, results may be presented at conferences or published in medical journals and as part of a thesis. This will be done in such a way that your child cannot be identified.

Information about your baby's participation in this research project may be recorded in your health records.

7. How can I access my information?

In accordance with relevant Australian and/or Victorian privacy and other relevant laws, you have the right to access the information collected and stored by the researchers. You also have the right to request that any information with which you disagree be corrected. Please contact one of the researchers named at the end of this document if you would like to access your information.

8. What are the possible benefits of participating in this research?

There is no direct benefit to your child taking part in this study.

We hope that learning more about how birth vaccines affect a baby's immunity, will help guide immunisation policies in the future.

9. What are the possible risks, side effects, discomforts and/or inconveniences?

<u>Risks of BCG vaccination</u>

BCG is one of the most widely used vaccines and is given to more than 120 million babies every year worldwide. BCG immunisation can occasionally cause adverse effects. Your baby may have none or some of the more common effects listed below, and they may be mild, moderate or severe. If your

baby has any of these effects, or you are worried about them, you should talk with your doctor and inform a member of the research team. You and your doctor are also able to contact us at any time with any concerns regarding the immunisation, and if necessary, your child can be seen at the RCH in one of our clinics by a paediatric specialist. Adverse effects of the vaccine may need to be reported to the Ethics Committees of the participating hospitals.

Adverse effects related to BCG immunisation

BCG immunisation can be associated with discomfort but is minimised when given by experienced immunisation staff. A pustule (small pimple) will form where the vaccination is given in the left upper arm, and later a small scar will develop as part of the normal vaccination reaction. Minor side effects usually get better by themselves, without requiring any specific treatment.

Almost all people who get BCG vaccine will get a red lump where they had the injection after about a week. This may become an ulcer (open sore) by about 2-8 weeks that subsequently heals leaving a small scar.

Rare side effects of BCG vaccine include:

- A large skin ulcer where the person had the injection (the most common side effect - about three people in 100)
- Infection of the lymph glands in the armpit (about one person in 100)
- Keloid scarring (very noticeable scarring on your skin). This does not happen very often.
- Severe immediate allergic reactions but these are rare
- Spread of the BCG infection to other sites in the body including bone is also rare but can be very serious and result in your baby becoming seriously unwell.

Risks of Hepatitis B vaccination

Side effects are mild and temporary after immunisation with Hepatitis B vaccine. Most commonly there is redness and soreness at the injection site (about 5 in 100) and a low-grade fever (3 out of 100).

If your baby gets a fever under the age of 6 weeks, he/ she should be seen by a doctor immediately. There are many other, more common causes of fever in very young babies that may need urgent attention and treatment.

Risks of blood tests

There are no major risks associated with a blood test. It is possible your child may feel some discomfort during the blood test. They may feel a sting when the needle is put in their arm to take the blood. We can give your baby some sucrose solution (sugar and water mixture) or you may give your breast milk before the blood is taken to help with pain relief. It is possible there may be some bruising, swelling or bleeding where the needle enters the skin.

10. What if new information arises during this research project?

During the research project, new information about the risks and benefits of the project may become known. If this occurs, you will be told about this new information and your doctor will discuss whether this new information affects you and your baby.

11. Can I have other treatments during this research project?

While your baby is participating in this research project, he/she will not be restricted from using any other medication or treatment for conditions which may arise incidentally during the research study period.
All other immunisations as recommended by the Australian Immunisation Program schedule will be given at the normal times.
However, it is important to inform us about any treatments or medications you or your baby may be taking, including over-the-counter medications, vitamins, pre/probiotic preparations or herbal remedies as these may interfere with the analysis of results.

12. What will happen when my participation in this research project ends?

At the end of the study, your baby will continue to have routine care as any other baby.

13. What are my child's alternatives to taking part?

Participation in a research project is voluntary. It is your choice to let your child take part in this research. You do not have to agree if you do not want to.

If you give your consent and change your mind, your child can withdraw from the project. You do not need to tell us the reason why you want to stop your child being in the project.

Whatever your decision, it will not affect any treatment or care your child gets, or your family's relationship with The Royal Children's Hospital, The Mercy Hospital for Women or any other organisation associated with the study.

It is recommended that your child get the BCG for protection against TB at least 1 month before you travel. If you would like your child to receive BCG, you will need to arrange this yourself.

14. What if I withdraw from this research project?

Please tell us if you and your baby plan to leave the research study so we can let you know if there are any health risks or special requirements linked to withdrawing.

If you decide to leave the project, we would like to keep the personal and health information about your baby and the samples that have been collected. This is to make sure that the results of the research can be measured properly. If you do not want this to happen, please tell us before you join the study.

15. Could this research project be stopped unexpectedly?

This research project may be stopped for a variety of reasons. These may include unusual or an unexpected high rate of side effects. Although BCG and Hepatitis B has an established safety record, the research team will monitor for any side effects.

16. Will we be informed of the results when the research project is finished?

We will send you a letter summarising the study's main findings, although individual results will not be given.

17. What happens if my child is injured during the project?

If your child suffers any injury or complication as a result of this research project, contact the study team as soon as possible. The study team will help to arrange appropriate medical treatment for your child. If your child is eligible for Medicare, they can get medical treatment to treat the injury or complication as a public patient in any Australian public hospital at no cost to you.

18. Will my child be able to claim compensation if injured?

By signing the consent form, you are not giving up any legal rights to seek to obtain compensation for injury.

If you would like more information about the project or if you need to speak to a member of the research team in an emergency, please contact:

Study Coordinator:	**Dr Lianne Cox**
Contact telephone:	(03) 8341 6387,
Principle Researcher:	**Professor Nigel Curtis**
Contact telephone:	(03) 9345 5522 (via The Royal Children's Hospital switchboard)

All other members of our research team can be contacted via the study coordinator:

Research Nurses & Midwives: Ms Veronica Abruzzo

Ms Clare Morrison

Ms Kate Wall

Ms Lea Daniher

Ms Natalie Papagiorcopulo

Ms Brooke O'Neill

Ms Clare Brophy

Ms Julie Quinn

Ms Monica Owlad

Ms Judith Spotswood

Ms Karen Bellamy

Ms Hannah Turner

Research Doctors:

Dr Dan Casalaz

Dr Lianne Cox

Dr Bridget Freyne

Dr Hayley Du Plessis

Dr Eva Sudbury

Dr Freya Harewood

If you have any concerns and/or complaints about the project, the way it is being conducted or your child's rights as a research participant, and would like to speak to someone independent of the project, please contact:

The reviewing HRECs approving this research:

1. HREC name: Human Research Ethics Committee Mercy Health

Telephone: 03 8458 4808 Email: ethics@mercy.com.au

2. HREC name: Human Research Ethics Committee RCH Melbourne

Telephone: 03 9345 5044 Email rch.ethics@rch.org.au

CONSENT FORM

HREC Project Number:

Research Project Title: A randomised, controlled trial investigating the influence of BCG and Hepatitis B immunisation at birth on neonatal immune responses

Version Number: 3 **Version Date:** 15th May 2015

- I have read, or had read to me in my first language, the information statement version listed above, and I understand its contents.
- I believe I understand the purpose, extent and possible risks of me and my child's involvement in this project.
- I voluntarily consent for me and my child to take part in this research project.
- I have had an opportunity to ask questions and I am satisfied with the answers I have received.
- I understand that this project has been approved by The Royal Children's Hospital Melbourne and Mercy Health for Women Human Research Ethics Committee and will be carried out in line with the National Statement on Ethical Conduct in Human Research (2007).
- I consent to the Research Ethics Committees, which approved this study, to access my information, or to contact me to ask about my research experience, in order to ensure that the project is being run in accordance with government standards.
- I understand I will receive a copy of this Information Statement and Consent Form.

OPTIONAL CONSENT

☐ I do	☐ I do not	consent to be contacted about future research related to this project
☐ I do	☐ I do not	consent to my baby's stool samples being analysed
☐ I do	☐ I do not	consent to my and my baby's nasal swabs being taken and analysed
☐ I do	☐ I do not	consent to the use of my child's blood samples for genetic analysis use in this project

Parent/Guardian and/Mother's Name	Parent/Guardian and /Mother's Signature	Date
Name of Witness to Parent/Guardian/Mother's Signature	Witness Signature	Date

Declaration by researcher: I have explained the project to the parent/guardian who has signed above, and believe that they understand the purpose, extent and possible risks of their child's involvement in this project.

Research Team Member Name	Research Team Member Signature	Date

Note: All parties signing the Consent Form must date their own signature.

H. ELVIS study protocol

VAC/01

A RANDOMISED, CONTROLLED TRIAL INVESTIGATING THE INFLUENCE OF BCG AND HEPATITIS B IMMUNISATION AT BIRTH ON NEONATAL IMMUNE RESPONSES

THE EARLY LIFE VACCINES AND IMMUNITY STUDY

VERSION 4.0 18th November 2015

CONFIDENTIAL

STATEMENT OF COMPLIANCE

This document is a protocol for a clinical research study. The study will be conducted in compliance with all stipulations of this protocol, the conditions of ethics committee approval, the NHMRC National Statement on Ethical Conduct in Human Research (2007) and the Note for Guidance on Good Clinical Practice (CPMP/ICH-135/95).

TABLE OF CONTENTS

1. PROTOCOL SYNOPSIS

Title	A randomised, controlled trial to determine the influence of BCG and Hepatitis B immunisation at birth on neonatal immune responses
Objectives	**Primary** To determine the influence of BCG and Hepatitis B immunisations shortly after birth on the neonatal immune responses to non specific antigens. **Secondary** To determine the immunological mechanisms underlying the non-specific effects of BCG and Hepatitis B vaccines by comparing markers of immunity. To evaluate the effect of BCG and Hepatitis B immunisations shortly after birth on intestinal microbiome structure by analysing stool samples in the first week of life. To describe the initial colonisation of the nasal microbiome in infants.
Study Design and Methodology	This will be a randomised, controlled trial in infants recruited in Melbourne over a two -year period from March 2015. **Recruitment (MHW) (Study point 1)** Pregnant women, attending antenatal clinics, and women who delivered a baby meeting eligibility criteria, will be given a copy of the participant information and consent form as well as verbal information about the study by a member of the research team and asked to consider their infant's involvement. **Enrolment (Study point 2)** If they agree to their infant's participation, they will sign and date a paper or electronic informed consent form prior to any study assessments/procedures being conducted. All mothers who have consented to the trial antenatally will have an alert in their medical record relating to their participation in the study. This alert will remind staff to avoid routine infant vaccine administration. A member of the research team will also conduct a brief interview with the participant's mother to collect information on demographic, environmental and other factors. These will be used to analyse any differences at baseline between the different groups. **Maternal blood testing (Study point 3)** A maternal blood sample will be taken to confirm negative Hepatitis B infection, unless the first antenatal hepatitis B screening test was performed within 7 days of delivery of the infant, and may also be used to evaluate factors known to influence neonatal immunity including vitamin and mineral levels and antibody titres to selected antigens. This

	will happen within 5 days of delivery of the infant, mostly commonly at the same time as study points 4 and 5. **Randomisation (Study point 4)** Participants will be randomised within 3 days of birth to one of the four study groups: study group 1 (BCG vaccine at birth, Hepatitis B vaccine at 7 days), group 2 (BCG vaccine and Hepatitis B vaccine at birth) group 3 (Hepatitis B vaccine at birth, BCG vaccine at 7 days) and group 4 (BCG vaccine and Hepatitis B vaccine at 7 days) **Intervention and administration (Study point 5)** Participants will receive vaccinations according to the group they have been randomised to within 3 days of birth. Should they have consented to stool and or nasal swab collection, these samples will be taken at this point. The mother will also be given stool sample collection kits. An appointment will be made for the study point 7 visit. **Maternal blood test result review (Study point 6)** The results of the blood test taken at Study point 3 will be checked to confirm the mother is Hepatitis B negative within 7 days of birth. Should the result be positive for Hepatitis B infection, the baby will be excluded from the study and both infant and baby will be referred for appropriate further management. **Study investigations (Study point 7)** A member of the research team, doctor or phlebotomist, experienced in paediatric blood collection, will collect an infant blood sample (up to 5 ml). This will occur at 7 (+/- 4) days post study point 5. If consent was obtained for stool and nasal swab collection, these samples will be obtained at this visit. Mother's will be taught how to collect nasal swabs from themselves and baby by the researcher conducting this visit. This visit is likely to take place at the Mercy Hospital for Women. In rare circumstances, this visit may be at the participant's home. The participants randomised to receive BCG immunisation at 7 days will be vaccinated at this visit after their blood has been drawn. Participants, who have been randomised to receive Hepatitis B vaccine at 7 days, will be offered this vaccine and should they want it, it will be administered after their blood has been drawn. Pre-paid self- addressed envelopes and appropriate equipment may be provided to collect the nasal swabs at study point 8. **Nasal swab collection (Study point 8)** An email or phone text message reminder with a linked electronic data form from REDcap will be sent to participants who agree to collection of a nasal swab between 4 and 12 weeks of age. The mother will collect a

	nose swab from herself and baby. The swabs will be posted by the family to the Royal Children's Hospital in pre paid self –addressed envelopes provided. **Schedule of Study Procedures** All eligible participants will have the following study procedures performed provided there are trained staff members to appropriately carry them out. **Randomisation (within 3 days of birth)** Soon after birth, all eligible participants will be randomised to group 1 (BCG vaccine at birth, Hepatitis B vaccine at 7 days), group 2 (BCG vaccine and Hepatitis B vaccine at birth) group 3 (Hepatitis B vaccine at birth, BCG vaccine at 7 days) and group 4 (BCG vaccine and Hepatitis B vaccine at 7 days). **Vaccination** Participants allocated to group 1 and group 2 will receive a single, 0.05mL intradermal dose of BCG vaccine in the left deltoid within 3 days of birth. Participants allocated to group 2 and group 3 will have an intramuscular dose of 0,5ml of Hepatitis B vaccine (H-B-Vax II) containing 5 micrograms of Hepatitis B surface antigen protein in the thigh within 3 days of birth. Participants allocated to group 1 and group 4 will be offered an intramuscular dose of 0,5ml of Hepatitis B vaccine containing 5 micrograms of Hepatitis B surface antigen protein in the thigh at Study point 7. Participants allocated to group 3 and group 4 will receive a single, 0.05mL intradermal dose of BCG vaccine in the left deltoid at study point 7. **Blood Collection** A maternal blood sample will be collected at study point 3. A blood sample will be taken from the infant at study point 7. **Stool Collection** The mother will be asked to collect a small sample of stool from the participant's nappy on each day that this is possible till the study point 7 visit. Standard stool collection sample containers will be supplied together with an airtight plastic container to store the samples until collection by the study team. (See Appendix C) Parents/LAR of study participants will be informed that they can opt out of the stool collection without exclusion from the trial. **Nasal swab collection** For those participants who have provided consent, a nasal swab will be taken at study point 5 and 7 from infant and mother. The mother will be taking a sample at study point 8 from herself and baby.

	Parents/LAR of study participants will be informed that they can opt out of the nasal swab collections without exclusion from the trial **Questionnaires** A member of the research team will complete a questionnaire at the study point 7 visit regarding the infant and mothers' diet, use of antibiotics and general health. Parents will be sent a reminder via email or text message to complete a very short online questionnaire prior to study point 8 if they provided consent for nasal swab collection.
Planned Sample Size	We plan to evaluate up to 300 infants (n = 50 to 75 in each group)
Study Population	Participants will be randomised within 3 days of birth, equally to study group 1, 2, 3 or 4 An infant must meet all of the following criteria in order to remain eligible for participation in the study: 1. Less than 3 days old; 2. English speaking parent; 3. Planned travel to a TB endemic country within the infant's first 5 years of life. 4. An informed consent form must be signed and dated by the infant's mother after the nature of the study has been explained and prior to any study assessments/procedures; 5. The infant's mother has screened negative for HIV during this pregnancy; 6. The infant's mother has screened negative for Hepatitis B during this pregnancy; 7. There is no known household contact infected with Hepatitis B 8. Born no earlier than eight weeks before estimated date of delivery; 9. Birth weight >1500g. 10. Delivered vaginally. 11. Singleton pregnancy 12. Not received Hepatitis B Vaccine prior to randomisation 13. Not received BCG vaccine prior to randomisation An infant meeting any of the following criteria will be excluded from study participation (including contraindications to receive BCG as per the NH&MRC Immunisation Handbook (10th edition, 2013): 1. Known or suspected HIV infection 2. Treatment with corticosteroids or other immunosuppressive therapy, including monoclonal antibodies against tumour necrosis factor-alpha (TNF-alpha) (e.g. infliximab, etanercept, adalimulab). 3. Born to a mother treated with bDMARDS (e.g. TNF-alpha blocking monoclonal antibodies) in the 3rd trimester;

	4. Congenital cellular immunodeficiencies including specific deficiencies of the interferon gamma pathway; 5. Malignancies involving bone marrow or lymphoid systems; 6. Serious underlying illness including severe malnutrition; 7. Medically unstable; 8. Generalised septic skin disease and skin conditions such as eczema, dermatitis and psoriasis; 9. Significant febrile illness; Also excluded are infants with: 1. A mother who is immunosuppressed; 2. A mother who has received Intravenous immunoglobulins during her pregnancy 3. A family history of immunodeficiency; 4. Consanguineous parents. 5. Mother who is having a planned Caesarean Section
Study Vaccines	The BCG vaccine used will be the BCG Vaccine SSI, a freeze-dried live vaccine prepared from an attenuated strain of Mycobacterium bovis. Study participants will receive a single 0.05mL intradermal dose. Study participants will be vaccinated with an intramuscular dose of 0,5ml of Hepatitis B vaccine (H-B-Vax II) containing 5 micrograms of Hepatitis B surface antigen protein.
Outcome Measures	**Primary Outcome measures** Cytokine responses to *in vitro* stimulation of blood with antigens at 7 days post vaccination **Secondary Outcome measures** Other *in vitro* measures of immune responses in infant blood Microbiome structure in stool samples Microbiome structure in nasal swab samples
Statistical Methods	For the primary outcome measure, Kuskal Wallis tests will be used to assess the null hypothesis. If there is evidence for a difference between groups, pairwise comparison between groups will be done using Mann-Whitney U tests.

2. GLOSSARY OF ABBREVIATIONS

ABBREVIATION	TERM
AE	Adverse Event
BAIR	BCG for Allergy and Infection Reduction
BCG	*Bacille Calmette-Guérin*
bDMARDS	Biological Disease-Modifying Anti-Rheumatic Drugs
CONSORT	Consolidated Standards of Reporting Trials
CRF	Case Report Form
GCP	Good Clinical Practice
GMP	Good Manufacturing Practice
HIV	Human Immunodeficiency Virus
HBV	Hepatitis B Vaccine
LAR	Legally Acceptable Representative
MIS	The Melbourne Infant Study
MCRI	Murdoch Children's Research Institute
MHW	Mercy Hospital for Women
NH&MRC	National Health & Medical Research Council
PICF	Parent/Participant Information Consent Form
RCH	Royal Children's Hospital (Melbourne)
RCT	Randomised, Controlled Trial
SAE	Serious Adverse Event
SSI	Statens Serum Institut
SUSAR	Suspected Unexpected Serious Adverse Drug Reaction
TB	Tubercle Bacillus (Tuberculosis)
TGA	Therapeutic Goods Administration
TNF	Tumour Necrosis Factor

3. INVESTIGATORS AND FACILITIES

3.1. Study Location/s

Murdoch Childrens Research Institute
50 Flemington Road
Parkville VIC 3052

The Royal Children's Hospital, Melbourne
50 Flemington Road
Parkville VIC 3052

Mercy Hospital for Women, Melbourne
163 Studley Road
Heidelberg VIC 3084

3.2. Study Management

The trial will be coordinated by the Principal Investigators who will lead a team of study doctors, research nurses and research assistants. Informed consent discussions will be delegated to trained members of the research team. The study coordinator will be delegated responsibility for overseeing data collection and maintaining study documentation. The handling and administration of the BCG and Hepatitis B study vaccines will be the responsibility of designated trained members of the research team.

3.3. Principal Investigators

3.3.1. Professor Nigel Curtis

Professor of Paediatric Infectious Diseases, Department of Paediatrics, The University of Melbourne;
Head of Infectious Diseases Unit, The Royal Children's Hospital Melbourne;
Leader, Infectious Diseases & Microbiology Group, Murdoch Childrens Research Institute

3.3.2. Doctor Dan Casalaz

Paediatrician
Neonatal Intensive Care Unit
Mercy Hospital for Women

3.3.3. Professor Susan Donath

Clinical and Epidemiological Biostatistics Unit
University of Melbourne and Murdoch Children's Research Institute

3.3.4. Doctor Lianne Cox

Paediatric advanced trainee: Postgraduate Research Fellow
PhD Student, University of Melbourne
Honorary fellow, Infectious Diseases & Microbiology Group, Murdoch Childrens Research Institute

3.4. Statistician

Prof Susan Donath
Clinical and Epidemiological Biostatistics Unit
University of Melbourne and Murdoch Childrens Research Institute

4. INTERNAL TRIAL COMMITTEES

Not applicable.

5. INDEPENDENT SAFETY MONITORING COMMITTEE

An Independent Safety Monitoring Committee will be established despite BCG and Hepatitis B vaccine having a robust and well-established safety profile in healthy infants. It will meet 6 monthly. This will comprise:

- Statistician
- Neonatologist
- Infectious diseases or vaccine physician

The role of this committee will be to review the SAEs and SUSARs

6. FUNDING AND RESOURCES

This study is funded through MCRI theme funds.

7. INTRODUCTION AND BACKGROUND

Neonatal mortality
Around 3 million children aged 0-28 days died in 2012, representing 43% of all the deaths of children under the age of 5. Infectious diseases contribute substantially to these deaths in this vulnerable group. (1)

Most of these deaths occur in developing countries, but in developed countries though the mortality rates are lower, the health implications associated with chronic morbidity as a result of infectious disease related inflammation is also of concern.

Despite overall reduction in under 5 mortality rates over the last decade, neonatal mortality rates continue to increase. It is therefore imperative that we continue to optimise current strategies and investigate novel ways to improve future health care to this particular subgroup.

Neonatal susceptibility to infection
It has long been the teaching that neonates are predisposed to sepsis because of a comparatively immature immune system resulting in the inability to defend against infection. However, emerging evidence suggests simple immune system immaturity does not fully explain this predisposition to infection. The explanation for the increased risk of infection in neonates is becoming much more complex.

This is because we understand the factors that influence immune responses to infections in neonates better, now that technology allows more detailed study in this area.
Factors influencing neonatal immune responses to infection

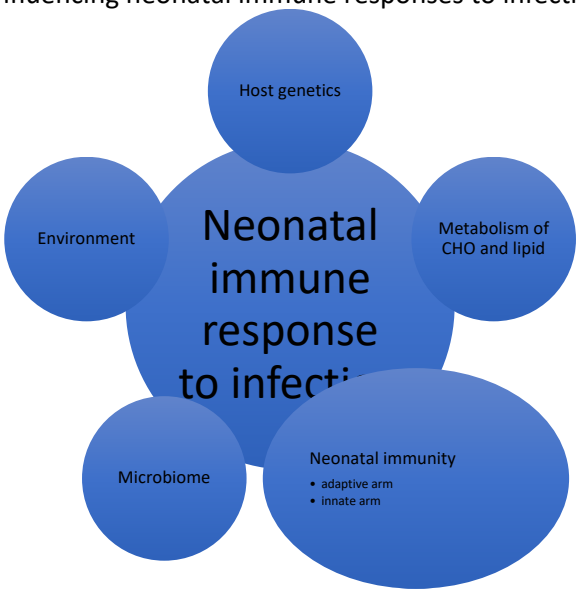


Figure 1 Factors influencing the neonatal immune responses to infection

This research will focus on the relationship between neonatal immunity and the microbiome in relation to the birth vaccines.

1. Neonatal immunity
The immune system has two arms.

The innate immune system represents the first line of defence against infection. It is composed of various cells, including natural killer (NK) cells and monocytes/macrophages. They sense pathogens

via pattern recognition receptors and ultimately produce effector molecules called cytokines. These cytokines regulate the adaptive arm of immunity.

The adaptive immune system is responsible for generating antibody responses via B-lymphocytes and cell-mediated responses via T lymphocytes. This arm protects against specific antigens. A key feature of the adaptive arm is the development of immunological memory, where an antigen is remembered and allows more effective killing on subsequent exposure.

There is increasing recognition of the importance of the innate immune response in the early neonatal period when the adaptive response is still immature.

The concept of immune tolerance is likely to be central in explaining these inadequacies in neonatal responses to infection. In the antenatal period, immune homeostasis must allow tolerance of maternal tissue and in the immediate postnatal period, it must allow rapid colonisation of organisms to establish the essential complex microbiome.

2. The microbiome

The microbiome is the collective term for the genomes of the microorganisms (microbiota) that share our body space.

The intestinal microbiome has been intensively studied in the last decade and has provided insight into the development and maturation of the infant immune system.

Through studies in animals and humans, it is becoming increasingly clear that intestinal microbiota influence the development of both Th1- and Th2-mediated diseases through their effect on regulatory T cells and pathways that, when intact, help prevent disordered immune responses associated with infections and diseases such as allergy and auto immunity.

3. Host genetics

Host genetics are believed to play a role in immune responses generated to invading organisms. It has long been observed that some neonates are more susceptible than others to infections.

There is minimal data for the neonatal population of the contribution of host genetics to susceptibility to infection. However, studies in older children have shown this association (2) and it has also been shown that immune responses to vaccines and non-vaccine antigens are influenced by host genetic differences. (3)

In summary, multiple factors influence neonatal immune responses to infection and our understanding continues to develop, as research in this area is ongoing.

Modulating neonatal immune responses to infections

Neonatal vaccination strategies would seem an obvious intervention and we have been successfully modulating the neonatal immune responses to specific infections since the 1940's.

The first neonatal vaccine used to prevent specific infection was BCG, now routinely used and recommended by the WHO to prevent severe tuberculosis in endemic countries. BCG is safely administered at birth.

Since 1982 hepatitis B vaccine has been used to prevent hepatitis B infection in neonates and was introduced into the routine schedule as a vaccine at birth in Australia in 2000.

The efficacy of trivalent oral polio vaccine and acellular pertussis vaccine have been studied and the results are promising showing that these vaccines are effective and safe given in very early life. (4) (5)

However, neonatal vaccination is limited due to several factors. For the same reasons that these neonates are susceptible to infection, impairments in their immune system does not allow the adequate formation of immune memory to the vaccinated antigen. Maternal factors such as antibody crossing the placenta and maternal nutrition also affect the ability of the neonate to generate effective responses to the vaccines to provide protection from specific infectious diseases.

Non Specific Effects of vaccines

There is now an expanding body of evidence for non-specific effects of various vaccines used in childhood. These non-specific effects relate to immunomodulatory capabilities of these vaccines that influence immune outcomes outside of the vaccine's specific targeted disease.

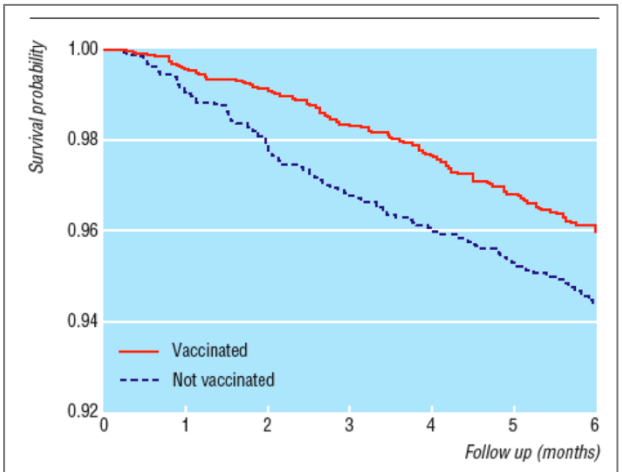
Of interest in this study are two vaccines given at birth ie BCG and Hepatitis B vaccine (HBV).
Evidence for non-specific effects of BCG

BCG has been used for decades and has allowed observations and study of its non-specific effects resulting in what is currently a large body of evidence.

BCG influences the developing infant immune system with significant clinical and population consequences (6) (7) (8).

BCG immunisation approximately halves neonatal mortality in high mortality regions. (9).

In randomised trials, BCG reduced total mortality from diseases other than tuberculosis by 25% (95% CI 6-41%) in the USA and the UK in 1948-61 (7), by 64% (1-87%) in Guinea-Bissau (10), and neonatal mortality by 45% (11-66%) when given to low-birth-weight babies in Guinea- Bissau (8) (11). The substantial reductions in child deaths in high-mortality regions occur as a result of BCG's non-specific beneficial anti-infection effects because the reduced mortality is attributable to decreased deaths from pneumonia, sepsis and diarrhoea (8) (11) (6).



Kaplan-Meier survival curves for children who did and did not receive BCG vaccine. Six months' follow up of 5274 children aged 0-6 months at initial visit, Guinea-Bissau, 1990-6

BCG protects against other infection (12) (13) (14), including mycobacteria (such *as Mycobacterium leprae*) (13), other bacteria (such as *Shigella flexneri*) (15), viruses (such as vaccinia virus) (16), and protozoa (such as *Plasmodium chaduadi*) (14).

BCG strongly influences the host response to other vaccines (10) (11) (17).

BCG administered at the time of primary immunisation markedly increases the cellular and antibody responses to hepatitis B vaccine (17). In addition, in high-mortality regions, BCG alters the way that other vaccines influence all-cause mortality (10) (11) (6) (18).

BCG's immunomodulatory properties are exploited for the treatment of carcinoma of the bladder in humans (19). BCG also reduces mortality in mice experimentally transplanted with tumours (20). A case-control study recently suggested that the risk of melanoma development was decreased in patients vaccinated with BCG (21). Importantly, vaccination in early life was associated with a better melanoma survival (22). It has been hypothesised that the beneficial effect of BCG on immune maturation in newborns(23) could be protective against leukaemia and melanoma, the former again linked to sub-optimal immune priming (24-26).

Evidence for non-specific effects of Hepatitis B vaccine
An observational study in Guinea Bissau found the mortality rate to be higher in infants receiving HBV, especially in females. (27)

Immunological mechanisms underlying non-specific effects of vaccines
Until recently, the underlying immunological mechanism responsible for these non-specific effects of vaccines was completely unknown. Even now these mechanisms are incompletely defined. However, evidence from animal and human studies is mounting for the innate immune system as the key to these observed non-specific effects.

Initial neonatal responses to invading pathogens involve the cells of the innate immune system, which recognizes them by groups of pattern recognition receptors including the Toll –like receptors (TLR) of which 11 different receptors have been described to date, and NOD receptors. These are differentially distributed in neonates compared to adults. When these are stimulated, cytokines are produced and induce protective responses.

For example, it has been shown that TLR-8 is a strong activator of immune responses in neonates. (28) Studies in mice investigating the effect of pre-treatment TLR ligands on outcomes of neonatal sepsis found survival increasing by 21% with TLR7/8 ligand and by 40% with a TLR4 ligand with decreased bacteraemia. (29)

Murine studies with TLR 9 ligand, CpG oligonucleotides, have shown protection from lethal Listeria infection and led to adaptive responses conferring long -term protection against Listeria. (30)
Murine and human studies have shown that BCG is recognized by some of these pattern recognition receptors. (31) (32). (33)

More recently, Kleinnijenhuis et al described that the mechanism that BCG exerts its non-specific effects is due partly to epigenetic reprogramming of cells of the innate immune system. (33)
To our knowledge no studies have been done investigating the effects of hepatitis B vaccine on neonatal innate immunity.

It is our aim to document the influence of these two birth vaccines on neonatal innate immunological responses to non-specific antigens and describe the mechanism responsible for the observed effects.

An opportunity to investigate the microbiome influencing neonatal immune responses to infection

The Intestinal Microbiome

The interaction between the intestinal microbiota and the systemic immune response is still incompletely defined. Few studies have specifically investigated how changes in the intestinal microbiota influence systemic immunity.

Measuring the systemic immune response to a vaccine is one way to evaluate immunity in the context of intestinal microbiota.

Probiotics (which temporarily alter the microbiota structure) have been shown to enhance antibody responses to oral vaccines. This includes rotavirus (with *Lactobacillus casei GG*) (34), Salmonella (with Lactobacillus GG) (35), polio (with *Lactobacillus rhamnosus GG* and *Lactobacillus acidophilus CRL431*) (36), and cholera (with multiple probiotic strains) (37). This beneficial effect was observed after a short period (1–5 weeks) of probiotic treatment prior to vaccination. This beneficial effect of probiotics on vaccine immune responses was also seen in systemically delivered vaccines against diphtheria and tetanus (with Lactobacillus F19), *Haemophilus influenzae* type B (with Lactobacillus F19) (38) and with 4 probiotic strains- *L. rhamnosus GG, L. rhamnosus LC705, Bifidobacterium breve Bbi99*, and *Propionibacterium freudenreichii ssp. Shermanii* (39), and Hepatitis B (with *Bifidobacterium longum* and *L. rhamnosus* (40) in infants after a 6-month period.

It would therefore be reasonable to suggest that optimal colonization of intestinal microbiota in the neonatal period leads to optimal immune priming at this vulnerable age.

Of great interest, it has recently been suggested that host immune responses also influence the composition of the intestinal microbiota. (41) This has led to interest in the ability of vaccines to act as immunomodulators to influence intestinal microbiota profiles.

One of the objectives of this study is to investigate whether birth vaccines have any influence over intestinal microbiome structure, which to our knowledge has never been investigated before.

The nasal microbiome

We will be collecting nasal swabs from participants and their mothers to document the normal nasal microbiome colonisation pattern. We will also investigate whether birth vaccines have any influence over the nasal microbiome structure in the first few months of life, which to our knowledge has also not been investigated before.

8. RESEARCH HYPOTHESIS AND AIM

Beyond the well-documented specific protective effect against infections, immunisations at birth also have non-specific immunomodulatory effects.

Our hypothesis is that giving an immunisation at birth changes innate immune responses to non-specific antigens.

The aim of this study is to determine the influence of BCG and Hepatitis B immunisation at birth on neonatal immune responses to non-specific antigens, by comparing infants given different immunisation regimens of these two vaccines at birth.

Our secondary hypothesis is that intestinal microbial structure is influenced by immunisations given at birth. We will be comparing intestinal microbial structure in the infants given different immunisation regimens of Hepatitis B vaccine and BCG at birth.

9. RATIONALE FOR CURRENT STUDY

Neonatal morbidity and mortality from infectious diseases is of global concern. Our current understanding of neonatal innate immunity is limited, but of growing interest as we postulate strategies of improving host defence against infection in this vulnerable population. Data from studies investigating the beneficial non-specific effects of vaccines, in particular BCG, is pointing towards a mechanism involving the innate immune system. (33) More studies in this field are needed.

Interestingly, some observational studies have reported negative non-specific effects of vaccines, including Hepatitis B vaccine. (27) We need higher levels of evidence for these observed effects, which have been noted in developing countries with high disease burden and among studies with many potential confounders.

To our knowledge the mechanism underlying these observations are as yet incompletely defined and not previously investigated in the neonatal population.

Where vaccine policy around the world differs in its recommended birth vaccination schedule, the summative effect of these vaccinations (BCG and Hepatitis B Vaccine) on innate immune responses is similarly unknown.

This study will investigate the influence of Hepatitis B and BCG vaccine on neonatal innate immune responses, in isolation as well as their summative effect. We will document these responses to non-specific antigens and investigate the underlying mechanism producing these effects.

This will improve our current understanding of the influence of vaccines on neonatal innate immunity and direct thinking towards developing strategies exploiting beneficial non-specific effects to improve resistance to infection in the very young.

In Australia, routinely, a funded dose of hepatitis B vaccine is offered to all babies at birth, but BCG vaccine is not.

Hepatitis B vaccine is offered routinely to all babies at birth to prevent vertical transmission (mother to child transmission) of Hepatitis B virus. The vaccine is effective for prevention of this transmission if given to the baby within the first week of life. All pregnant women are screened for Hepatitis B

infection during pregnancy, as the result dictates certain management for mother and infant at the time of birth. This screening test is usually done in the first trimester. A negative result in the mother negates the need for Hepatitis B vaccine for the infant for protection against vertical transmission of the virus.
There is a chance, though extremely small, that a pregnant mother may become infected with hepatitis B after her initial screening test. (eg sexual contact with a partner infected with Hepatitis B). In our study we will recheck mothers shortly after delivery of the infant for Hepatitis B infection to ensure that a late acquisition of the virus did not occur after the previous test. We may not retest the mother for hepatitis B infection if her first antenatal hepatitis B test is performed within 7 days of delivery of the infant.
If the Hepatitis B vaccine is given after the first week of life it is still effective at preventing horizontal transmission (transmission from infected household contacts) of Hepatitis B virus.

There is no Australian standard as to when BCG should be administered prior to travel other than a recommendation that it is administered at least 1 month prior to travel. Our decision is to vaccinate at birth, as this is the most common time for BCG to be administered to infants in countries where TB is endemic. There is no evidence that vaccinating at birth or at day 7 with BCG alters its efficacy against tuberculosis.

We are therefore presented with an opportunity to study these important non-specific effects of vaccines on neonatal immunity without changing standard care these infants would have received. Further to this, this study falls under the umbrella of a much larger study currently underway at the Mercy Hospital for Women, led by the same research team, also investigating aspects of neonatal innate immunity: The MIS BAIR Study. We will be utilising the same staff, recruitment and enrolment strategies. We will be using the same systems for both paper and electronic consent. The home visits will be captured using the same infrastructures currently in use. The laboratory immunological tests are the same as in MIS BAIR. We don't foresee a significant extra burden to staff.

10. STUDY OBJECTIVES

10.1. Primary Objective

To determine the influence of BCG and Hepatitis B immunisations shortly after birth on the neonatal immune responses to non specific antigens.

10.2. Secondary Objectives

To determine the immunological mechanisms underlying the non-specific effects of BCG and Hepatitis B vaccines by comparing markers of immunity.

To evaluate the effect of BCG and Hepatitis B immunisations shortly after birth on intestinal microbiome structure by analysing stool samples in the first week of life.

To evaluate the effect of BCG and Hepatitis B immunisations shortly after birth on nasal microbiome structure by analysing nasal swab samples in the first few months of life.

11. STUDY DESIGN

11.1. Type of Study

This is a randomised, controlled trial in 200 infants.

11.2. Study Design Diagram

Antenatal Clinics/ Postnatal Ward	**Study point 1**	Recruitment			
	Study point 2	Enrolment			
Postnatal ward	**Study point 3**	Maternal blood sample (5 ml of blood) (Day 0-5)			
	Study point 4	Randomisation (Day 0-3)			
		Group 1	Group2	Group 3	Group 4
	Study point 5	Infant Vaccination (Day 0-3)			
		Group 1	Group 2	Group 3	Group 4
		BCG	BCG Hep B	Hep B	Nil
		Infant stool collection*			
		Maternal and infant Nasal swab collection*			
		Make study point 7 appointment			
		Give stool specimen collection kit			
Post delivery	**Study point 6**	Confirm recent maternal negative hepatitis B test (Day 0 -7)			
Mercy Hospital (Day 7+/- 4 from study point 5)	**Study point 7**	Researcher completes questionnaire			
		Infant Blood sample (up to 5 ml)			
		Infant Stool collection*			
		Maternal and infant Nasal Swab collection*			
		Infant Vaccination			
		Group 1	Group 2	Group 3	Group 4
		(Hep B)	Nil	BCG	(Hep B) BCG
Home	**Study point 8**	Self administered maternal and infant nasal swab*			

* *If consent has been obtained*

For groups 1 and 4, if study point 7 occurs beyond 7 days of age, these babies will still be offered Hepatitis B vaccine. We have established, by checking maternal blood that these infants are not at risk of acquiring Hepatitis B infection from their mothers. However, the vaccine will be effective against horizontal transmission of the virus. This risk is also extremely low, but the parents may choose whether to vaccinate or not. (Just as all parents consent to the routine birth dose or not) These infants, regardless of whether they have had Hepatitis B vaccine or not, go on to receive the normal vaccines according to Australian immunisation guidelines. They are considered fully immunised after 3 doses at 2, 4 and 6 months of age and do not require any catch up vaccines for a missed dose at birth. (ie birth to 1 week of life)

The immune function tests we are conducting are time critical. The blood samples need processing 4 hours (+/- 10%) after collection. We will arrange transport of samples to the lab at MCRI. These samples do not require refrigeration for transport.
Due to this restriction blood samples will only be collected when there are members of the laboratory research team available to process the samples.
This will not limit the ability for us to analyse our primary outcome (immunological differences between the 4 groups at 7 days of life)

11.3. Number of Subjects

Up to 300 healthy infants. Allows for a minimum of 50 participants in each group with complete samples and results.

11.4. Expected Duration of Study

Recruitment of study participants will occur over one-year period from March 2015.
The duration of each participant's individual participation in the study will be up to 14 days, unless consent for nasal swab collection has been obtained, which extends this to up to 3 months.

12. OUTCOME MEASURES

All outcome measures will be compared between the four groups.

12.1. Primary Outcome Measures

The cytokine response to *in vitro* stimulation with a range of antigens, including killed pathogens, at 7-days (+-4) post vaccination.
Four hours (+/- 10% or 24minutes) after samples are collected they will be stimulated with different concentrations of infective antigens (e.g. killed *S. aureus, E. coli, S. pneumoniae*, RSV, Rotavirus, group B streptococcus, *C. albicans*,) for twenty hours (+/- 10% or 2 hours). Cytokine expression will be analysed in supernatants by *Luminex*-based multiplex assays (IFN□γ□□TNF-α, IL-1 β, IL-6, IL-10, IFN-α, IL-4, IL-12, IL-17, IL-18).

12.2. Secondary Outcome Measures

Other *in vitro* measures of the immune response including flow cytometry, transcriptomics and proteonomics.

The analysis of the microbiome will be done using non-culture dependent sequencing methods, in order to identify which bacteria are present in each sample. This will be done by collaborators. The analyses methods used for this aspect of the project will be confirmed once the final methodology has been decided.

13. STUDY TREATMENTS

13.1. Treatment Arms

The vaccines, which will be evaluated, are the BCG Vaccine SSI and the Hepatitis B vaccine. Study participants will be randomised in a 1:1:1:1 ratio to one of four treatment arms: group 1, group 2, group 3 or group 4. Those participants randomised to group 1 and 2 will receive a single dose of BCG

vaccine within 3 days of birth. Those randomised to group 2 an 3 will receive Hepatitis B vaccine within 3 days of birth.

13.2. Study drugs

BCG Vaccine SSI and Hepatitis B vaccine

13.2.1. Descriptions

The **BCG Vaccine SSI** is a freeze-dried (lyophilised) live vaccine prepared from an attenuated strain of Mycobacterium bovis. It will be supplied in multi-dose vial and reconstituted prior to vaccination. Unreconstituted (lyophilised) vaccine must be stored at + 2°C to +8°C and protected from light. Reconstituted vaccine must be stored at 2°C to +8°C and discarded 4 hours after reconstitution.

A label, which complies with the requirements of Annex 13 of the Australian Code of Good Manufacturing Practice (GMP) for Medicinal Products, will be affixed to each vial of study vaccine.

The **Hepatitis B vaccine** is a recombinant DNA vaccine. It is formulated as a monodose vial or prefilled syringe.

13.2.2. Dosage and Route of Administration

Study participants randomised to all groups will receive a single 0.05 mL intradermal injection of BCG Vaccine SSI over the distal insertion of the deltoid muscle approximately one third down the upper left arm as follows:

The skin is stretched between thumb and forefinger;

The needle should be almost parallel with the skin surface and slowly inserted (bevel upwards), approximately 2 mm into the superficial layers of the dermis;

The needle should be visible through the epidermis during insertion;

The injection is given slowly;

A raised, blanched bleb is a sign of correct injection;

The injection site should be left uncovered to facilitate healing.

Study Participants in groups 2 and 3 will receive an intramuscular dose of 0,5ml of Hepatitis B vaccine (H-B- Vax II) containing 5 micrograms of Hepatitis B surface antigen protein.

Participants in group 4 will receive BCG prior to Hepatitis B vaccine (should the parents choose to) to decrease pain as evaluated in a previously published study.(42)

13.2.3. Preparation and Administration of Study Drug

The investigator or delegate will be responsible for administration of the BCG and Hepatitis B vaccine to all participants in accordance with the provisions of this protocol and applicable NH&MRC guidelines. Only designated trained members of the research team or clinical staff will administer vaccines.

BCG Vaccine SSI should be administered with a syringe of 1 mL subgraduated into hundredths of a mL fitted with a short bevel needle of 25G/0.50 mm or 26G/0.45 mm.

13.2.4. Dispensing and Product Accountability

BCG and Hepatitis B vaccine will be stored in the pharmacies of the participating hospitals using standard cold chain precautions. An additional small supply will be stored in the RCH pharmacy or laboratory in logged/alarmed fridges. The vaccines will be dispensed only to designated trained members of the research team or clinical staff.

The investigator or delegate will maintain accurate records of the receipt of the study vaccines. In addition, for BCG, accurate records will be kept regarding the reconstitution and administration of each dose of vaccine.

At the end of the study, there will be a final reconciliation of study vaccines received, reconstituted, administered and destroyed. Any discrepancies will be investigated, resolved and documented by study personnel. Unused vials of study vaccine will be donated to the Pharmacy Department of the RCH, Melbourne for non-study use.

13.2.5. Excluded medications and treatments

None.

14. SUBJECT ENROLLMENT AND RANDOMISATION

14.1. Recruitment

Pregnant women, attending antenatal clinics or women who have delivered an infant vaginally at the Mercy Hospital for Women in Melbourne will be approached by specially-trained members of the research team who will give them information, in the form of the parent information and consent form, about the study and explain what participation involves. After the initial conversation with a member of the research team, the parents or legally acceptable representative (LAR) will be given the opportunity to ask any questions. The research team will answer any question about the study and then ask the woman to consider their infant's involvement. The copy of the information statement will be provided electronically (eg email) or in paper form.

Additional methods of recruitment, including print articles, may also be used as required (ethics approval will be sought prior to their use).

If they agree to their infant's participation, the infant's LAR and/mother will sign and date an informed consent form prior to any study assessments/procedures being conducted. The consent form will describe the purpose of the study, the procedures to be followed and outline the risks and benefits of participation. The member of the research team will ensure that the infant's LAR and/mother understands the information provided and has had the opportunity to direct any questions to the investigator. Consent will be voluntary and free from coercion.

The member of the research team that conducted the informed consent discussion will also sign an electronic or paper consent form. A copy of the consent form will be given to the participant's LAR and/mother. If paper consent forms are used, they will be kept in a locked filing cabinet and will only be accessible by the researchers. The paper consent forms will also be scanned and uploaded into the participant's (password-protected) REDcap record. See appendix B regarding the process of electronic consent. Though participants can choose whether to give electronic or paper consent, we would encourage electronic consent as this has advantages over paper consent.
These advantages include:

- Electronic format reduces the risk of lost documents
- Centralised storage decreases time to access document
- Readily accessible anywhere, anytime on REDCap
- Access is password protected on REDCap, less likely to inadvertently being seen by non-researchers
- Indefinite lifespan of the document (no fade or degradation with time)
- Space saving/ decrease storage space requirements
- Families have an electronic copy (same advantages)
- Proof that families were provided with a copy (the electronic trail)
- No paper, no ink. Cheaper and more environmentally friendly.

14.2. Randomisation

Randomisation will take place after the LAR and/mother has provided consent to participation in the study within 3 days of the infant's birth, provided adequately trained staff are available to carry out the randomisation procedure.

Researchers involved in the randomisation will be trained in the use of the REDCap randomisation function in addition to the other aspects of the study. The REDCap function prompts staff to answer

simple questions pre-randomisation to ensure eligibility and automatically completes the random group allocation. It alerts staff if a participant is no longer eligible. Researchers will not be given REDCap access until they have been adequately trained. Post randomisation, staff will be prompted once again to answer questions to ensure eligibility and access the group to which the participant has been randomised.

15. ELIGIBILITY CRITERIA

15.1. Inclusion Criteria

An infant must meet all of the following criteria in order to be eligible for inclusion in the study:

- Less than 3 days old;
- Planned travel to a TB endemic area in the first 5 years of life;
- English speaking mother;
- An informed consent form must be signed and dated by the infant's mother after the nature of the study has been explained and prior to any study assessments/procedures.
- The infant's mother has screened negative for HIV and Hepatitis B during this pregnancy (routinely performed during the antenatal period)
- There is no known household contact infected with Hepatitis B
- Born no earlier than eight weeks before estimated date of delivery
- Birth weight >1500g
- Delivered vaginally
- Singleton pregnancy

15.2. Exclusion Criteria

An infant meeting any of the following criteria will be excluded from study participation (including contraindications to receiving BCG as per the NH&MRC Immunisation Handbook (10th edition, 2013)):

- Treatment with corticosteroids or other immunosuppressive therapy, including monoclonal antibodies against tumour necrosis factor-alpha (TNF-alpha) (e.g. infliximab, etanercept, adalimulab);
- Born to a mother treated with bDMARDS (e.g. TNF-alpha blocking monoclonal antibodies) in the 3rd trimester;
- Congenital cellular immunodeficiencies including specific deficiencies of the interferon gamma pathway;
- Malignancies involving bone marrow or lymphoid systems;
- Serious underlying illness including severe malnutrition;
- Medically unstable;
- Generalised septic skin disease and skin conditions such as eczema, dermatitis and psoriasis;
- Significant febrile illness;
- Received Hepatitis B vaccine prior to randomisation
- Received BCG vaccine prior to randomisation

Also excluded are infants with:

- a mother who is immunosuppressed;
- a mother who has received Intravenous immunoglobulins during her pregnancy;

- a family history of immunodeficiency;
- consanguineous parents;
- a mother who is having a planned Caesarean Section

16. RANDOMISATION PROCEDURES

Study participants will be randomly assigned to one of 4 groups within 3 days of birth.

A statistician not directly involved in the analysis of the study results will prepare the randomisation schedule. A web-based computerised system (REDcap) designed and administered by the Clinical Epidemiology & Biostatistics Unit (CEBU) at the RCH will be used to ascertain each infant's allocation.

Study personnel will randomise eligible study participants by connecting to the CEBU web-based system online and providing the relevant participant identification details including initials and ID number. The participant ID number will consist of a sequential four-digit number representing the participant's order of enrolment at the site; i.e. in the format 3001. The CEBU web-based system will then state the participant's study group allocation, and will store an electronic record of the randomisation.

17. STUDY PROCEDURES SCHEDULE POST ENROLMENT

Visit 1 (Study point 3, 4 and 5)	**Day 0 -3 post delivery**	
	Mother	**Baby**
	Blood sample (up to 5ml) [a]	Randomisation to one of 4 groups
		Vaccination of Group 1,2 and 3
		Collect stool sample*
	Nose swab collection*	Nose swab collection*
Visit 2 (Study point 7)	**7 days from visit 1**	
	Mother	**Baby**
	Interviewed	Blood sample (up to 5ml)[b]
		Vaccination group 1, 3 and 4
		Collect stool sample*
	Nose swab collection*	Nose swab collection*

[a] To check for Hepatitis B infection [b] Optional consent for epigenetic analysis
* Optional sample collections

BCG is indicated for all babies recruited as they plan to travel to a TB endemic country. It is recommended that the vaccine be given at least 1-month prior to travel. Hepatitis B vaccine is routinely given to all babies in Australia between 0-7 days of life. This is to protect the infant from possible infection from an (unknowingly) infected mother. We will try not to deviate from giving Hepatitis B vaccine to these infants within this time frame, even though we will exclude every infant who we cannot confirm whether his/her mother is infected with Hepatitis B or not. This is done by testing all mothers for Hepatitis B infection. If the first antenatal hepatitis B test occurs prior to 7 days before delivery of the infant, we will retest the mothers within 5 days of delivery (likely within 3 days), which acts as a second precautionary test for infection in mothers enrolled in this study.

18. LABORATORY ASSESSMENTS

18.1. Blood analysis

Innate and adaptive immune responses will be assessed initially by cytokine analysis. The remaining sample will be stored until we analyse these results.

Based on the results of the cytokine analysis we will potentially analyse other measures of the immune response using flow cytometry, transcriptomics, proteomics or metabolomics.

The genes in DNA act as instructors on how to build proteins. When a certain protein is needed, a copy of the gene on the DNA is made, which is called RNA. When we extract RNA from blood cells, we analyse which genes are actively in use at that time. This is called transcriptomics. We will undertake transcriptomic analysis to help understand the molecular mechanisms underlying the immunomodulatory effects of BCG and Hepatitis B vaccine and other factors.

Where consent is obtained, we will extract DNA from the baby's blood samples. We will investigate features in participants' DNA that might be associated with the immune response to BCG or Hepatitis B vaccination or other factors.

The DNA tests will include single nucleotide polymorphism (SNP) analyses that will be used only for research purposes. There will be no attempt to genotype individuals for SNPs associated with high-risk disease (such as those underpinning monogenetic disorders like Cystic Fibrosis, cancer risk etc.) The information obtained from the SNP analyses will not be clinically relevant.

DNA has slight chemical modifications that are not associated with changes in the DNA sequence but which can alter the way genes are expressed. These are called epigenetic changes. They may be caused by environmental factors. We will investigate epigenetic changes that may allow us to understand how BCG and Hepatitis B vaccine affects infant's immunity.

The genetic analysis of DNA and RNA that we are doing is for research purposes only and the significance of the results relating to the immune system is unknown. Therefore we will not be providing individual results to participants.

Epigenetic analysis is an optional part of the study.

18.2. Stool analysis

Microbial composition will be assessed using non-culture dependent sequencing methods.

Parents will be given a pack containing stool specimen collection containers and an information sheet on how to collect the sample. They will be asked to place an amount of stool into the container when the infant has a dirty nappy. Parents can choose to collect samples as often as they feel they can. eg every dirty nappy or 1 sample every day or 1 sample in total. They will be provided with a plastic container in which to deposit these stool specimens. This container can be placed in their home freezer till collection by our research staff at the second visit. These samples will be placed in a cool environment for transport to the laboratory. This part of the study is optional. See Appendix C at the end of this document.

Researchers will collect these samples form the parent and transport it to the MCRI lab on a frozen ice brick. Once at the lab sample containers will be placed into the minus 80-degree freezer till ready for processing.

18.3. Nasal swab analysis

Microbial composition will be assessed using non-culture dependent sequencing methods.

19. ADVERSE EVENT REPORTING

19.1. Definitions

19.1.1. Adverse Event (AE):

Any untoward medical occurrence in a participant enrolled in this study regardless of its causal relationship to study treatment.

19.1.2. Serious Adverse Event (SAE)

Adverse events are classified as serious or non-serious.

An SAE is defined as any AE that:

1) Results in death; or
2) Is immediately life-threatening; or
3) Requires inpatient hospitalisation; or
4) Results in prolongation of existing hospitalisation; or
5) Results in persistent or significant disability/incapacity

Significant medical events will be considered an SAE when, based upon appropriate medical judgment, they may jeopardise the participant and may require medical or surgical intervention to prevent one of the outcomes listed above.

19.1.3. Suspected Unexpected Serious Adverse Reaction (SUSAR):

A SUSAR is any SAE that is both suspected to be related to the study treatment and is unexpected (that is, not consistent with the applicable product information).

19.2. Assessment and Documentation of Adverse Events

For the purposes of this study the investigator or delegate will be responsible for recording AEs regardless of their relationship to the study vaccines, with the following exceptions:
Conditions that are pre-existing at enrolment and do not deteriorate will not be considered AEs.
Abnormal laboratory values will not be considered AEs unless deemed clinically significant by the Investigator and documented as such.

The documentation of each AE on the CRF will include:

- A description of the AE;
- The onset date, duration and date of resolution;
- Severity (mild, moderate, severe);
- Seriousness (SAE or not);
- Any action taken;
- The outcome;
- The relationship to the study vaccine (Unrelated, Possible, Probable, Definite).

The severity of an AE and its causal relationship to the study vaccines will be assessed in accordance with the provisions of Appendix A.

Changes in the severity of an AE will be documented.

All AEs will be followed to resolution, where possible.

19.3. Eliciting Adverse Event Information

The period of observation for AEs will extend for the period between vaccination at study point 5 and the 7-day visit at 7 (+/- 4days) from study point 5. After this the parent will have access to a telephone number and email address to contact a member of the research team to inform of a suspected adverse event up to 3 months post immunisations. (Study point 5 and/7).

19.4. Serious Adverse Event Reporting

An independent safety monitoring committee will be established despite the expected low risk of adverse events in this study. It will meet 6 monthly. This will comprise:

- Statistician
- Neonatologist
- Infectious diseases or vaccine physician

The role of this committee will be to review the SAEs and SUSARs

19.4.1. SAEs

Any SAE occurring in a study participant will be reported to the RCH HREC. This will occur using the RCH periodic SAE form and will be submitted in the unlikely event that it should occur. Any SAEs suspected to be related to participation in the study will be highlighted to the HREC by the principle investigator or delegate. The HREC specific safety reporting form will be completed signed and submitted by the investigator or delegate.
Any SAE occurrence in a participant during their initial admission at the Mercy, will be reported within 72 hours of a member of our research team being notified (or being made aware of) the event in accordance with the reporting policy of the HREC.

19.4.2. SUSARs

All SUSARs occurring in a study participant will be reported to the hospital HREC and will be notified within 72 hours of occurrence, in accordance with the safety reporting policy of the HREC.

Experimental Drugs section, Drug Safety and Evaluation Branch of the TGA within 15 calendar days of first knowledge for non-fatal events and 7 calendar days for fatal or life-threatening events. The investigator or medically qualified delegate will complete, sign and submit the SUSAR form.

20. STATISTICAL METHODS

20.1. Sample Size Estimation

The chosen sample size is in keeping with similar published immunological studies by us and other groups. Based on the results of cytokine assays from previous infant studies, a sample size of 50 infants in each group will have 80% power to detect an effect size of 0.634 using a two group t-test log- transformed data with a 0.05 two-sided significance level. We expect that the data may be too skewed to perform log-transformed analyses and that the analysis will have to be based on non-parametric methods. The power of the study may therefore be somewhat less, but it is not possible to estimate this accurately.

Likely recruitment: As there are ~1000 babies born each year at MHW who meet the eligibility criteria for our study, we need only 20% recruitment.

20.2. Population to be analysed

All randomised participants with outcome data will be included in the analysis.

20.3. Statistical analysis:

Blood will be stimulated with 16 different antigens in separate analyses. We will evaluate 17 different cytokine responses for each antigen. For each of these 17 cytokines, for each of the 16 antigens, the non-parametric equality of medians (Kruskal- Wallis) test will be used to assess the null hypothesis that median cytokine concentration is the same in the four groups.

For each cytokine and antigen, if there is evidence for a difference between the four groups, the following pair-wise comparisons will be done using Mann Whitney U tests:

- Group 3 with Group 4.
    - i.e. what is the effect of a birth dose of Hepatitis B vaccine on innate immune responses? This is currently the Australian birth immunisation practice.
- Group 1 with Group 4.
    - i.e. what is the effect of a birth dose of BCG on innate immune responses? This will allow comparison with an international study (clinicaltrial.gov identifier number NCT01694108)
- Group 2 with Group 3.
    - i.e. What is the effect of a birth dose of BCG on the immune response of babies vaccinated with Hepatitis B?
- Group 2 with Group 4.
    - i.e. What is the cumulative effect of these 2 vaccines on immune responses?

		Hepatitis B vaccine at birth	
		+	-
BCG vaccine at birth	+	Group 2	Group 1
	-	Group 3	Group 4

Given the large number of comparisons, p values will be evaluated conservatively.

21. INTERIM ANALYSES

No interim analyses are planned.

22. DATA MANAGEMENT

22.1. Data Collection

The Principal Investigator or delegate will maintain an individual file and source documentation for every participant enrolled in the study. The file will document the participant eligibility as well any serious adverse reactions experienced and other notes as appropriate. The participant file will be

kept up to date so that it reflects the latest observations on the participant. The original, signed Parent/LAR Information Statement (P/GIS) will be filed with each participant's file. Any paper documents will be filed and kept in a locked cupboard that is only accessible to the research team.

22.2. Data Storage

All study information collected by study personnel will be entered into an electronic database. Access to the database will be restricted by unique password and username. Data will be stored in a re- identifiable form.

22.2.1. Study Record Retention

Data will be stored securely until the youngest participant has turned 25 years old or 15 years after the last study publication, whichever is longest.

23. ADMINISTRATIVE ASPECTS

23.1. Confidentiality

Participant confidentiality will be strictly held in trust by the investigator, study personnel, the Murdoch Children's Research Institute (MCRI) and their agents. This confidentiality is extended to cover testing of biological samples. The study protocol, documentation, data and all other information will be held in strict confidence. No information concerning the study or the data will be released to any unauthorised third party, without prior written approval of the sponsoring institution. Authorised representatives of the MCRI may inspect all documents and records required to be maintained by the Investigator, including but not limited to, medical records and pharmacy records for the participants in this study. The investigator will permit access to such records. Any laboratory specimens, evaluation forms, reports or other records that leave the study site will be identified only by the participant identification number in order to maintain participant confidentiality. Participant information will not be disclosed without written permission of the participant's parent/legal representative, except as necessary for monitoring by the relevant HREC or regulatory authorities.

23.2. Independent HREC Approval

This protocol and the informed consent documentation and any subsequent modifications will be reviewed and approved by the relevant Human Research Ethics Committee (HREC). A letter of protocol approval by the HREC will be obtained prior to the commencement of the study, as well as approval for other study documents subject to HREC review.

23.3. Modifications of the Protocol

This study will be conducted in compliance with the current, ethically approved version of the protocol. Any change to the protocol or informed consent documentation that affects the scientific intent, study design, participant safety, or may affect the willingness of a parent/LAR to continue to allow their child's participation in the study will be considered an amendment and will therefore be prepared and filed as an amendment to this protocol and/or informed consent documentation, all such amendments will be submitted to the relevant HREC for approval prior to becoming effective.

23.4. Protocol Deviations

The investigator and study personal will exercise due diligence to avoid protocol deviations. However, any protocol deviations will be recorded in the participant's source document and CRF. Any deviations deemed to have a potential impact on the integrity of the study results, participant safety or the ongoing ethical acceptability of the study will be report to the relevant HREC within the specified timelines.

All significant protocol deviations will be documented in the Clinical Study Report (CSR). Where deviations to the protocol identify issues for protocol review, the protocol will be amended in accordance with the provisions outlined in section 12.3.

24. USE OF DATA AND PUBLICATIONS POLICY

Any publication or presentation of the study results will be in accordance with the revised NH&MRC policy for the dissemination of research findings, which came into effect on 01 July 2012. Published data will summarise group outcomes only. Individual study participants will not be identified.

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26. APPENDICES

APPENDIX A
Causality of Serious Adverse Events (SAEs)

The relationship of serious adverse events to the BCG or Hepatitis B vaccines will be assessed as follows:

- **Unrelated**:

There is no association between the BCG vaccine or Hepatitis B vaccine and the reported event. SAEs in this category do not have a reasonable temporal relationship to exposure to the test product, or can be explained by a commonly occurring alternative aetiology.

- **Possible**:

The BCG vaccine or Hepatitis B vaccine could have caused or contributed to the SAE. SAES in this category follow a reasonable temporal sequence from the time of exposure to the vaccine and/or follow a known response pattern to the vaccine, but could also have been produced by other factors.

- **Probable:**

The association of the SAE with the BCG vaccine or Hepatitis B vaccine seems likely. SAEs in this category follow a reasonable temporal sequence from the time of exposure to the vaccine and are consistent with the known mode of action of the vaccine, known or previously reported adverse events to the vaccine, or judgment based on the investigators clinical experience.

- **Definite:**

The SAE is a consequence of administration of the BCG vaccine or Hepatitis B vaccine. SAES in this category cannot be explained by concurrent illness, progression of disease state or concurrent medication reaction. Such SAEs may be widely documented as having an association with BCG vaccine or Hepatitis B vaccine, or occurring after rechallenge.

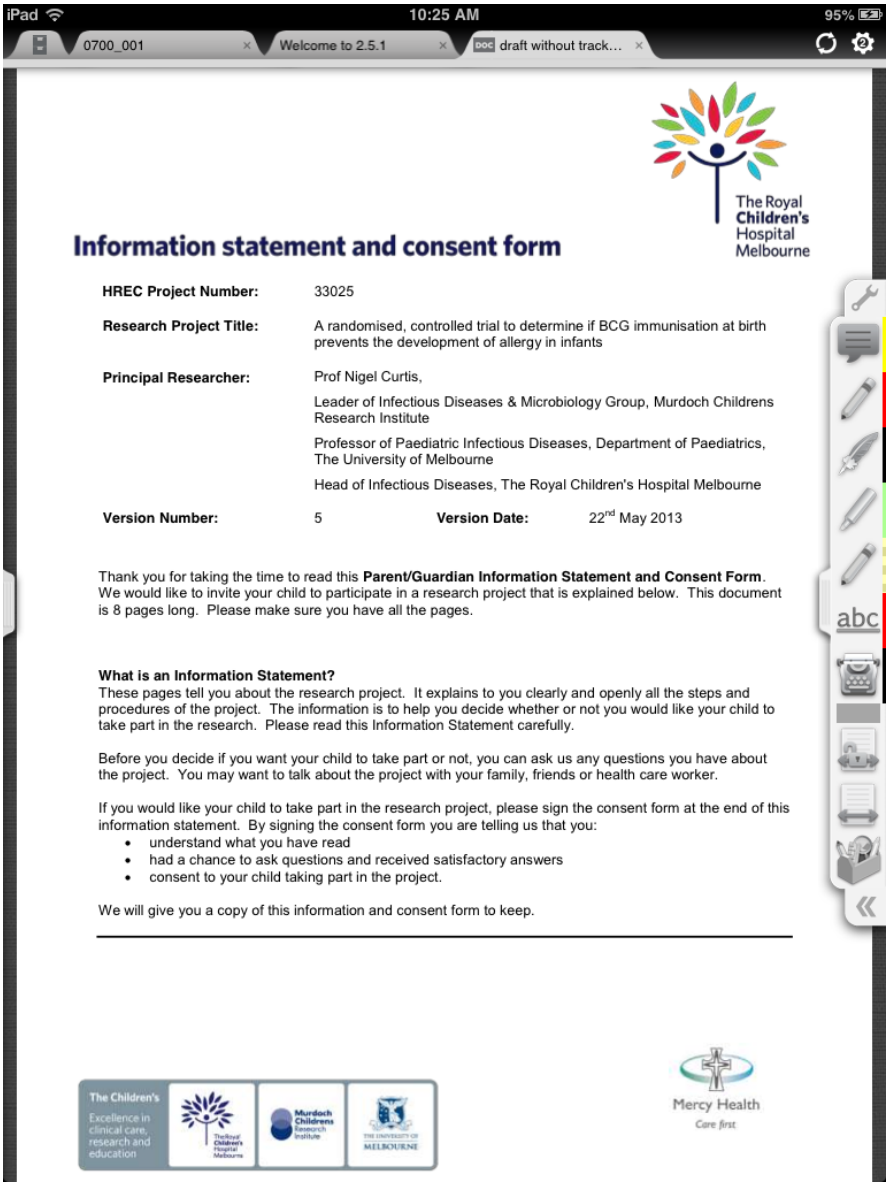
APPENDIX B
Obtaining signed consent using an iPad

The process for using an iPad to obtain families electronic consent is as follows;

1.1 The parent's/LAR's will be provided all information required for them to provide informed consent as per the National Statement on Ethical Conduct in Human Research (2007).

1.2 After the initial conversation with a member of the research team, the parent or LAR will be given the opportunity to read the information statement and consent form and ask any questions. They will be provided with a copy of the information statement electronically (e.g. email) or in paper form. This will enable them time to discuss the study with others before making an informed choice about participating in the study.

1.3 Once they have had time to consider participating, if they would like to provide informed consent they will be given access to a study iPad to view the document and sign in the presence of a researcher. (See photo)

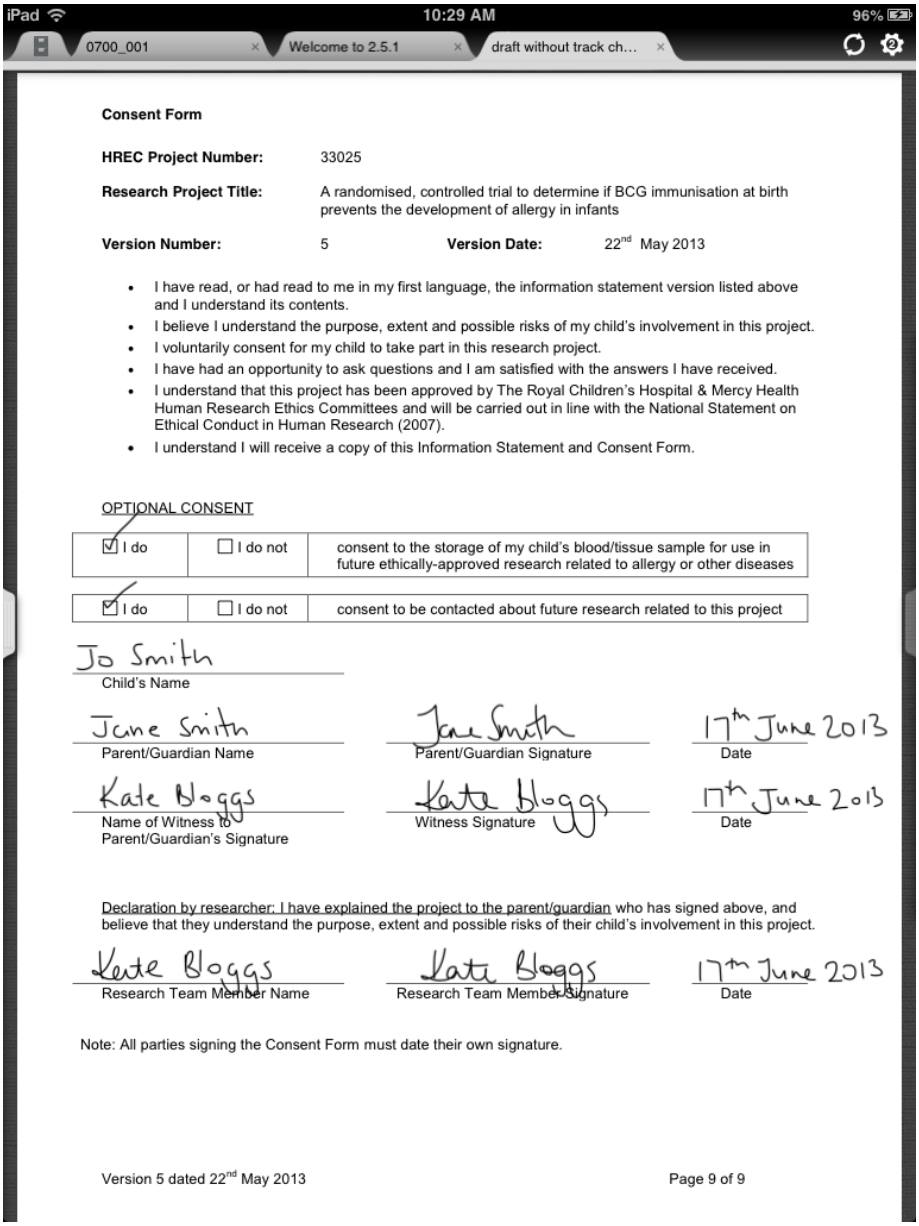


1.4 Whilst with a member of the research team, they will sign the PDF file directly onto the document using a stylus pen using the iPad application *iAnnotate*. A new file will

be created for each consent form and will be labelled with the participants study number.

1.5 There is no added risk for taking a copy of any signatures on the form greater than the normal possibility of scanning a form into PDF format.

1.6 The email address for the parent or LAR will be obtained at the time of consent, and checked with them prior to sending the form to ensure that it is sent to the correct address.



iPad 10:29 AM 96%

0700_001 | Welcome to 2.5.1 | draft without track ch…

Consent Form

HREC Project Number: 33025

Research Project Title: A randomised, controlled trial to determine if BCG immunisation at birth prevents the development of allergy in infants

Version Number: 5 **Version Date:** 22nd May 2013

- I have read, or had read to me in my first language, the information statement version listed above and I understand its contents.
- I believe I understand the purpose, extent and possible risks of my child's involvement in this project.
- I voluntarily consent for my child to take part in this research project.
- I have had an opportunity to ask questions and I am satisfied with the answers I have received.
- I understand that this project has been approved by The Royal Children's Hospital & Mercy Health Human Research Ethics Committees and will be carried out in line with the National Statement on Ethical Conduct in Human Research (2007).
- I understand I will receive a copy of this Information Statement and Consent Form.

OPTIONAL CONSENT

☑ I do	☐ I do not	consent to the storage of my child's blood/tissue sample for use in future ethically-approved research related to allergy or other diseases
☑ I do	☐ I do not	consent to be contacted about future research related to this project

Jo Smith
Child's Name

Jane Smith
Parent/Guardian Name

Jane Smith
Parent/Guardian Signature

17th June 2013
Date

Kate Boggs
Name of Witness to Parent/Guardian's Signature

Kate Boggs
Witness Signature

17th June 2013
Date

Declaration by researcher: I have explained the project to the parent/guardian who has signed above, and believe that they understand the purpose, extent and possible risks of their child's involvement in this project.

Kate Boggs
Research Team Member Name

Kate Boggs
Research Team Member Signature

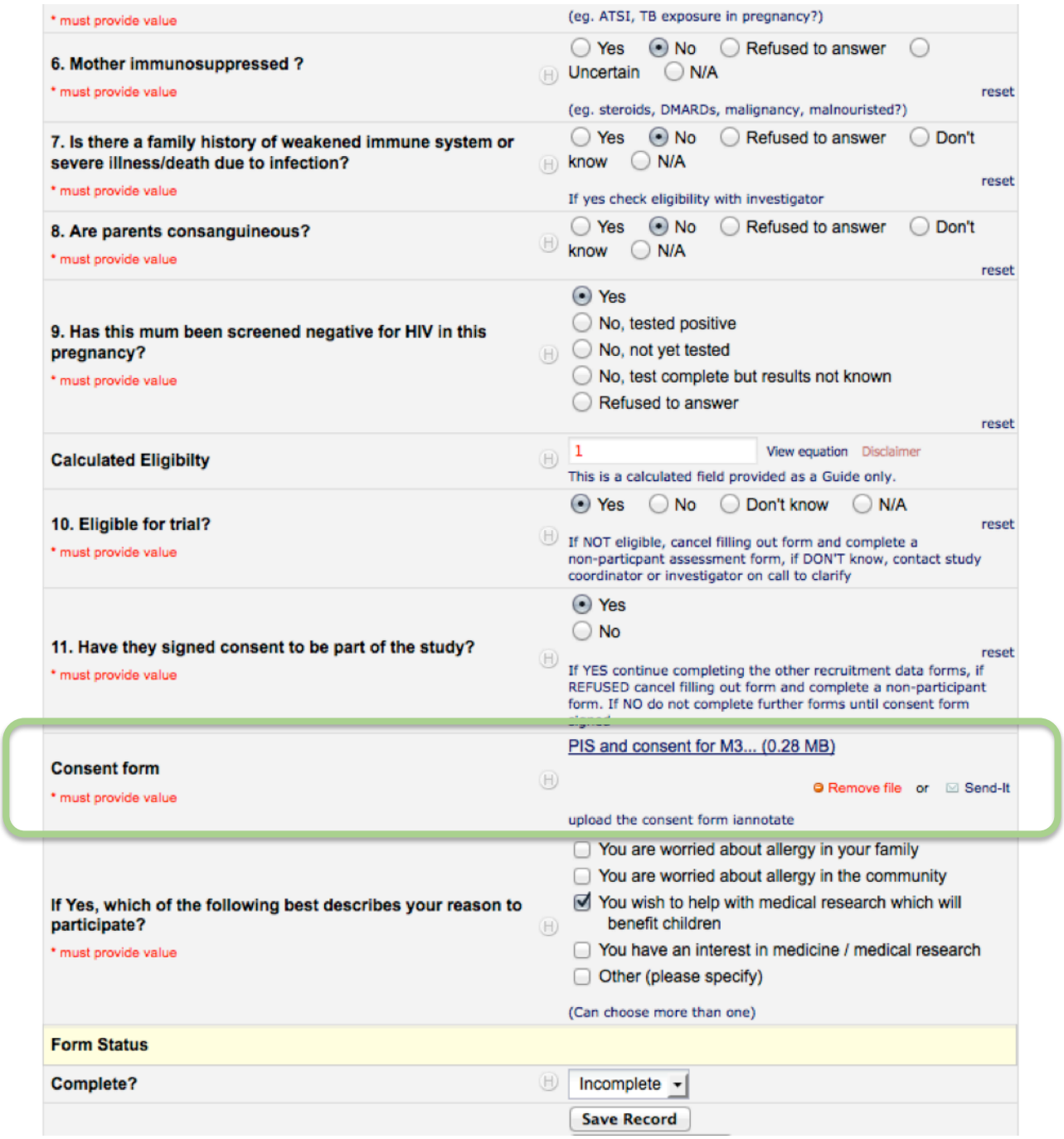
17th June 2013
Date

Note: All parties signing the Consent Form must date their own signature.

Version 5 dated 22nd May 2013 Page 9 of 9

1.7 Once the form is signed by; the parent or LAR, witness and researcher, the signed form will be emailed directly to the parent or LAR, and study coordinator from a RCH or MCRI email account. If the LAR requests a paper copy of the signed parent/participant information consent form (PICF), they will be provided with a printed copy.

1.8 The study coordinator will then load the file directly into the participant's REDcap record where it will be stored. (See photo below)



1.9 Version control of the electronic version of the PICF will be no different from current paper copy documents. The date and version numbers are on the front page of the PIS, the consent form and the footer of each.

APPENDIX C

PARENT INSTRUCTIONS FOR COLLECTION OF INFANT STOOL SAMPLES

Equipment Required and Supplied

- Pre-labelled specimen jars x7
- Biohazard bag x7
- Pen

Procedure

Infant stool samples are to be collected daily, or as often as possible, from birth to 7 days of age.

1. Wash your hands well with soap and dry thoroughly before taking the sample
2. Lay the nappy out flat

3. Open the specimen jar

4. Use the built in scoop in the lid to collect a small amount of stool (about the size of a twenty cent coin) from the nappy

5. Discard the rest of the stool with the nappy in the usual way
6. Place the scooped stool sample into the specimen jar

7. Firmly screw the lid closed

8. Label the specimen jar with the your study ID, date and time
9. Wash your hands well with soap and dry thoroughly
10. Place the specimen jar in the biohazard bag and store in the freezer.

If you have any questions about the collection of samples please call a member of Research team on 03 9926 6042 or 0409 846 988 (Monday-Friday (9am till 5pm)