Investigating the Non-Specific Effects of BCG in Neonates

Sarah Prentice

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Research group affiliations: The Co-Infection Studies Programme, MRC/Uganda Virus Research Institute, Uganda.

The Dockrell Group, Department of Immunology and Infection, LSHTM.

I, Sarah Prentice, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Abstract

Animal models, epidemiological studies and a small number of randomised controlled trials suggest that BCG might protect infants against diseases other than tuberculosis. The hypothesis remains contentious because a mechanism to explain such protection has not been proven in infants. Adult studies suggest that BCG acts via epigenetic modifications to 'train' the innate immune system, enhancing its pro-inflammatory cytokine response to non-tuberculous pathogens. This thesis describes two randomised controlled trials, in Uganda and The Gambia, of early vs. delayed BCG vaccination in neonates. These explored the impact of BCG on the innate immune system through; 1) histone modifications at the promoter region of pro-inflammatory cytokines, 2) *in vitro* pro-inflammatory cytokine production following non-specific stimulation and 3) the inflammatory-iron axis response following *in vivo* heterologous stimulation. Clinical data were collected to explore the global applicability of the non-specific effects of BCG.

These studies showed that infants BCG vaccinated at birth had significantly reduced all-cause infectious disease incidence in the first 6 weeks of life compared to infants who had not received BCG (Incidence Rate Ratio 0.71 95%CI (0.53-0.95)). This was particularly pronounced in male infants (IRR 0.57 (0.36-0.88)). A corresponding trend toward reduced H3K4me3 (stimulatory) and H3K9me3 (inhibitory) epigenetic modification at the promoter region of pro-inflammatory cytokines in PBMCs collected at 6 weeks of age from BCG vaccinated infants was demonstrated. This was most significant for H3K9me3 at the TNFα promoter region (p=0.001), suggesting a potential for greater cytokine production in response to heterologous pathogen challenge. Pro-inflammatory cytokine concentrations following *in vitro* and *in vivo* non-specific stimulation were significantly increased in BCG vaccinated male infants at the 6 week time-point subsequent to receipt of Expanded Programme of Immunisation vaccinations. This thesis, therefore, provides strong evidence for a beneficial non-specific effect of BCG in healthy neonates, likely mediated through epigenetic training of the innate immune system.

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1. Glossary

A	ALRI	Acute lower respiratory tract infection
B	BCG	Bacille Calmette Guérin
	BD	Becton Dickinson
	BSA	Bovine serum albumin
C	CD	Cluster differentiation
	CFR	Case fatality rate
	Cfu	Colony forming units
	ChIP	Chromatin immunoprecipitation
	CpG ODN	CpG oligodeoxynucleotides
	CRF	Case report form
	CSF	Cerebro-spinal fluid
	CuI	Cumulative incidence
	CV	Coefficient of variance
D	DHS	Demographic health survey
	DNA	Deoxyribonucleic acid
	DSMB	Data safety monitoring board
	DTH	Delayed type hypersensitivity
	DTP	Diptheria-tetanus-pertussis
	DTwP-Hib-HepB	Diptheria, tetanus, whole cell pertussis, *Haemophilus influenza* and hepatitis B vaccination (aka 5-in-1)
E	EDTA	Ethylene diamine tetraacetic acid
	EGF	Epidermal growth factor
	EGTA	Ethylene glycol-bis(β-aminoethyl ether) tetraacetic acid
	ELISA	Enzyme linked immunosorbant assay
	EPI	Expanded programme of immunisations
	EV	Ectromelia virus
F		
G	GBS	Group-B Streptococcus
	GMCSF	Granulocyte-macrophage colony stimulating factor
	GMR	Geometric mean ratio
	GPS	Global positioning system

H

H3K4me3	Histone 3 lysine 4 trimethylation
H3K9me3	Histone 3 lysine 9 trimethylation
Hb	Haemoglobin
HCL	Hydrochloric acid
HCT	Haematocrit
HEPES	4-(2-hydroxyethyl)-1-piperazneethanesulfonic acid
Hib	*Haemophilus influenza* type b
HIV	Human immunodeficiency virus
HK	Heat killed
HR	Hazard ratio
HRP	Horseradish peroxidase
HSV	Herpes simplex virus

I

ID	Intradermal
IFN	Interferon
Ig	Immunoglobulin
IO	Intraoccular
IP	Intraperitoneal
IM	Intramuscular
IN	Intranasal
IR	Incidence rate
IRR	Incidence rate ratio
IV	Intravenous

J

K

L

LBW	Low birthweight
LPS	Lipopolysaccharide
LRTI	Lower respiratory tract infection
LSHTM	London School of Hygiene and Tropical Medicine
LTBI	Latent tuberculosis infection

M

MCH	Mean cell haemoglobin
MCHC	Maternal and child health clinic
MCHC	Mean cell haemoglobin concentration
MCP	Monocyte chemoattractant protein
MCV	Mean cell volume
MIF	Macrophage inhibitory factor
MIP	Macrophage inflammatory protein
MR	Mortality rate
MRI	Magnetic resonance imaging

	MRR	Mortality rate ratio
	MRC/UVRI	Medical Research Council/Uganda Virus Research Institute
	MS	Multiple sclerosis
	MTB	Mycobacterium tuberculosis
N	NaCl	Sodium chloride
	$NaHCO_3$	Sodium bicarbonate
	NDA	National Drugs Authority
	NG	Nasogastric
	NK	Natural killer
	NOS	Not otherwise specified
	NSE	Non-specific effects
O	OFC	Occipito-frontal circumference
	OPV	Oral polio vaccine
	OR	Odds ratio
P	PBMC	Peripheral blood mononuclear cell
	PCR	Polymerase chain reaction
	PCV10	Pneumococcal conjugate vaccine 10-valent
	PDGF-AB/AA	platelet derived growth factor-AB/AA
	PHA	Phytohaemaglutinin
	PI	Principal investigator
	PIC	Protein inhibitor complex
	Poly I:C	Polyinosinic:polycytidylic acid
	PPD	Purified protein derivative
Q		
R	RBC	Red blood cells
	RCT	Randomised controlled trial
	RDW	Red cell distribution width
	REC	Research ethics committee
	RES	Reticuloendothelial system
	RNA	ribonucleic acid
	RPMI	Roswell Park Memorial Institute
	RR	Relative risk
	RSV	Respiratory syncytial virus
S	SAE	Serious adverse event
	SAM	Severe acute malnutrition
	SC	Subcutaneous

	SCID	Severe combined immunodeficiency
	SD	Standard deviation
	SDS	Sodium dodecyl sulfate
	SEB	Staphylococcus enterotoxin B
	SK/SD	Streptokinase/streptodornase
	SSI	Statens Serum Institut
	sTFR	serum transferrin receptor
	STGG	Skimmed-milk, tryptophan, glucose, glycerol
	SUSAR	Suspected unexpected serious adverse reaction
T	T1DM	Type 1 Diabetes Mellitus
	TB	Tuberculosis
	Th	T-helper cell
	TIBC	Total iron binding capacity
	TLR	Toll-like receptor
	TMB	Tetra-methyl benzidine
	TNF	Tumor necrosis factor
	Tris	tris(hydroxymethyl)aminomethane
	TSAT	Transferrin saturation
	TST	Tuberculin skin test
	TT	Tetanus toxoid
U	UIBC	Unbound iron binding capacity
	UK	United Kingdom
	UNCST	Ugandan National Council for Science and Technology
	UNICEF	United Nations Children's Fund
	URTI	Upper respiratory tract infection
	USA	United States of America
	UTI	Urinary tract infection
V		
W	WBC	White blood cells
	WHO	World Health Organization
X		
Y		
Z		

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3. Introduction

The Heterologous Effects of Vaccines

That vaccines may produce effects beyond protection against the targeted disease has been recognised since the first days of vaccinology. Indeed, the observation in 1768 that infection with cowpox provided protection against smallpox lead to the development of the first widely used vaccination, and ultimate eradication of the disease.[1] In this case, the smallpox and cowpox viruses are related, and sufficiently similar to induce immunological cross-protection. Further examples of this type of cross-protection include the use of *Mycobacterium bovis* in Bacille Calmette Guérin (BCG) for protection against *Mycobacterium tuberculosis*, *Mycobacterium leprae*[2] and *Mycobacterium ulcerans.*[3] The ability for vaccines to have effects on unrelated diseases is also widely recognised. Idiosyncratic reactions following vaccinations are a simple example of this, for instance myopericarditis after small-pox vaccine.[4] Reductions in vaccine-preventable diseases can also have down-stream effects on pathogens that commonly cause super-infections, for instance the prevention of influenza-associated secondary bacterial pneumonias by influenza vaccination.[5]

The possibility that commonly used vaccines may have widespread and durable effects on non-vaccine targeted diseases, resulting from long-term impacts on the immune system, is a more contested theory. These effects have variously been described as 'off-target effects', 'heterologous effects', or 'non-specific effects' (NSE).

The studies described in this thesis were designed to investigate whether BCG vaccination in neonates can protect infants against heterologous invasive infectious diseases by non-specifically enhancing the innate immune system. This research was intended to help clarify some outstanding, contentious issues from the literature to date. The current evidence surrounding the NSE of BCG is discussed below. Evidence regarding the NSE of BCG on clinical disease morbidity and mortality outcomes in humans and animals is discussed first, followed by a review of immunological/mechanistic studies and finally a discussion of potential modifiers of a NSE of BCG.

3.1 Human studies investigating the NSE of BCG on clinical outcomes

Since BCG vaccination was first introduced for protection against tuberculosis (TB) in the 1920's, researchers have suggested that it may produce beneficial effects against heterologous diseases. During the period of introduction of universal BCG vaccination in Sweden from 1927-1931, Carl Naeslund observed that infants who had received BCG vaccination had an almost 3-fold lower all-cause mortality compared to unvaccinated infants.[6] This reduction was largely due to reductions in non-tuberculous related deaths occurring in the neonatal period. As BCG introduction in Sweden was not randomised, it may be argued that these results served merely as an indicator of the health or socio-economic status of the vaccine recipients. Subsequent to this a large number of epidemiological studies, and several randomised controlled trials, have been conducted to investigate whether BCG may have non-specific beneficial effects. These are described below. The main focus will be on the effect of BCG in reducing all-cause mortality and infectious mortality/morbidity, although the evidence for BCG affecting other diseases will also be reviewed.

3.1.1 The NSE of BCG on all-cause mortality and morbidity

Observational studies

More than twenty epidemiological studies have been published investigating the potential non-specific effects of BCG on all-cause mortality (Table 3.1). Although heterogeneous in design, these largely report beneficial effects on all-cause mortality when assessed by documented BCG vaccine status[7-17] or BCG scar/PPD response.[18-20] Point estimates for the reduction in all-cause mortality associated with BCG vaccination in these studies range from 0.18-0.70. Three published studies have not reported significant beneficial effects overall of BCG on all-cause mortality,[21-23] although in all of these studies a trend toward protection was seen, with point estimates for effects ranging from 0.47-0.68. Observational studies investigating indicators of all-cause morbidity such as hospitalisations[24, 25] and stunting,[26] have all been reported to be lower in BCG vaccinated infants compared to unvaccinated infants, although the latter was only significant in infancy. In all cases the protection afforded by BCG was greater than would be expected from specific protection against tuberculosis.

Table 3.1. Epidemiological studies and randomised controlled trials investigating the effects of BCG on all-cause mortality and hospitalisations

Epidemiological studies						
Country	**Study design**	**Participant characteristics**	**Outcomes**	**Results**	**Limitations**	**Reference**
All-cause mortality, hospitalisations or health centre use						
Benin 1983-87	Case-control	74 children aged 4-35mths who died and 230 controls matched on sex, date of birth and place of residence.	Primary health-care utilization comparing children who died with those surviving.	RR 0.68 (0.38-1.23) of mortality if BCG vaccinated Measles vaccination reduced RR of mortality, DTP had no effect on mortality. Low weight for age increased mortality and low health centre utilization associated with increased risk of death.	Vaccination status may just be a proxy marker for health centre utilization ('healthy vaccine bias'). Lack of a similar effect of DTP argues against this.	Velema 1991[27]
Guinea-Bissau 1990-96	Prospective cohort. Landmark updating approach for vaccination status	5274 infants Followed up to 13mths of age. Adjusted for cluster, age and other vaccines.	MR	BCG associated with MR 0.55 (0.36-0.85) from 0-6mths of age DTP associated with increased MR 1.84 (1.10-3.10). No changes after adjustment for background factors	Underlying differences in vaccinated vs. unvaccinated subjects noted (mothers of vaccinated children had more frequent health centre contact and the children had larger arm circumference than unvaccinated).	Kristensen 2000[13]* Jensen 2005[28]* Jensen 2007[29]*
Guinea-Bissau 1989-2001	Prospective cohort. Cross-sectional analysis for scar at start of study then followed up for 12mths (1996-1998)	1813 6mth old children examined for scar. 813 vaccinated children also tested for PPD responses.	MR from 6mths to 18mths	MRR 0.41 (0.25-0.67) in children with BCG scar vs. no scar. Stronger PPD responses in vaccinated infants also associated with reduced mortality 0.46 (0.23-0.94) Remained after adjusting for sex, demographic variables, and	All infants in study had documented BCG vaccination. Lack of scar/PPD response maybe a marker of general ill health. However also tested DTH response to diphtheria and tetanus toxoids and no relationship found with these. Authors also note previous studies showing BCG scar more related to strain of BCG and vaccine technique.	Garly 2003[18]*

				birth weight. Stronger results for first 6mths of follow-up, little after 12 mths		
	Prospective cohort. As above (1998-2001)	1617 children from 3mths to 5yrs	MR over 12mth follow-up. Causes of death from verbal autopsy	MRR 0.45 (0.25-0.91) if BCG scar. Reduced deaths from malaria 0.32 (0.13-0.76) Combined MR with above cohort = 0.43 (0.28-0.65)		Roth 2005[19]*
Guinea-Bissau 1984-87	Prospective cohort Vaccination status assessed by cards or given by study team, 6mth intervals. Landmark approach to vaccine status assignment	1657 children aged 0-8mths Adjusted for sex, age, season, region and other vaccinations	MR	MR 0.63 (0.30-1.33) if BCG vaccinated. Inverse of those receiving DTP (MR 1.95 (1.07-3.57))		Aaby 2004[21]*
Burkino-Fasso 1985-93	Prospective cohort Vaccination status assessed by looking at cards. If no card seen then children analysed as un-vaccinated. Retrospective updating approach.	9085 children followed from 6mths-2yrs of age	MR	MR 0.50 (0.34-0.75) No impact of adjustment for health service utilisation, nutritional status and demographic variables. No sex-differential effect	Children only selected for participation if they had survived to 6mths (first follow-up). Vaccination cards tend to be discarded upon death of infant. Infants vaccinated in the intervening period would be miss-classified as un-vaccinated, exaggerating the beneficial effects of vaccinations. 10-15 times the mortality in unvaccinated infants than vaccinated. Independence of vaccination programme from follow-up and strong effects suggests high risk of vaccine bias.	Vaugelade 2004[9]†■
Bangladesh	Prospective	37894 children from	Mortality (HR)	HR 0.88 (0.67-1.16) for effect	Maternal education independently associated with	Breiman

1986-2001	cohort. Information on vaccine status updated on day of vaccination	6wks – 9mths.	from 6wks to 9mths of age	of BCG on mortality with any age of vaccination. Beneficial effect with early BCG HR 0.59 (0.47-0.73) Stronger effect of DTP at any age on mortality HR 0.76 (0.67-0.88). No differential effect of sex or vaccination order. Age and educational status of mother and birth order of child independent predictors of mortality. Re-analysis by Aaby et al 2017 looking at impact of vaccination order. BCG administered first associated with higher mortality to 9 months of age (MRR 1.78(1.03-3.03)) than BCG+DTP co-administered.	risk of death and vaccination status of children, thus likely confounder. Likely 'healthy vaccinee' effect, with very unwell children less likely to receive vaccines but more likely to die. Children not regarded as vaccinated until 30d after dose to try to account for this. BCG vaccination very frequently given with DTP or measles vaccine, not alone.	2004[10†]■ Aaby 2017[15]*
Papua New Guinea 1989-94	Prospective cohort. Vaccine status and mortality assessed at monthly intervals. Retrospective updating of vaccine status, but immunisation cards held at clinic so less chance of	4048 children followed from birth to 2yrs	Mortality (HR)	Mortality if BCG vaccinated HR 0.40 (0.25-0.66) to 2yrs HR 0.17 (0.09-0.34) for 1-5mths. Mortality if at least 1 DTP vaccination HR 0.48 (0.22-1.09) No significant sex-differential effect	Children dying before 29d of age were excluded from analysis. Pigbel vaccine also given at DTP vaccination times.	Lehmann 2005[12†]

	survival bias.					
	Sub-study of above	2079 hospitalised infants aged 5-17mths.	CFR of hospitalised children.	No significant differences in CFR by vaccination status. In BCG vaccinated infants there was no sex-differential mortality. In vaccinated children the F:M MR was reversed between DTP and measles vaccinations (higher post DTP for girls, lower post-measles).		Verium 2005[30]*
Senegal 1996-99	Two prospective cohorts. Retrospective updating	7796 + 3573 children receiving either BCG+DTP together or no vaccinations. Followed up to 2yrs of age.	MR (HR) for the effect of vaccinations. Adjusted for gender and various socio-demographic factors.	MR for recipients of BCG/DTP 0.59 (0.46-0.74) and 0.70 (0.50-0.97) in two cohorts. No significant effect on mortality of measles vaccination. No documented sex-differential effect	Vaccinated infants also provided with a 3mth supply of malaria chemoprophylaxis so would have extra protection against malaria. BCG vaccination provided at 2mths with DTP, not at birth.	Elguero 2005[14]
	Reanalysis of above study. Landmark updating	4133 children	MR and F:M MRR. Controlled for age birth year birth season and village.	BCG+DTP had lower mortality than unvaccinated children MRR 0.69 (0.53-0.89) but BCG first did not. DTP before BCG associated with non-significantly increased MRR 1.34 (0.8-2.3). F:M MRR 1.45 (1.0-2.1) after DTP.	Unclear why only 4133 children of the above data set included in the analysis. Lack of an effect of BCG first (which was not the recommended schedule at the time) suggests that BCG=DTP is a marker of parents adherent to the recommended protocols.	Aaby 2015[31]*
India 1998-2002	Prospective cohort nested within an RCT of vitamin A supplementation.	10274 infants	MR <6mths of age (hazard ratio). Controlled with various confounders (vaccine propensity	Receipt of either DTP or BCG reduced mortality by 30-50% compared to either getting both or neither vaccinations. Reduction in beneficial effect of BCG in females vaccinated with DTP.	Deaths <1wk not included. Vitamin A supplementation could have interacted (although as an RCT it should have been balanced) Unclear how vaccine status was updated.	Moulton 2005[16]

			score created).			
Malawi 1995-97	Prospective cohort. Analysed by both landmark and retrospective updating	803 children followed from birth to 18mths of age.	MRRs by last vaccination received, analysed by sex.	Non-significant trend toward reduced mortality with any vaccination. Female MR increased post-DTP vaccination (p=0.1) but decreased post measles vaccine (p=0.01).	Rainy season noted to have affected vaccine uptake and may also have impacted on death rates.	Aaby 2006[22]*
Ghana 1998-2004	Prospective cohort. Immunisation status updated annually (unclear how)	17967 followed up to 5yrs of age	Time-conditional HRs for mortality. Adjusted for poverty indicators.	Receipt of any vaccination is strongly protective against death in a time dependent manner (BCG 0.18 (0.17-0.20)) Not reported by sex.	By 1yr most infants received BCG and DTP so unable to untangle effects. Full immunisation by 1yr significantly better survival than partial, which is significantly better than none. Differential effects do not appear to alter by poverty indicators.	Bawah 2010[17]■
Guinea-Bissau 2003-2004	Prospective cohort. Vaccine status confirmed by vaccine card review. Children with no information about vaccination were excluded.	11949 children attending paediatric outpatient clinic appointments	Hospitalisation risk ratio by last vaccination received. Controlled for various socio-demographic factors.	Hospitalisation risk ratio 1.99 (1.37-2.89) comparing BCG unvaccinated with vaccinated children. More significant in first 8-30d of life (2.25 (1.42-3.58)). No difference in hospitalisation between children receiving DTP after BCG compared to those with BCG alone. No sex-differential effect for BCG vaccination.	No adjustments for birthweight made (BCG delayed until children >2.5kg in Guinea-Bissau).	Biai 2011[24]*
India 1987-89	Prospective cohort. Vaccine status assessed every 3mths. Landmark approach	4138 children	MR to 5yrs of age.	MRR 0.60 (0.18-1.97) Children with BCG+DTP or BCG as most recent vaccination had lower mortality than with DTP MRR 0.15 (0.03-0.70). 2-fold higher F:M mortality in the post-DTP age group (2-8mths).	No adjustment for age. Unvaccinated group included those with missing information.	Hirve 2012[11]*
India	Prospective	12142 children from	HR for	No difference in F:M MRR after	No adjustment for age. Differing times in study by	Krishnan

2006-11	cohort. Determination of vaccination age not specified	birth to 36mths	mortality by vaccination status. Adjusted for demographic variables.	BCG vaccination (F:M MRR 1.06 (0.67-1.67)). Significantly increased after DTP (F:M MRR 1.65 (1.17-2.32)).	vaccination status. Children receiving two vaccines together excluded. 35% underlying differential F:M MR. Low number of unvaccinated participants.	2013[23]■
Uganda 2006-14	Prospective cohort Landmark analysis.	819 children followed-up to 7yrs of age	MR (adjusted for multiple confounders using multiple correspondence analysis).	Non-significant reduction in mortality rate in post-neonatal children (MR 0.47 (0.14-1.53) associated with BCG vaccination. Significant decrease in children aged 1-5yrs (MR 0.26 (0.14-0.48). No sex-differential effect.	No information about timing of BCG vaccination with respect to HIV status of mother (likely to be delayed and mortality 34 times increased in HIV positive mothers in this study).	Nankabirwa 2015[32]
Guinea-Bissau 2009-2011	Prospective cohort	15911 known BCG vaccinated infants followed-up to 5yrs of age	MRR	BCG vaccinated infants with a scar associated with 0.48 (0.26-0.90) reduction in mortality to 12 months. No significant sex-differential effect. Effect only in children vaccinated in neonatal period.		Storgaard 2015[20]*
33 Sub-Saharan countries 1998-2014	Retrospective and cross-sectional data collected in demographic surveys. Retrospective updating approach	368,450 children	OR of stunting in children under-5. Controlled for various child, maternal and household co-variates.	Overall BCG vaccination status did not affect stunting OR 1.0 (0.98-1.03). Early BCG vaccination associated with decreased stunting OR 0.92 (0.89-0.94)) compared to later vaccination (OR 1.64 (1.53-1.76)). Trend held for timing of other vaccinations. Sex-differential effect not reported.	Trend of reduced stunting with early receipt of vaccinations suggests that children who receive vaccines at the right time are different to those who receive them delayed. Children <1mth old excluded. Significant variance of groups for measured confounders (though adjusted for in the analysis), suggesting vaccinated infants healthier than unvaccinated.	Berendsen 2016[26]
Denmark 1971-2010	Case-cohort Retrospective from Copenhagen	47622 Danish school children born 1965-76. Comparison of children	MRR (Hazard rates)	aHR 0.58 (0.39-0.85) for non-accidental mortality in BCG vaccinated vs. unvaccinated subjects.	As BCG was phased out and optional, there may have been unmeasured confounders associated with BCG uptake that created a spurious beneficial effect, but no differences by social class argues against this,	Rieckmann 2017[7]*

	School Health Record Register, but information collected prospectively. The period studied covered the phasing out of free BCG provision on school entry.	receiving BCG only vs. vaccinia only vs. BCG and vaccinia vs. one only of BCG and vaccinia. Adjusted for sex, social class, birth by C-section, immigration status and eczema.		Deaths due to accidents were not significantly associated with BCG vaccination status. Effects remained when stratified by social class. No sex-differential effect.	as does the lack of effect of BCG on accidental deaths.	

Trials							
Country	**Participant characteristics**	**Intervention**	**Randomised?**	**BCG strain and dose**	**Outcomes**	**Results**	**Reference**
USA	Children and adolescents aged 0-16yrs	566 BCG vaccinated compared to 528 unvaccinated	Semi: Alternately allocated	Unknown	All-cause mortality	48% reduction in all cause mortality (-4 to 75%) with BCG vaccination.	Levine 1946
USA 1935-98	Children and adolescents aged 0-20yrs	1551 BCG vaccinated 1457 placebo vaccinated	Semi: Alternately allocated	0.1mg Pasteur	All-cause mortality over 9-11yrs of follow-up	19% reduction in all-cause mortality (-21 to 46%) with BCG vaccination.	Aronson 1948
Canada 1933-45	Native Canadian Indian populations between 0-13yrs with no prior BCG vaccination	306 BCG vaccinated 303 controls	Yes	0.2mg Pasteur	All-cause mortality over 60mths of follow-up	12% reduction in all-cause mortality (-33% to 42%) with BCG vaccination.	Fergusen 1949
USA 1937-1960	Neonates exposed to TB	311 BCG vaccinated 250 Unvaccinated	Semi: Alternately allocated	Pasteur or Tice	All-cause mortality	4% increase in all-cause mortality in BCG vaccinated infants.	Rosenthal 1961
Guinea-Bissau	Previously vaccinated	BCG revaccination at 19mths = 1437	Yes	BCG SSI ID 0.1ml	1˚ Mortality 2˚ Hospitalisation,	No significant difference in mortality (HR 1.20 (0.77-1.89)) or hospitalisations (IRR 1.04 (0.81-	Roth 2010[33]

2002-2006	infants with PPD reaction <15mm diameter Aged 19mths-5yrs	control =1434				analysis by sex, exploratory analysis by timing of DTP immunisation	1.33)). No differential effect by sex. Cluster of deaths seen in BCG arm in infants likely to have received booster DTP after BCG during revaccination campaign.	
Guinea-Bissau 2002-2004	Low birthweight (<2.5kg) neonates born out of hospital	BCG at first health centre contact = 51 BCG at >2.5kg (around 6wks of age) = 54	Yes	BCG SSI ID 0.05ml BCG Russia in control group	1.	MRR to 12mths of age	Note: early version of the Aaby 2011 trial below, stopped due to concerns with randomisation in the hospital part. No concerns with randomisation at local health centres so reported.	Biering-Sorensen 2012
Guinea-Bissau 2004-2008	Low birthweight (<2.5kg) neonates	BCG at birth (median age 2d) = 1182 BCG at ~ 6wks (median age 49d) = 1161	Yes	BCG SSI ID 0.05ml BCG Russia in control group	1˚ 2˚	All-cause mortality up to 12mths of age MRR at 12mths BCG at birth vs. later: 0.83 (0.63-1.08)	Significant beneficial effect seen in neonatal period, prior to BCG receipt in control group: MRR 0.55 (0.34-0.89). Beneficial effect greater in infants <1.5kg at birth MRR 0.43 (0.21-0.85). Reduction in deaths due to reduced sepsis, respiratory illness and febrile illness.	Aaby 2011
						Growth in first year	No significant difference in weight, length, MUAC or head circumference at 2, 6 or 12mths of age. Trend at 2mths toward early BCG being more beneficial for girls for growth parameters (p=0.04 for interaction).	Biering-Sorensen 2015[34]
Guinea-Bissau 2008-2014	Low birth weight (<2.5kg) neonates	BCG at birth = 2083 BCG at discharge from maternity ward or first health centre contact = 2089	Yes	BCG SSI ID 0.05ml in early group BCG Russia in control group		Neonatal all-cause MR (<28d) All-cause MR at 12mths of age.	MRR for neonatal period 0.70 (0.47-1.04). For infectious deaths MRR associated with BCG was 0.57 (0.35-0.93). Effects most pronounced within first 3 days after randomisation. Non-significant 12% MR reduction at 6 and 12mths after birth. No sex-differential effect.	Biering-Sorensen 2017[35]

Denmark 2012-2015	BCG within 7d of birth = 2129 Control (no BCG) = 2133	Yes	BCG SSI ID 0.05ml	All cause hospitalisations to 15mths of age.	No significant difference in hospitalisations in BCG vaccinated vs. controls (HR 1.05 (0.93-1.18)). No differences when analysed by sex or prematurity.	Stensballe 2017[36]
				1˚ Psychomotor development at 12mths (Ages and Stages Questionnaire)	No significant differences in Ages and Stages Questionnaire score by BCG vaccination status.	Kjaergaard 2016[37]
				2˚ Psychomotor development in premature infants at 6, 12 and 22mths		

d, days; wks, weeks; mths, months; yrs, years; F, Female; M, Male; MR, Mortality Rate; MRR, Mortality Rate Ratio; HR, Hazard Rate; HRR, Hazard Rate Ratio; IRR, Incidence Rate Ratio; OR, Odds Ratio; RR, Relative risk; ID, Intradermal; MUAC, mid-upper arm circumference; CFR, case-fatality ratio; BCG, Bacille Calmette Guerin; SSI, Statens Serum Institut, DTP, Diptheria-Tetanus-Pertussis; DTH, Delayed Type Hypersensitivity; PPD, Purified Protein Derivative; HIV, Human Immunodeficiency Virus, * Studies with direct involvement from the Aaby group †WHO commissioned studies ■Studies excluded from the WHO commissioned systematic review (Higgins et al[38]), due to high risk of bias.

Although the epidemiological evidence largely supports a non-specific beneficial effect of BCG against all-cause mortality, the studies have a number of methodological flaws that have hindered their acceptance as proof of such an effect by the research community. These flaws are also common to the other observational studies of the NSE of BCG, which are discussed below.

- **Unmeasured confounders:** Multiple studies have confirmed that children who receive vaccinations in a timely manner are a fundamentally different population to children who do not (reviewed in[39]). Vaccinated children tend to be born to mothers of higher socio-economic status, who are older, more experienced, have higher educational attainment and are more proactive in their health-seeking behaviour. Vaccinated children are also likely to be healthier as health-care staff are generally unwilling to vaccinate ill children (the 'healthy vaccine effect' or frailty bias), although some have argued that this effect may work in reverse, with unwell children being seen in clinics more often and therefore having greater opportunities for receiving routine immunisations.[40] Also, in Guinea-Bissau BCG is deferred in low birthweight (LBW) infants until they reach >2.5kg. LBW infants have higher mortality rates than normal weight infants, and birthweight was rarely available in these epidemiological studies sufficiently to allow for adjusted analyses (only adjusted for in two studies[18, 19]). These confounders would tend to exaggerate a beneficial non-specific effects of vaccinations. Although most studies described in Table 1 have attempted to adjust for potential confounders, the possibility of residual confounding remains one of the strongest arguments against the observational evidence for the NSE of BCG. Evidence suggesting differential effects of vaccinations by sex (where unmeasured confounders would be acting in a similar fashion for boys and girls) and opposing directions of non-specific effects for live vs. inactive vaccines have been used as counter arguments against the major influence of unidentified confounders[39] (see "Effect Modifiers' section below).
- **Misclassification of vaccination status:** The correct determination of the exposure of interest (BCG vaccinated or not) has been challenging in most of the described epidemiological studies. Although mainly prospective in design, BCG status was often determined retrospectively, at intervals, by observation of vaccination cards, parental recall, or observation of a BCG scar. Each of these approaches has limitations, which may result in misclassification of BCG status. Vaccination cards may be lost or unreadable and recall bias may affect parental reporting of vaccine

status. BCG scar is known to be an imperfect marker of vaccination, with up to 50% of infants failing to develop a scar following BCG administration in some studies,[41, 42] with scar development affected by BCG strain and vaccinator technique.[43] In only 1 study was date of BCG vaccination fully known as it was provided by the study team,[14] or reasonably assumed to be complete as it was entered into primary care records at the time of vaccination,[8, 10, 12] or phased out of use over a known time period.[7] Studies with retrospective determination of BCG status have variously used a retrospective updating approach (status changed to BCG vaccinated on date vaccination received),[9, 14, 26] or a landmark approach (status changed to BCG vaccinated on date of study visit)[11, 13, 21, 24, 31, 32] in their analysis. Vaccination cards are often destroyed upon the death of a child and scars cannot be assessed or parents may be less willing to be interviewed following their child's death. As a result the retrospective updating approach tends to over-estimate the beneficial effects of vaccines, with children vaccinated and then dying between study visits being misclassified as unvaccinated or 'no information' (which are often analysed together). In effect, this approach introduces 'immortal person time' for vaccinated individuals; as to be classified as vaccinated they have to have survived to the next follow-up visit, and thus introduces a survival bias.[29] In contrast, the landmark approach of updating vaccine status from the date the vaccination card was reviewed tends to nullify any effect of vaccinations. As neither will accurately represent the true effect of vaccinations such as BCG, it is recommended that both approaches be reported in observational studies of vaccine effects.[39]

- **Selection bias:** Several epidemiological studies may have introduced selection bias into the estimates of the NSE of BCG by not including children during the neonatal period when BCG vaccination is received. This would mask any early positive or negative effects of BCG (see "Effect modifiers' section), and could mean that surviving infants in either group may be different to those who died prior to study enrolment. One study may also have introduced selection bias by selecting participants based on their future DTP status.[12]
- **Reporting and/or publication bias**: As with all studies, there is a risk of reporting/publication bias away from results showing null or negative effects of vaccinations. This is known to occur in randomised controlled trials[44] and is likely to be even stronger in epidemiological studies, due to the lack of formalised registries of on-going studies. This may exaggerate the perceived beneficial effects of BCG.

- **Experimenter bias** (+/- confirmation bias and reporting bias): Of particular concern in the NSE field is the possibility of experimenter bias. More than half of the epidemiological papers (and many of the trials) investigating the NSE of BCG on all-cause mortality have been published by the Aaby group, who first formulated the hypotheses regarding the impact of routine immunisation schedules on all-cause mortality. This has led to questions regarding the global applicability of their findings as the majority of evidence comes from Guinea-Bissau, a country with an extremely high infant mortality rate, although the group has also published studies from Denmark, Malawi, Bangladesh, India and Senegal. In a number of cases, several papers have been published from the same cohort, including re-analyses of old data and including multiple post-hoc hypotheses without corrections for multiple testing. This could have the effect of over-representing the diversity of evidence for the NSE of BCG. It should be noted, however, that whilst the Aaby group has produced a large amount of the supporting evidence, they have also published results from several studies that do not wholly confirm their theory. This argues against undue confirmatory/reporting bias from the group. It should also be noted that cohort studies conducted by the Aaby group use the more conservative 'landmark approach' to their analysis, which would tend to diminish perceived effects of vaccinations, rather than exaggerate them.

Clinical trials

A number of randomised or semi-randomised studies conducted in North America and the UK in the 1940s and 50s provided early evidence for a NSE of BCG (Table 3.1). Randomly allocated vaccination of native Indian children aged 0-13 years in Saskatchewan led to a 12% reduction in mortality rate from diseases other than TB;[45] alternately allocated vaccination of people aged 0-20 years in the US produced a 19% mortality rate reduction from non-tuberculous disease;[46] children aged 0-16 years alternately allocated BCG in New York City had a 48% reduction in mortality from diseases other than TB;[47] and adolescents given *Mycobacterium microti*, the vole bacillus, (as opposed to *Mycobacterium bovis*) in the UK showed a 35-53% non-TB mortality rate reduction.[48] One published study, however, investigating the impact of BCG vaccination on mortality in TB exposed neonates, showed a small (4%) increase in mortality rate ratio in BCG vaccinated infants.[49] Although individually the non-tuberculous mortality rate changes in these studies were not statistically significant,

meta-analysis of these gives a combined estimate for the non-specific mortality rate reduction of BCG as 25% (95%CI 6%-41%).[50] However, the heterogeneity of the studies and particularly strong weighting (due to large participant numbers and strong estimates of effects) given to the studies using *Mycobacterium microti* should caution against over-interpretation of these results.

The best available evidence for BCG having NSE on all-cause mortality comes from a large randomised controlled trial (RCT) conducted by the Aaby group, comparing BCG vaccination (Staten Serum Institute (SSI) 1331 strain) at birth with BCG given around 6 weeks of age in 2343 low birth LBW infants in Guinea-Bissau.[51] This study reported a 45% reduction in all-cause mortality (Mortality Rate Ratio (MRR) 0.55 (0.34-0.89)) in BCG vaccinated infants prior to 6 weeks of age (when non-vaccinated infants were vaccinated and all infants received Diphtheria-Tetanus-Pertussis (DTP) vaccination). The authors also reported a non-significant 17% mortality rate reduction (MRR 0.83 (0.68-1.08)) at 1 year of age, although this reduction is almost entirely accounted for by the early mortality reductions. The beneficial effects appeared strongest in the lowest birthweight infants. A smaller RCT in 105 LBW infants comparing BCG at first health centre contact or delayed to 6 weeks of age showed a similar non-significant trend (MRR 0.41 (0.14-1.18) p=0.098).[52] A third trial conducted by the group, essentially replicating the first trial but recruiting more participants, has recently confirmed the original findings.[53] Meta-analysis of all three trials suggests an overall reduction in neonatal mortality of 38 % (MRR 0.62 (0.46-0.83)) associated with BCG at birth, and a 16% reduction in all-cause mortality at 12 months (MRR 0.84 (0.71-1.0)).[53] No differential effects by sex were reported initially in these studies but a recent reanalysis of these studies suggests that non-specific beneficial effects of BCG may be stronger in males in the first week following vaccination, and stronger in females thereafter (see 'Effect Modification' section below).[35]

Although these studies provide strong supporting evidence for a NSE of neonatal BCG on all-cause mortality, a number of concerns remain. The Aaby group in Guinea-Bissau, where much of the epidemiological data has been produced, performed all of the studies, leading to suggestions that this may be a localised effect. The study populations were particularly high-risk LBW infants, in a country with one of the highest neonatal mortality rates in the world, leading to uncertainty as to whether NSE will be clinically relevant on a global scale. Indeed, an RCT conducted by the same group in Denmark investigating the impact of neonatal BCG vaccination on all-cause hospitalisations other

than injuries in children <15 months old, did not show any beneficial effect,[36] although a secondary analysis suggested benefit in children born to mothers with BCG vaccination (data not reported).[36] Changes in the growth rate of the BCG strain used (see 'Effect Modification' section below), as well as lower infectious exposures and genetic differences, may account for the lack of overall benefit of BCG in this study. Another RCT conducted by the Aaby group in Guinea-Bissau did not show any reduction in all-cause mortality or hospitalisations with BCG revaccination at 19 months of age, although the authors believe the results may have been confounded by a national DTP immunisation campaign that occurred concurrently.[54]

A thorough systematic review of observational and trial evidence up to January 2014, commissioned by the World Health Organization, concluded that BCG at birth appeared to reduce mortality by more than would be expected by disease specific mortality reductions.[38] However, it did not find enough evidence to determine optimal timing of BCG in comparison to other vaccinations, and did not comment about strain effects (see 'Effect Modification' section below). It concluded that evidence was not sufficient to recommend any change in BCG vaccination policy in countries that have phased out its routine use in neonates, or that routinely delay administration beyond the neonatal period.

It should be noted that a number of small studies investigating the immunological effects of BCG in infants have randomised infants to BCG at birth or delayed vaccination. Several of these have documented all-cause mortality, although it was not investigated as a specific outcome (Table 3.2). These studies were mainly very small, reported no deaths in either arm and have not contributed to meta-analyses of the clinical NSE of BCG.

Table 3.2. Other randomised controlled trials of neonatal BCG vaccination reporting mortality data

Country	Participant characteristics	Intervention	BCG Strain and dose	Documented deaths	References*
Lithuania	Term neonates >3kg	BCG at <6d vs at 3 months of age	BCG SSI 0.05ml	0/159 (birth) vs. 0/150 (delayed) followed to 1 year	Sucillienne 1999[55]
India	Premature infants (<34/40 weeks)	BCG at 34-35/40 weeks post-conceptional age vs. at 38-40/40 weeks	BCG SSI 0.1ml ID	1/30 (early) vs. 0/31 (late) followed to 6 months	Thayyil-Sudhan1999[56]
The Gambia	Neonates >2.5kg	BCG at birth vs BCG at 2mths or 4.5mths	BCG Pasteur 0.05ml ID	0/35 (birth) vs. 0/64 (delayed) followed to 4.5 months	Ota 2002[57]
South Africa	Neonates weighing >2.5kg. HIV unexposed	BCG at birth vs. at 10 weeks	BCG SSI 0.05ml ID	0/25 (birth) vs. 0/21 (delayed) followed to 50 weeks	Kagina 2009[58]
The Gambia	Neonates >2.5kg	BCG at birth vs. at 4.5 months	BCG Russia 0.05ml ID	1/53 (birth) vs. 2/50 (delayed) followed to 9 months	Burl 2010[59]
South Africa	HIV-exposed uninfected infants	BCG at birth vs. at 8 weeks	BCG SSI 0.05ml ID	0/71 (birth) vs. 0/69 (delayed) followed to 14 weeks	Tchakoute 2015[60] and Hessling 2016[61]

BCG, Bacille Calmette Guerin; SSI, Statens Serum Institut; ID, intradermal; HIV, Human Immunodeficiency Virus.

A number of other randomised studies delaying BCG from birth in neonates have also been conducted (see 'Mechanistic Studies section) but made no specific mention of death rates. In most cases a comment was made about baseline and follow-up variables being comparable between groups.

3.1.2 The NSE of BCG on infectious disease incidence and morbidity

Most of the studies showing a beneficial effect of BCG on all-cause mortality were conducted in areas where infectious causes of death predominate, making protection against non-tuberculous pathogens a likely causal mechanism. As such, a number of studies have investigated whether BCG vaccination alters the incidence of infectious disease morbidity (Table 3.3).

Observational studies

A case-control study carried out by the Aaby group in Guinea-Bissau reported that children hospitalised for acute lower respiratory tract infection (ALRI) had higher odds of being BCG unvaccinated (assessed by vaccination cards or scar status) than age, sex and district matched controls (adjusted odds ratio (aOR) 2.87 (1.31-6.32)).[62] The potential for confounding is high in this study, but it did also report that in children documented to have received BCG vaccination, the odds of not having a BCG scar were higher in children hospitalised for ALRI than in the community, although this was not statistically significant (aOR 1.54 (0.86-2.75)). Although this may simply be a marker of the responsiveness of the underlying immune system, rather than the efficacy of BCG vaccination *per se,* the authors report unpublished studies in the same population which show that BCG scar is affected most by BCG strain and vaccination technique, rather than by infant characteristics.

In a case-control study of Bangladeshi children admitted with severe acute malnutrition and sepsis, lack of BCG vaccination was associated with an aOR of identifiable bacteraemia of 7.69 (1.67-32.73), which in itself was strongly associated with mortality.[63] It is unclear whether the effect of BCG vaccination was an *a priori* hypothesis in this study. Numbers of bacteraemic infants were small in this study (18/405) and, as with all observational studies, the potential for residual confounding remains. However, as both cases and controls were severely malnourished with pneumonia, the difference being presence or absence of bacteraemia, it is hard to hypothesise a causal link between a demographic factor that reduces the likelihood of BCG vaccination that also increases the likely haematogenous spread of bacteria above effects on malnutrition.

Table 3.3. Epidemiological studies and randomised controlled trials investigating the effects of BCG on non-mycobacterial infectious disease.

Infectious disease incidence						
Country	**Study design**	**Participant characteristics**	**Outcomes**	**Results**	**Limitations**	**Reference**
Acute Lower Respiratory Tract Infection (ALRI)						
Guinea-Bissau 1994-1995	Case-control Matched on sex-age and district	386 case-control pairs Adjusted for background factors including birthweight, season of birth, and other vaccinations.	OR of ALRI by vaccination/scar status	OR 2.73 (1.37-5.44) for risk of ALRI if unvaccinated. Association only significant amongst girls OR 5.25 (1.8-15.3)	Very small numbers of birthweights known. Much higher proportion of LBW infants in unvaccinated cases. Non-significant due to numbers but likely to be confounder. Non-significant trend to socio-economic indicators being higher in vaccinated infants. Scar status assessed by study nurses aware of hypothesis.	Stensballe 2005[62]*
33 countries 2000-2010	Retrospective cohort from DHS data. Retrospective updating of vaccination status.	58021 +93301 children <5 years old	RR for ALRI. Adjusted for a vaccine propensity score using determinants of vaccine use	17-37% RR of ALRI associated with BCG vaccination. DTP significantly modified this effect ($p<0.001$) BCG before DTP RR 0.78 (0.70-0.89) BCG with DTP 0.82 (0.71-0.94) BCG after DTP 1 (0.87-1.13). Also modified by vaccine strain used.	ALRI definition = cough and rapid breathing reported by parents in preceding 2 weeks. HIV status unknown, but effects strongest in areas of low HIV burden. Countries contributing data to the study not listed.	Hollm-Delgado 2014[64]

Spain 1992-2011	Retrospective cohort	464611 hospitalizations due to respiratory infections and sepsis of children < 15 years of age	Hospitalisation rates for ALRI (not TB) and sepsis by BCG status. Documented as preventable fraction (PF).	ALRI: PF 41.4% (40.3-42.5) p<0.001. PF increases with age. Sepsis: <1 year old PF 52.8% (43.8-60.7) p<0.001	Different communities received or did not receive BCG (Basque County vs. rest of Spain). Comparisons with neighbouring regions done to try and control for this and results comparable. No socio-economic status adjustment. Stratified by age.	De Castro 2015[25]
Greenland 1989-2004	Retrospective cohort using electronic health-care records	19363 children followed from 3 months to 3 years of age. The period included 5 years where routine neonatal vaccination was stopped.	All-cause infectious disease hospitalisations and ALRI hospitalisations IRR comparing BCG vaccinated and unvaccinated children	All-cause hospitalisations IRR 1.07 (0.06-1.20) ALRI: 1.10 (0.98-1.24) No sex-differential effects	Infants not recruited until after 3mths of age, therefore early effects of BCG would not be seen.	Haahr 2016
Sepsis						
Bangladesh 2011-2012	Unmatched case-control	405 hospitalised children <5 years with severe acute malnutrition (SAM) and pneumonia. Cases = bacteraemia + SAM + pneumonia Controls = SAM + pneumonia	OR of having bacteraemia by BCG status	OR for lack of BCG vaccination in bacteraemic patients 7.39 (1.67-32.73) p<0.01.after adjustment for potential confounders	Unclear if vaccination status was a pre-defined end-point for the study.	Chisti 2015[63]
HIV						
Denmark 1971-2010 and Guinea-Bissau 2004-2007	a) case-cohort in Denmark as above, comparing b) cross-sectional study in Guinea-Bissau	a) 47622 Danish school children born 1965-76. b) 1751 adults (>15 years) in Guinea-Bissau (10% of randomly selected houses in DSS area)	HIV-1 prevalence by BCG and vaccinia vaccination status, documented (study a) or scar status (study b)	aOR for HIV-1 with BCG vaccination Danish study 0.7 (0.41-1.18) Guinea-Bissau: 0.5 (0.23-1.10) Combined: aOR 0.63 (0.41-0.98)	• Small numbers of HIV+ve cases • BCG scar used as proxy for vaccination in GB but may just be marker of immune system integrity	Rieckmann 2017[8]*

					Adjusted for various social class indicators. No sex-differential effect.	• BCG correlated with immigrant status, which correlates with HIV in Denmark, but would expect this to reduce observed protective effect of BCG.	
Enteropathogens							
Guinea-Bissau 1996-1998	Prospective cohort	200 children from birth to 2 years		IR of enteropathogens F:M IRR	No significant differences in IR or F:M IRR of enteropathogens by vaccination status. Trend toward lower F:M IRR after BCG and higher after DTP (interaction p=0.02 for RSV 0.01 for Cryptosporidium)	Many sub-analyses. Unclear if these were pre-specified. Children censored if samples taken within 2 weeks of vaccination (as assumed that it would take some time for vaccinations to have an effect).	Rodrigues 2006[65]* Valentiner-Branth 2006[66]*
Trials which included infectious disease end-points							
Country	**Participant characteristics**	**Intervention**	**Randomised?**	**BCG strain and dose**	**Outcomes**	**Results**	**Reference**
Guinea-Bissau ***2002-2006***	Previously vaccinated infants with PPD reaction <15mm diameter Aged 19mths-5yrs	BCG revaccination at 19 months = 713 control = 720	Yes	BCG SSI ID 0.1ml	1˚ Malaria incidence 2˚ Hospitalisations, mortality, analysis by sex and Mantoux reaction	No significant difference in malaria incidence IRR 1.22 (0.99-1.51). No sex-differential effect All-cause	Rodrigues 2007[54]

					hospitalisations significantly more in revaccinated children IRR 2.13 (1.10-4.13) No significant difference in overall mortality, or clinic presentations. No difference by Mantoux reaction.	
Denmark ***2012-2015***	BCG within 7d of birth = 2129 Control (no BCG) = 2133	Yes	BCG SSI ID 0.05ml	Parent-reported childhood infections	No significant difference in infectious illness episodes or GP visits by BCG status. BCG vaccinated children born to BCG vaccinated mothers had reduced illness episodes up to 3mths of age IRR 0.62 (0.39-0.98)	Kjaergaard 2016[37]

BCG, Bacille Calmette Guerin; PPD, Purified Protein Derivative; SSI, Statens Serum Institut; ID, Intradermal; IR, Incidence rate; IRR, Incidence Rate Reduction; OR, Odds Ratio; RR, Relative Risk; F, Female; M, Male; HIV, Human Immunodeficiency Virus; TB, Tuberculosis; SAM, Serious Acute Malnutrition; PF, Preventable Fraction; ALRI, Acute Lower Respiratory Tract Infection; DHS, Demographic Health Survey; DTP, Diptheria-Tetanus-Pertussis; TB, Tuberculosis

An analysis of data collected by Demographic Surveillance Systems (DSS) across 33 countries (largely from Sub-Saharan Africa), suggested a 17-37% relative risk reduction of ALRI hospital admissions associated with BCG vaccination, which was modified by subsequent DTP vaccination.[64] Although adjusted for a composite 'vaccine propensity score' the potential for residual confounding in this study is high. Particularly concerning is the lack of documentation about the Human Immunodeficiency Virus (HIV) status of the mother, as HIV may lead to delays in BCG vaccination of the child in some settings and is associated with increased morbidity in both HIV-infected and HIV-exposed uninfected children. However, the observation that the effects of BCG remained, and in fact were strongest, in areas of low HIV incidence argues against maternal HIV status unduly confounding the results.[8]

A large retrospective cohort study comparing two regions of Spain with differing BCG vaccination policies suggested that if unvaccinated children had received neonatal BCG the preventable fraction for ALRIs would be 41.4% (40.3-42.5) in children <15 years old and 52.8% (43.8%-60.7%) for sepsis admissions in infancy.[25] Although demographic and health-care system differences may underlie these regional differences in hospitalisations, the results remained when only adjacent geographical regions were compared. However, as BCG vaccination was given throughout Spain prior to the time period used in this study, it should have been possible to do a comparison of hospitalisations between regions when under universal BCG to see if there were pre-existing differences. This was not conducted (or reported) by the study authors.

In 1991 Greenland stopped routinely administering BCG vaccination to neonates at birth, a policy that was reversed in 1996. A retrospective cohort study has recently been published comparing all-cause hospitalisation rates, and ALRI-specific hospitalisation rates, in infants born before, after and during the period of BCG vaccination stoppage.[67] This study failed to confirm any benefits of neonatal BCG vaccination on either all-cause hospitalisations (Incidence Rate Ratio (IRR) 1.07 (0.06-1.20)) or ALRI related hospitalisations (IRR 1.10 (0.98-1.24)). However, this study did not include infants dying before three months of age and could therefore have missed a significant early effect of BCG vaccination.

Notably two case-control studies of enteropathogen incidence in Guinea-Bissau failed to show any significant effect of prior BCG vaccination (although there was a trend toward lower male incidence rate compared with female incidence rate following

BCG).[65, 66] As children were censored within 2 weeks of vaccination, however, an early effect of BCG would not have been shown.

Clinical trials

No clinical trials of neonatal BCG vaccinations have been conducted with infectious disease incidence or morbidity as primary outcomes. The Aaby group did collect verbal autopsy data in their two LBW infant clinical trials, which suggested that the reduction in deaths shown with neonatal BCG was primarily due to a reduction in neonatal sepsis, ALRI and all-cause febrile illness.[51] The diversity of pathogenic protection supports the assertion that BCG mediates its heterologous effects by a NSE on the immune system, as opposed to antigenic cross-protection against specific pathogens. A trial providing BCG revaccination to infants at 19 months of age showed no difference in malarial incidence compared to unvaccinated infants.[54]

Thus, although limited in quantity and with some methodological issues, the observational and trial evidence largely point to any NSE of BCG being mediated through reductions in ALRI and sepsis in neonates in high mortality settings.

3.1.3 The NSE of BCG on non-infectious disease

In parallel to studies conducted in low-income settings suggesting that BCG may modulate the immune system to respond in an enhanced manner to infectious pathogens, studies in high-income settings have been conducted exploring a possible role for BCG in prevention of allergic, autoimmune and inflammatory diseases. The hygiene hypothesis suggests that reductions to pathogen exposure in early life resulting from socio-economic development, bias the immune system to more Th2-mediated responses and predispose to inflammatory and atopic conditions.[68] It has been proposed that the strong Th1-immune responses induced by BCG vaccination may modulate propensity to these diseases in the long-term.

3.1.3.1 The NSE of BCG on allergy, atopy, asthma and wheeze

Observational studies

Twenty-eight observational studies have investigated an association between BCG vaccination and the risk of allergic diseases including eczema and asthma.[69-71] These are a combination of retrospective studies utilizing health-care records or parent recall for determination of clinical outcome,[72-76] cross-sectional studies of the prevalence of clinical and immunological atopic indicators such as skin prick testing and serum IgE[69, 77-83] and case-control studies.[74, 84-89] Systematic reviews of these studies suggest that BCG reduces the likelihood of asthma/wheeze (OR 0.73 (0.56-0.95)),[69, 70] although this is not associated with a significant change in serum IgE or skin prick testing.[69] This may indicate that protection is against non-atopic/intrinsic asthma only, possibly due to reductions in under-lying respiratory pathologies common in early-onset wheeze. Longer-term follow-up of a cohort from Manchester suggested that protection was only transient, with difference by BCG status shown at 6-11 years of age but not at 13-17 years.[69] No clear protection against eczema, allergic rhinitis or food allergies was identified on systematic review of observational studies.[71] The studies are limited by the heterogeneity of BCG timings and strains used, difficulties with accurate clinical diagnosis of wheeze, asthma and eczema, and the varying methods of assessing BCG status (parental recall, scar observation and PPD response), which all have their drawbacks.

Clinical trials

There are currently two published randomised controlled trials investigating the effect of neonatal BCG vaccination on atopic outcomes,[90, 91] with one further on-going study waiting to report.[92] The first study investigated the prevalence of allergic diseases (eczema, wheeze, allergic rhinitis and food allergies) at 4 and 18 months of age in 121 high-risk infants randomised to BCG SSI or placebo at 6 weeks of age.[90] This showed no overall reduction in allergic disease but a trend toward reduced eczema incidence (0.72 (0.5-1.0) p=0.06) and reduced use of eczema medications (0.58 (0.3-1.0) p=0.04) at 18 months. However the study was only powered to show a 50% reduction in outcomes. Also, 74% of the BCG vaccinated group did not show a tuberculin reaction at 4 months of age, and 32% had no scar. These infants were subsequently revaccinated

with BCG, confounding the interpretation of the results, and also leading to concerns regarding the immunisation techniques used in the study.

The Aaby group conducted a large investigator-blind randomised controlled trial comparing neonatal BCG vaccination-SSI, given at <7 days of age, with no vaccination in 4262 Danish children.[91] The primary outcomes of this study were all-cause hospitalisations (described above), but wheeze, eczema and medication use were measured as secondary outcomes. The study showed a small trend toward reduced risk of atopic dermatitis in BCG vaccinated infants (Relative Risk (RR) 0.90 (0.8-1.0)), becoming significant in infants with a familial allergic predisposition (RR 0.4 (0.74-0.95)),[93] but no reduction in food allergy[94] or recurrent wheeze in the first year of life.[95] In contrast to the infectious disease outcomes in this study, maternal BCG status was not an effect modifier for the effect of BCG on any of the atopic outcomes. The study was limited by the lack of blinding of parents, which may have influenced their health-care seeking behaviour and recall of illness episodes, although clinical interviewers were blinded to vaccination status.

A trial investigating BCG vaccination as an immunotherapeutic for asthmatic children in Mexico failed to show any changes in asthma severity or emergency department attendances compared to placebo.[96]

3.1.3.2 The NSE of BCG on autoimmune and other Inflammatory diseases

Observational studies

Murine models have shown protection against autoimmune and inflammatory diseases such as multiple sclerosis (MS) and type 1 diabetes mellitus (T1DM) with prior BCG vaccination. In contrast, anecdotal observations linking the timing of certain vaccinations with onset of diseases such as T1DM have led to concerns, particularly amongst the lay public, about a causal link between the two. Studies investigating associations between BCG and chronic inflammatory disorders in humans have been equivocal.

A recent systematic review identified seven case-control studies investigating the odds of MS debut or relapse by BCG status.[97] No study showed a significant difference in the odds of MS debut with prior BCG vaccination. These studies were generally small (the largest involving 140 cases) and were heterogeneous in the age of BCG vaccination. All

the studies used questionnaires to assess BCG vaccination status, which may lead to recall bias, although significant effects, either positive or negative, of BCG would be expected if this had a differential effect in cases and controls. None compared the risk of relapse or disease progression in cases according to BCG vaccination status. A recently published study of 97 Japanese patients with various inflammatory demyelinating disorders suggested a protective effect of BCG vaccination, as evidenced by greater positivity for anti-BCG IgG levels (p=0.005).[98]

A meta-analysis of observational studies investigating an association between childhood immunisations and inflammatory bowel disease identified eight case-control and three cohort studies.[99] No association between childhood BCG vaccination and later inflammatory bowel disease was found (RR 1.04 (0.78-1.38)). Again these studies were limited by sample size, the possibility of recall bias for vaccine status and heterogeneous age of BCG vaccination. A Danish cohort study that analysed the risk of inflammatory bowel disease by timing of BCG vaccination did suggest a small reduction in risk with BCG given before 4 months of age (HR 0.43 (0.20-0.93)).[100]

Relatively few studies have assessed the impact of BCG vaccination on T1DM. Two large retrospective cohort studies using heath records (in Canada and in Sweden) showed no association between BCG vaccination at birth or in the first year of life and later T1DM.[101, 102] A UK-based case-control study showed no association between childhood immunisations and later T1DM, although BCG was not analysed independently from other routine immunisations.[103] One case-control study in Canada suggested a later onset of T1DM in BCG vaccinated infants, possibly pointing to a temporary protective effect on the immune system.[104] However, a prospective cohort of German children born to mothers with T1DM suggested that BCG vaccination reduced progression to clinical disease in autoantibody positive children (54% vs. 27% progression by 5 years, p=0.03),[105] although the numbers of BCG vaccinated autoantibody positive children were very small.

Clinical trials

No randomised controlled trials have been conducted to investigate the effect of neonatal or infant BCG vaccination on the development of autoimmune disorders. This is presumably due to the relative rarity of the outcomes of interest in the general population, the lag-time from infancy to usual onset of the outcomes, and the absence of

suggested protection in observational studies. A number of trials have been performed to investigate whether BCG given early in the course of autoimmune disease may be used as an immunotherapy to modulate its course.

One group in Denmark has conducted two randomised controlled trials investigating the potential for BCG to act as an immunotherapeutic agent to alter the course of MS in adults. One small crossover pilot study carried out in 12 MS patients suggested that BCG given early after diagnosis reduced the degree of disease activity (as assessed by Gadolinium MRI scans) in the short term, and reduced the risk of developing persistent T1 hypo-intense lesions when followed up for 2 years.[106, 107] This led to a larger double-blind placebo-controlled randomised study[108] of 82 patients with clinically isolated syndrome, which showed reduced lesion development in the first 6 months following Pasteur BCG (RR 0.54 p=0.03), with significantly reduced clinical severity and reduced requirement for disease modifying therapies at 60 months post vaccination.[108] Further larger studies will be required to see if BCG may have a role in disease-modification in progressive MS.[109]

Due largely to murine models suggesting that BCG may reduce pancreatic islet cell destruction and even restore insulin secretion when given in the early stages of the disease, several small studies have investigated its effect in early T1DM. In 1994 Shehadeh *et al*. described a small study of BCG vaccination (Connaught strain) in 17 newly diagnosed patients with T1DM, with 29 clinic patients used as historical controls. Patients receiving BCG went into remission significantly more often than historical controls (65% vs. 7% p<0.0001), although most of these patients relapsed again after 1-8 months.[110] A subsequent alternately allocated placebo-controlled trial showed no changes to C-peptide level or clinical course of T1DM in 26 adult patients when followed-up over 18 months.[111] These findings were confirmed in an RCT of 94 children vaccinated with BCG or placebo within the first four months of T1DM diagnosis and followed-up for 1 year.[112] More recently, however, there has been interest in the potential for BCG to restore islet cell function in long-term T1DM, possibly through its TNFα stimulating abilities. A small proof-of-concept RCT gave BCG (Sanofi-Pasteur) to 3 long-term T1DM patients and showed transient improvements in C-peptide levels, but it is unclear whether this would have any clinical utility.[113]

Observed reductions in delayed type hypersensitivity in patients with Crohn's disease lead to two small trials of comparing oral BCG (Institut Pasteur) with placebo in established inflammatory bowel disease.[114, 115] Neither reported improvements in

clinical or laboratory markers of the disease. No studies have been performed using intradermal BCG as immunotherapy for inflammatory bowel disease.

3.1.3.3 The NSE of BCG and malignancy

It was noted in the early 20th century that patients with tuberculosis rarely developed malignancies.[116] This, combined with the observation that local or systemic bacterial infections could induce remission of lymphosarcomas, led to interest in the use of BCG to prevent or treat malignancies.[117]

Observational studies

Since the 1970s epidemiological studies have variously suggested a decrease in childhood leukaemia and lymphomas incidence with prior BCG vaccination[118, 119, 120, 121, 122] or no effect.[123-130] Heterogeneity as to age at BCG vaccination may be partly responsible for these differing results, with neonatal BCG appearing most protective,[131] although population based differences cannot be ruled out. A meta-analysis of these, largely case-control studies, showed reduced odds of childhood leukaemia with any vaccination received in the first year of life (OR 0.58 (0.36-0.91)) with BCG having the strongest point estimate of an effect (OR 0.73 (0.50-1.08)).[132]

A large European multi-centre case control study reported both decreased incidence of melanoma[133] and increased survival[134] in melanoma patients who have had prior BCG (or smallpox) vaccination. Epidemiological evidence for early-life BCG providing non-specific protection against the later development of cancers other than melanoma and haematological malignancies, is lacking.

As with all observational work, the potential for there being unmeasured environmental confounders remains high in these studies. The particularly strong association between attendance at day care and reduction in haematological malignancies may suggest that diverse immunological challenges in early life could affect the maturation of the immune system and alter the later propensity to tumour development, rather than it being a unique NSE of BCG.[135]

Clinical trials

One controlled study of BCG vaccination in nearly 35,000 people in USA in the 1950s was followed up 30 years later to investigate cancer rates in the two groups.[136] Overall no difference in cancer incidence was shown by BCG vaccination status, and there was even a suggestion of increased risk of Hodgkin's Lymphoma. The study was limited by the relatively small numbers of individual types of cancer, and the fact that all participants were aged >5 years on BCG vaccination – limiting the ability to detect an effect of early BCG or the effect of BCG on childhood malignancies.

The use of BCG as an immunotherapeutic agent for superficial bladder cancers and melanomas is well known, and a review of the clinical trial evidence supporting this practice is beyond the scope of this thesis.[137] It is worth noting, though, that initial trials suggesting a use for systemically administered BCG in treatment of cancers[138] were not subsequently borne-out.[139] In fact Zbar and colleagues defined a number of features required for BCG use in cancer treatment, including long-lasting contact between live BCG (at a dose of 10^6-10^8 colony forming units) and the tumour cells.[140, 141] Thus, although the use of BCG as an immune-modulating agent in cancers is proof that BCG can have non-disease-specific influences on the immune system, they might act via different mechanisms to those linking intradermal BCG administration with reduced all-cause mortality and protection from infectious disease.

3.1.4 Summary: Human studies of the clinical NSE of BCG

Clinical trial and epidemiological data are supportive, though not conclusive, of there being a non-specific immunological effect of BCG vaccination. Evidence suggests that this effect may reduce all-cause mortality in high-mortality settings, likely mediated through reductions in infectious disease. Evidence for a non-specific beneficial effect of BCG on disease outcomes in high-income, low-mortality settings is more equivocal.

3.2 Animal studies investigating the NSE of BCG on heterologous pathogen morbidity and mortality

In the mid-20th Century, a large number of animal studies were conducted investigating the ability of BCG to provide protection against heterologous pathogens. Studies investigating the heterologous effects of BCG on the clinical outcomes of infection, morbidity and mortality in animals are summarised in Table 3.4, and comprehensively reviewed by Freyne *et al*.[142]

As outlined in Table 3.4, the majority of published animal studies show at least some protection against infection, morbidity or mortality from a wide range of pathogen types, following pre-treatment with BCG. These NSE appear to be conserved across a range of animal models and experimental conditions used (with heterogeneity in route, strain and dose of BCG inoculum, age and sex of animals used, duration between BCG administration and pathogen challenge, route, type and dose of pathogen administration). In fact, only two studies of trypanosomiasis in mice[143, 144] and one of *Treponema pallidum* in rabbits[145] described no clinical benefits associated with any form of BCG pre-treatment, although reporting bias in favour of positive results is acknowledged to be particularly problematic with animal studies.[146]

Animal studies can be useful in understanding human disease, due to the ability to standardise procedures, manipulate a variety of experimental conditions and perform more invasive mechanistic studies than may be possible in human populations. However, extrapolation from animal studies to humans is also notoriously fraught with difficulties. Many of the described studies varied significantly from the likely conditions of BCG vaccination in humans. The intravenous (IV) or intraperitoneal (IP) route of administration was commonly used, as opposed to the intradermal (ID) route used with human BCG vaccination. BCG inoculating doses were generally much higher (in the range 10^6-10^8 colony forming units (cfu)) than those received by human infants (3-4 x 10^5 cfu). Studies investigating various doses of BCG pre-treatment tended to show dose-dependent responses, with heterologous effects only observable above 10^6 cfu.[147-149] Indeed some studies showed trends toward increased mortality e.g. from *Staphylococcus aureus*[147] and infection from *Escherichia coli*[148] following lower inoculating doses of BCG. The higher BCG inoculums used, combined with the smaller weight of the animals, results in a much increased cfu/kg inoculating dose in the animal models which could argue that any heterologous effects of BCG are unlikely to be

observed at the doses used in humans. The challenge doses of organisms used in the studies were also much greater than would normally occur in human disease, which may influence the degree to which BCG-induced heterologous protection is clinically relevant. Of most concern, however, may be the variation in protective effects of BCG observed with different durations of time from vaccination to pathogen challenge, with some studies describing decreased survival benefit or enhanced susceptibility to infection with certain durations of pre-treatment.[147, 148] The lack of significant protection against schistosomiasis in primate models of prior BCG vaccination, contrasting with the good protection shown in earlier murine models, exemplifies the caution needed when transposing results from animal studies to primates or humans.[150] Taken together, however, the wealth of studies in different animal models and of various pathogen types provides, at the very least, compelling supporting evidence to continue investigating the heterologous effect of BCG in humans that have been suggested by the epidemiological studies and trials.

Table 3.4. Animal studies investigating the NSE of BCG vaccination

Pathogen	Animal model	BCG intervention details	Numbers	Time to pathogen challenge	Results	Reference
Bacteria						
***Staphylococcus aureus* (IV)**	Mice Male and female, 4wks old	BCG Philadelphia (IP). Live 0.1mg or HK 2.5mg	HK=8 Live=8 Controls=8	13-19d	Reduction in average mortality and survival time in BCG treated mice compared to controls. HK more effective than live-attenuated.	Dubos 1957[151]
***Salmonella enteritidis* (IV)**	Mice Male, adult	BCG Pasteur (IV). 0.25mg	Immunised=141 Controls=85	14d	Increased average survival time, increased phagocytic index (colloidal carbon and LPS clearance) but increased susceptibility to endotoxin in BCG vaccinated animals vs. controls.	Howard 1959[152]
***Listeria monocytogenes* (IV)**	Mice Female, 6-8wks old	BCG Rosenthal (route and dose not specified)	High dose=5 Low dose=5 Controls =5 At each challenge interval	3d intervals up to 28d, then at 35d or 56d	Significant increase in host resistance to Listeria in the liver and spleen following high-dose BCG vaccination, with corresponding increase in clearance of Listeria from the blood stream. Similar trend but non-significant following low-dose BCG. Rapid onset of liver and spleen resistance (within first 48hrs).	Blanden 1969[153]
***Salmonella typhimurium* (IP)**	Mice Female. Age not specified	BCG (IV) 10^8 cfu. Strain not specified.	Immunised=363 Controls=343	10d	Reduction in mortality (37% vs. 63% $p<0.001$) and reduction in Salmonella infected cells in BCG pre-treated group. Salmonella antibody levels at 5d post Salmonella infection where no different in BCG pre-treated group compared to controls.	Senterfitt 1970[154]
***Shigella flexneri* (topical to keratoconjunctiva)**	Rabbits Age and sex not specified	BCG Pasteur (IV). 10^7cfu	Immunised=8 Controls=8	22d	Reduced Shigella growth in the eye from 2d post-infection (3x), but no subjective reduction in the severity of conjunctivitis symptoms (large infecting dose of Shigella noted). Correlation between the intensity of the DTH reaction to BCG and the ability to control Shigella multiplication in the eye. BCG pre-treatment produced Shigella endotoxin hyper-reactivity.	Nakamura 1972[155]
Streptococcus	Mice	BCG (strain not	Immunised=3		BCG vaccination followed by tuberculin challenge lead to the	Salvin 1974[156]

***faecalis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* (and *Candida albicans*) culture**	Female, adult	specified) (IV $3x10^6$cfu) + 50µg tuberculin challenge IV 3wks later	Controls=3		acute production of soluble lymphokines: MIF and IFNγ. Serum from BCG vaccinated animals inhibited bacterial growth in-vitro, but not candida.	
Staphylococcus aureus (IV)	Mice. Adult, male Immuno-competent and supressed.	BCG Brazil Various doses	Immunised=25 Controls=25 For each experimental condition	3, 7, 14 or 28d	Reduced mortality with BCG pre-treatment at any interval prior to challenge when given at 10^6cfu (but not at lower doses, in fact some evidence of increased mortality compared to controls at 7 and 28d), in immuno-competent and immuno-suppressed mice.	Sher 1975[147]
***Treponema pallidum* (ID)**	Rabbits Age and sex not specified	BCG (IV) 2mg Strain not specified	Immunised =6 Controls =6	28d (+/- BCG booster on 0d)	No modification of syphilitic lesions with either 1 or two doses of BCG pre-treatment.	Graves 1979[145]
***Escherichia coli* (surgical wound model – IM implantation of suture coated in *E.coli*)**	Mice Adult, male	BCG (SC) Various doses	Immunised=401 Controls=167	3.5, 6 or 13d	Immunisation 13d prior to infection significantly reduced *Escolar* growth from surgical infection sites ($p<0.004$) when given at $2\text{-}16x10^{17}$cfu. No significant differences following lower BCG pre-treatment doses given at 3d or 6d of age, with a trend toward enhanced bacterial growth.	Fogelman 1981[148]
***Legionella pneumophillia* (inhaled)**	Guinea pigs Adult, female	BCG (IP) Glaxo $5x10^6$cfu	BCG alone = 12 Controls =15 BCG+MTB infection = 22	5-6wks post BCG (3, 6 or 10d post MTB)	No survival benefit with BCG pre-treatment (0/12) compared to controls (0/15). 100% of animals challenged with *L.pneumophilia* 3d after MTB infection with BCG pre-treatment survived. Survival decreased with time from MTB infection (6d and 10d).	Gibson 1985[157]
***Nocardia seriolae* (IP)**	Japanese flounder (*P. olivaceous*) Adult, no sex specified	BCG. Strain not specified. $9.4x10^6$ cfu	Immunised = 28 Control = 30	28d	BCG pre-treated fish had lower mortality at 34d (21.4%) than controls (56.7%). Weak up-regulation in BCG vaccinated fish. Strong up-regulation of macrophage associated enzymes e.g. lysozymes.	Kato 2012[158]
Viruses						
Herpes simplex	Rabbits	BCG Pasteur	Immunised=30	4wks	Reduction in encephalitis-related mortality with BCG pre-	Larson 1972[159]

virus-2 (intravaginal and intracorneal)	Age and sex not-specified	(IV). 10^7cfu	Controls=30		treatment (33% mortality cf. 83% mortality). Route of HSV-2 infection dependent: best mortality benefit with corneal scarification rather than injection.	
Herpes simplex virus (McKrae strain)	Rabbits Age and sex not specified	BCG Pasteur (ID) 0.7ml of 7.5mg/ml solution	Immunised=75 Controls=75	14d	Significant reduction in corneal lesions at 7-14d post-HSV infection with BCG pre-treatment, but at no other point up to 5wks.	Kaufman 1975[160]
Herpes simplex virus-2 (IP)	Mice Neonatal, sex not specified	Viable BCG Tice (IP and ID). 0.05ml of 1-8x10^8cfu/ml	Immunised=31 Controls=31	2, 4 or 6d	Significant reduction in mortality ($p<0.0005$) when BCG given 6d prior to HSV infection, but not at 2d or 4d. Both IP and ID routes protective. No mortality benefit from pre-treatment with typhoid or Brucella vaccines (non-live).	Starr 1976[161]
Encephalomyocarditis, murine hepatitis, HSV-1 and 2, foot and mouth disease and influenza viruses	Mice Age and sex not specified	BCG Pasteur Inoculation route not specified	Not specified	Not specified	Significant improvement in survival with BCG pre-treatment (41% survival with BCG pre-treatment, 18% survival in controls). Improvement particularly marked for influenza, HSV-1 and encephalomyocarditis.	Floc'h 1976[162] (abstract only, available)
Influenzae A	Mice Adult, female	BCG (IP or IN) dose and strain not specified	BCG IP = 16 BCG IN=18 Control = 18	Various intervals. +/- BCG booster 2d prior to challenge	Significant survival benefit of mice with BCG pre-treatment 4 and 6wks prior to challenge ($p<0.01$), but not at 12wks. IN BCG administration more effective than IP. Booster doses also provided protection ($p<0.05$).	Spencer 1977[163]
***Ectromelia* virus (IP)**	Mice Female, 8wks old	Viable BCG (IP). Strain not specified. 1mg wet weight	Immunised=18 Control=21	21d	Reduction in EV mortality with BCG pre-treatment (6/16 vs. 14/16). Reduction in EV growth in peritoneal exudate and spleen cells. Interferon production significantly higher with BCG-pre-treatment in peritoneal exudate, but lower in liver, spleen and blood (possibly due to lower growth of EV in these organs). Spleen cells from BCG infected animals had an 8-fold greater capacity for in vitro IFN production than controls.	Suenaga 1978[164]

***Vaccinia* virus (IP)**	Mice Male, adult	BCG Connaught (IP/NG). 10^7cfu	Immunised=25 Control=25	7d or 12d	Reduced mortality with IP BCG pre-treatment (3/25 cf. 25/25). No effect with NG BCG pre-treatment. No difference in mortality benefit by time to challenge. Increased vaccinia virus antibody titres with BCG pre-treatment (1:128 vs. 1:512).	Werner 1979[165]
Ectromelia virus (IP/IV)	Mice Female, 8-12wk old	BCG Japan (IP). Viable and heat-killed. 1mg wet weight	Immunised=60 Control=60	4wks or 3mths	BCG pre-treatment improves survival from EV in both splenectomised and normal mice, compared to controls. The survival benefit persists at 1mth post-BCG inoculation but wanes by 3mth. Both HK and viable BCG provide resistance to EV compared to controls. Significantly increased carbon clearance (RES activity), and splenic IFNγ production with BCG pre-treatment. The increased IFN production with BCG was reduced by a) splenectomy, b) anti-thymocyte serum and c) anti-macrophage serum, with the combination reducing IFNγ to control levels.	Sakuma 1983[166]
Fungi						
***Candida albicans* (IV)**	Mice Adult, male Immuno-competent and supressed	BCG Brazil (live attenuated). 10^2, 10^4, 10^6 cfu	Not specified	3, 7, 14 or 28d	Increased mean survival time with BCG pre-treatment in immune-competent and supressed mice, but no decreased overall mortality.	Sher 1975[147]
***Candida albicans* (IV)**	Mice Adult, male	BCG Denmark (IV $5x10^6$cfu) with IP PPD 50μg		1-7d	Reduction in candida in the liver and spleen ($p<0.01$), increased H_2O_2 production (6-fold) and reduced germ tube length ($p<0.01$) in BCG/PPD stimulated macrophages, with BCG pre-treatment 1d-7d before.	Van t'Wout 1992[167]
***Candida albicans* (IV)**	SCID Mice Age and sex not specified	BCG Pasteur 10^6 cfu IV	Immunised=15 Control=15	14d	BCG pre-treatment significantly increased survival from disseminated candidiasis (100% vs.30%, $p<0.005$), decreased fungal burden in the kidney ($p<0.01$) and increased splenic TNFα production following LPS stimulation ($p<0.01$).	Kleinnijenhuis 2012[168]
Candida albicans	SCID Mice vs.	BCG Pasteur	SCID	14d	BCG pre-treatment significantly increases survival from lethal	Kleinnijenhuis

(IV)	NOD/SCID/IL2 Rγ mice (T,B and NK cell deplete) Female mice 6-8wks old	10^6 cfu IV	Immunised=15Control=15 NSG Immunised= 15 control=15		disseminated candidiasis in SCID mice. The protective effect of BCG was partly lost in NSG mice, suggesting a role for NK cells in BCG conferred protection.	2014
Protozoa						
***Trypanosoma cruzi* (IP)**	Mice Adult	Live BCG (strain not specified) (IV). 3mg wet weight	Immunised=10 Control=10	21d	No significant differences in mean survival time or peak parasitaemias. Radiolabelled parasite distribution significantly more in kidneys and spleen with BCG pre-treatment, vs. liver in controls.	Kuhn 1975[144]
***Trypanosoma cruzi* (IP)**	Mice 4-6 week old	BCG Mexico (IV) $4x10^6$cfu	Immunised=10 Controls=10	10d	Decreased mortality (100% vs. 60%), increased survival time (mean 31d vs. 19.4d), reduced blood stream trypanomastigotes in BCG pre-treated group.	Ortiz-Ortiz 1975[169]
***Trypanosoma cruzi* (IP)**	Mice Female, age not specified	BCG Glaxo (IP) 10^5cfu	Immunised=6 Control=6	3d or 18d	Increased *in vitro* macrophage killing of *T. cruzi* in mice pre-treated with BCG ($p<0.05$). No *in vivo* protection against mortality or increased time to mortality in BCG pre-treated mice.	Hoff 1975[143]
***Toxoplasma gondii* (supra-choroidal injection)**	Rabbits Male and female	BCG (IV and retrobulbar)	Immunised IV=10, retrobulbarly =10 Controls=10	14d	Delayed onset and severity of toxoplasma choroidal retinitis following IV BCG pre-treatment.	Tabbara 1975[170]
***Echinococcus multilocularis* (IP)**	Cotton rats Sex and age not specified	BCG Montreal (IP) $26.4x10^6$cfu	Immunised=12 Control=12	1wk 8 animals also received BCG 2wks after pathogen challenge	BCG pre-treatment reduced growth ($p<0.01$) and metastasis ($p<0.005$) of *E. multilocularis*. BCG treatment after established infection does not affect *E. multilocularis* growth, but does significantly reduce metastasis (as measured by number of cystic foci) ($p<0.005$), though to a lesser extent than BCG pre-treatment ($p<0.025$).	Rau 1975[171]
***Babesia microtii* and *rodhaini* (IP)**	Mice Female, 6wk old	BCG Glaxo (IV) $2x10^7$cfu	Immunised=57 Control=57	14d or 28d	BCG pre-treatment protected mice from parasitaemias and lead to rapid clearance at all inoculating doses of Babesia spp. and at 14d and 28d post BCG.	Clark 1976[172]

					BCG pre-treatment reduced circulating Babesia specific antibody levels, likely due reduced parasitaemias.	
***Leishmania donovani* (IV)**	Mice Female, 6wk old	BCG Pasteur (IV/IP). Various doses		30d+14d or 14d+0d	Significantly lower parasite levels in spleens and livers of BCG pre-treated mice ($p<0.01$). BCG given 14d/0d prior to challenge more effective than 30d/14d. Protection greater at when BCG dose 10^7 rather than 10^6, and with IV rather than IP BCG. BCG booster inoculation was also effective therapeutically at reducing parasite burdens of previously infected mice.	Smrkovski 1977[149]
***Schistamsoma mansoni* (percutaneous)**	Mice Female, adult	Viable BCG Tice (IV/IP/SC). Vs. viable BCG Pasteur vs. heat-killed BCG Tice $2x10^7$cfu	Immunised=6 Control=6 For each experimental condition	Various	Halving of schistosomule recovery from the lung and adult worm recovery from the circulation with IV BCG-pre-treatment ($p<0.01$). Rapid protection from *S.mansoni* infection for up to 8wks, following viable BCG pre-treatment. IV BCG given at time-points from 14d before to 3d after challenge protected against *S.mansoni* infection ($p<0.01$). BCG given >10wks before cercarial infection conferred no protection. Protection only induced with high-dose ($2x10^7$ cfu), viable, IV BCG administration. No protection if a) lower dose ($<2x10^5$) b) heat-killed or c) IP/SC administration.	Civil 1978[173]
Schistosoma mansoni	Baboons (Kenyan) 8-10kg, sex not specified	BCG Chicago (SC, IM or ID) dose varied	Immunised = 13 Control = 9	4 or 11d	Sub-cutaneous BCG administration 4 days prior to cercarial challenge lead to a significant reduction in worm burden (38%). IM or ID BCG administration 11 days prior to challenge did not. No sex differential effects. No differences in the ability of monocytes to kill shistosomulae.	Sturrock 1985[150]

***Plasmodium yoelii* (IP)**	Mice Age and sex not specified	BCG Pasteur (SC) 10^6 cfu	Immunised=30 Control=30	2wks or 2mths	BCG pre-treatment 2mths prior to challenge produced significant protection against *P. yoelii* infection ($p<0.05$) and parasitaemia (93% reduction at d16 compared to controls) but not at 2wks prior to challenge. Elimination of CD8 T-cells, reduces the BCG-induced protection ($p<0.05$). Up-regulation of 15 genes including chemokines, antimicrobial peptides and IL-1, following plasmodium infection in BCG-treated mice compared to controls. Treatment with two of these gene products (lactoferrin and cathelicidin-type peptide) reduced plasmodium parasitaemias in the absence of BCG pre-treatment ($p<0.05$).	Parra 2013[174]

IV, intravenous; IP, intraperitoneal; SC, subcutaneous; ID, intradermal; IN, intranasal; IM, intramuscular; NG, nasogastric; HK, heat killed; PPD, Purified protein derivative; BCG, Bacille Calmette Guerin; SCID, Severe Combined Immunodeficiency Disorder; d, days; wks, weeks; mths, months; yrs, years; cfu, colony forming units; MIF, macrophage inhibitory factor; IFN, interferon; TNF, Tumor Necrosis Factor; RES, Reticularendothelial system; DTH, Delayed Type Hypersensitivity; LPS, Lipopolysaccharide; NK, Natural Killer; NSG, NOD SCID gamma mice; HSV, Herpes Simplex Virus;

3.3 Mechanistic studies

There are a large number of published studies in humans that provide information regarding potential immunological mechanisms to explain the observed NSE of BCG vaccination against non-tuberculous pathogens. Many of these studies were designed to investigate the effect of BCG on mycobacteria-specific cytokine production, but also provide information about non-specific stimuli responses from their positive and negative control data. More recently, a number of studies have been designed purposely to investigate the impact of prior BCG on immunological responses to heterologous stimuli. Although there is also an abundance of animal data investigating the immunological mechanisms underlying the NSE of BCG,[175] these are not reviewed here because of the known difficulties in translating animal-based immunological findings to humans, and because of the wealth of more applicable human data available.

3.3.1 The NSE of BCG reported in studies designed to investigate mycobacterial-specific responses.

The majority of studies designed to investigate the effect of BCG on mycobacteria-specific immunogenicity, report no significant differences with heterologous positive control stimuli or with un-stimulated samples:

- Marchant *et al.* compared BCG Glaxo given at birth vs. 2 months of age vs. 4 months in Gambian infants.[176] They showed no difference in interferon-gamma (IFNγ), IL-4, IL-5 and IL-13 production or lymphocyte proliferative responses following 5-day whole blood stimulation with phytohaemaglutinin (PHA), when comparing BCG vaccinated with unvaccinated infants, or early vs. delayed BCG.[177]
- A subsequent RCT conducted in The Gambia comparing BCG Russia given at birth compared to 4.5 months of age also showed no differences in IFNγ, IL-10, IL-13, IL-6 and IL-17 cytokine production, or in CD4+ T-cells, CD4+CD25+ activated T-cells or CD4+CD25+FOXP3+nTreg cells, following 5-day whole blood stimulation with Staphylococcus enterotoxin B (SEB), PHA or Roswell Park Memorial Institute (RPMI) unstimulated growth medium as control stimuli.[178]
- Black and Weir *et al.* report results from several RCTs comparing BCG Glaxo to placebo in Malawian and UK adolescents. They report no significant differences in IFNγ responses in lymphocyte cultures stimulated for 5 days with PHA,

streptokinase/streptodornase (SK/SD) and RPMI up to 12 months post-vaccination[179-181] or TNFα and IL-1β levels from lipopolysaccharide (LPS) stimulated 24-hour whole blood cultures.[182]

- In South Africa, Hussey *et al*. conducted an RCT to investigate the influence of different BCG strains (Danish SSI vs. Japan), vaccination routes and vaccination timings (birth vs. 10 weeks) on mycobacterial-specific immunogenicity.[183] PHA and tetanus toxoid (TT) were used as positive controls in lymphocyte stimulation assays with no significant differences in IFNγ, IL-5 and IL-10 or lymphoproliferative responses shown at 10 weeks post vaccination.
- A subsequent South African study comparing BCG SSI at birth with 10 weeks of age also reported no differences to intracellular TNFα, IFNγ and IL-2 expression, or numbers of polyfunctional T-cells, following 12-hour whole blood simulation with RPMI or SEB, either at 10 weeks (comparing BCG vaccinated vs. unvaccinated infants) or at 12 months (comparing early vs. delayed BCG).[58] Similarly, a large study comparing the mycobacterial-specific immunogenicity of BCG SSI given at birth or 8 weeks of age in HIV-exposed infants in South Africa largely showed no differences in intracellular cytokine staining for IL-2, IL-13, IL-17 and IFNγ following 6-day whole blood stimulation with *Bordetella pertussis*, SEB and TT at either 8 weeks of age (BCG vaccinated vs. unvaccinated) or 14 weeks (early vs. delayed BCG).[184] A tendency toward lower *Bordetella pertussis* stimulated IL-13, and increased CD4+ T-cell proliferation to SEB, was reported at 14 weeks in the early BCG group.
- A small RCT conducted in Turkey investigating the impact of timing of BCG Pasteur vaccination (birth vs. 2 months of age) showed no differences in the production of IFNγ and IL-10 from PBMCs collected at 2 and 8 months of age and stimulated for 5 days with PHA, although a non-significant trend toward higher production of both with BCG vaccination at birth was seen.[185]
- Two UK based case-control studies, one in infants and another in adolescents, also reported no significant differences in the concentrations of 42 different cytokines and chemokines following 7-day whole blood culture with PHA, although a trend toward higher responses for all analytes from BCG vaccinated participants was seen at one month post-vaccination.[186]
- A study conducted in the USA in adults investigating mycobacterial-specific responses to two different BCG strains (Connaught and Tice) in adults, showed no

increased lymphoproliferation following *in vitro* stimulation with tetanus toxoid or RPMI at 1 or 2 months post immunisation.[187]

- An Australian cohort study comparing allergic responses in 7-14 year olds from two adjacent Sydney districts, one providing BCG Glaxo at birth for high-risk individuals and one not, mainly showed no significant differences for IL-4, IL-5 and IFNγ in whole blood stimulated for 48 hours with PHA or RPMI.[76] A significant reduction in IL-10 secretion in response to house dust mite was shown in BCG vaccinated infants, however. The authors also reported significantly lower total IgE levels in BCG vaccinated infants born to atopic parents, compared to those with no history of atopy.

Only a few studies not designed purposely to investigate the NSE of BCG have reported significant differences in non-mycobacterial outcomes. A case-control study conducted in Indian 5-7 year olds showed significantly increased IFNγ production from lymphocyte cultures stimulated with Concanavalin A ($p<0.01$) in children reported to have received BCG (strain unknown) at birth compared to unvaccinated children.[188] This study is at high risk of confounding, however, as it does not describe how cases and controls were chosen or matched, and whether underlying socio-demographic variables were comparable between groups. A longitudinal cohort study of Indonesian infants receiving BCG Pasteur (median age 5 weeks), showed significantly increased IFNγ and significantly decreased TNFα and IL-10 production in whole blood stimulated with PHA, comparing pre-BCG samples with 2 years post-BCG.[189] However, as similar effects were seen following PPD stimulation, and there was no control group, the possibility that these results reflect developmental changes as opposed to BCG-induced NSE is high.

3.3.2 Immunological effects reported in studies designed to investigate NSE of BCG

Studies designed intentionally to investigate the impact of BCG on cytokine responses to heterologous stimuli have tended to report significant effects, although the magnitude and timing of these effects vary.

The first such study was conducted by Ota *et al.* in The Gambia and was designed to investigate interactions between BCG and other routine vaccinations.[57] In this study

infants randomised to receive BCG Pasteur either at birth or at 2 months had significantly increased IL-5, IL-13, IFNγ, lymphoproliferation and antibody levels at 4.5 months of age, in whole blood cultures stimulated for 6 days with Hepatitis B surface antigen, compared to unvaccinated infants. Increased IL-13 was seen in response to tetanus toxoid stimulation at 4.5 months and increased polio antibodies at 2 months in BCG vaccinated compared to unvaccinated infants. No differences in any cytokines were found following PHA stimulation and no lymphoproliferative or antibody changes in response to tetanus or diphtheria toxoids. As Hepatitis B vaccine was given at the time of BCG vaccination, the authors suggest that the results may reflect a priming-ability of BCG when co-administered with other vaccines, a theory that has been backed up by recently published *in vitro* and murine studies.[190] A subsequent study in The Gambia, using BCG Russia instead of BCG Pasteur and comparing BCG vaccination at 6 weeks vs. 18 weeks of age, did not confirm these results, with no significant differences in any expanded programme of immunisation (EPI)-vaccine antibodies shown at 18 weeks of age, comparing BCG vaccinated with unvaccinated infants.[177] A panel of heterologous stimuli including heat-killed pathogens and Toll-like receptor (TLR)-ligands were also used in 16-hour PBMC stimulation assays in this study, with no overall differences in innate cytokines by BCG status shown. Minimal sex-differential effects of BCG vaccination were shown, but these did not persist long-term. The differing results between the two studies could reflect the different BGC strains used and the different BCG vaccine timings. A case-control study conducted in Australia comparing infants receiving neonatal BCG (Japan or SSI) with unvaccinated infants, also failed to confirm the findings of the initial Ota study, showing reduced anti-Hepatitis B surface antigen antibodies at 7 months of age in infants who had received BCG at birth ($p=0.03$).[191] The study did suggest some heterologous effects of prior BCG vaccination on EPI vaccine responses, however, as there was a trend toward increasing anti-pneumococcal, anti-*Haemophilus influenzae* type B (HIB) and anti-tetanus toxoid; this was significantly raised for the pneumococcal capsular polysaccharide antigens 9v ($p=0.01$) and 18c ($p=0.04$). Different blood sample timings, BCG strains and routine immunisation schedules may account for these differences, although it should be noted that the hepatitis B schedule was the same in both studies. As the Australian study was a case-control study, the possibility of unaccounted for population differences between the BCG vaccinated and unvaccinated infants explaining differences in antibody levels remains. Infants in the study were only BCG vaccinated if their parents were originally from a country with high TB-incidence

whereas BCG unvaccinated infants were recruited from routine Australian vaccination clinics. No information about the ethnicity or sociodemographic characteristics of the two groups was reported. Another case-control study conducted in The Philippines compared infants receiving standard BCG at birth (strain not reported), with BCG received after the first set of routine immunisations, also showed some effect of BCG on responses to EPI vaccine antigens, though differing again from both The Gambian and Australian results.[192] Infants with prior BCG vaccination showed significantly increased IFNγ production on 48-hour whole blood stimulation with TT and inactivated polio vaccine antigens (p=0.046), but no differences following PHA, hepatitis B Surface antigen or RPMI stimulation. Increased IFNγ+/TNFα+/CD4+/CD45RO+ T-cells (p=0.0018) and a trend toward lower circulating FoxP3+CR45O+regulatory CD4+ T-cells was also seen in BCG vaccinated infants following *in vitro* PMA/ionomycin stimulation. The authors suggest that this provides evidence of a Th1-polarising effect of neonatal BCG vaccination upon heterologous stimulation, but the results need to be interpreted with caution as the infants not receiving BCG at birth were out-born and from communities living far from health-care facilities which may be very different to children receiving BCG vaccination at birth (as is standard in The Philippines). The children were age and sex-matched, however, and there were no significant differences in infant weight-for-age, maternal age at delivery or educational attainment. Other studies looking at the impact of prior BCG vaccination on EPI vaccine antibody responses include studies from Denmark, Guinea-Bissau and South Africa, all of which showed no effect.[61, 193, 194] Two recent randomised controlled trials using live-attenuated viral vaccines as pathogen challenge models to assess the NSE of prior BCG SSI vaccination in Dutch adults have also shown contrasting results in terms of antibody production. Leentjens *et al.* reported significantly increased haemaglutinin antibodies and a tendency toward more rapid seroconversion in participants receiving BCG SSI 14 days prior to trivalent influenza vaccine.[195] However, no differences in circulating yellow fever antibody levels were shown in adult males given BCG SSI or placebo 1 month prior to live-attenuated yellow fever vaccine, despite lower levels of viraemia in the BCG vaccinated group.[196] Thus, the evidence for BCG mediating its NSE through alterations in specific antibody production is not clear.

A number of studies have used panels of *in vitro* heterologous stimuli, including heat-killed pathogens and specific TLR-ligands, to investigate the NSE of BCG on cytokine

production. The Aaby group conducted several of these studies, in Guinea-Bissau and Denmark. The first such study investigated the effects of BCG revaccination in 19-month old infants in Guinea-Bissau.[197] This showed no significant differences overall in IFNγ, IL-13, tumour necrosis factor α (TNFα) or IL-10 following PHA or LPS stimulation *in vitro* from whole blood samples taken either 11 weeks or 5-9 months post-vaccination, comparing BCG SSI revaccination at 19-months of age with none, although a trend toward increased IL-10 levels was suggested. During the course of the study a national DTP-immunisation catch-up campaign occurred, meaning that some study participants also received DTP during the study. A significant reduction in the TNFα/IL-10 (pro/anti-inflammatory) ratio was seen in male infants who had received a DTP booster by the time their blood was sampled, compared to those who had not (test for interaction p=0.03), suggesting that in male infants DTP vaccination may reduce a pro-inflammatory effect of BCG vaccination. This differential effect of DTP was not seen in female infants.

As part of a further RCT of the impacts of early vs. delayed BCG in low birth weight infants in Guinea-Bissau (original study described in 'Clinical trials' section above), Jensen *et al.* conducted an immunological sub-study investigating the heterologous effects in blood samples taken 4 weeks post-BCG SSI at birth or none (prior to any EPI vaccinations).[193] The production of IL-1β, IL-5, IL-6, IL-10, IL-17, TNFα and IFNγ in whole blood following 24-hour stimulation with a panel of TLR agonists was assessed. BCG vaccinated infants had significantly increased IL-6, TNFα and IFNγ following Pam3CSK4 (TLR2/1) stimulation and IL-6/IFNγ production following PMA/ionomycin stimulation. Levels of TNFα and IFNγ were also higher in the unstimulated cultures of BCG vaccinated compared to unvaccinated controls. There was a tendency toward stronger effects in BCG vaccinated females, although only IL-1β in response to Pam3CSK4 showed a statistically significant sex-differential effect. The ratios of pro:anti-inflammatory cytokines were also significantly increased following heterologous stimulation, for both monocyte-derived cytokines (TNFα:IL-10) and T-cell derived cytokines (IFNγ:IL-5), most significantly for Pam3CSK4, but also for CLO75 (TLR7/8) for monocyte-derived cytokines and PMA for T-cell derived cytokines. There was no overall difference in leucocyte differentials by BCG vaccination status, although BCG vaccinated females had significantly increased total leucocyte, monocyte and basophil counts. The study, therefore, suggests that BCG may mediated its non-specific beneficial effects in neonates by increasing pro-inflammatory

cytokine production and the pro:anti-inflammatory cytokine ratio following secondary stimulation with TLR2/1 and TLR7/8 agonists, and some suggestion of an increased effect in girls.

In contrast, studies conducted more recently by the group in Denmark have overall reported no significant differences in heterologous stimulated cytokine production or EPI-antibody production at 4 days, 3 months or 1 year post-randomisation, comparing neonatal BCG SSI vaccination with none.[194, 198] The authors did report a tendency toward increased TNFα:IL-10 ratio in the BCG vaccinated group to all non-specific stimuli, similar to their study in Guinea-Bissau, although this was not statistically significant and did not vary by sex. The authors also reported a tendency toward increased cytokine production to all non-specific stimuli and trend toward increased *Bordatella pertussis/*pneumococcal antibodies in infants who received their neonatal BCG vaccination between 2-7 days of age rather than 0-1 day, although this was a post-hoc analysis. The lack of significant immunological results in this study correlates with a lack of clinical findings. Interestingly, IFNγ induction by BCG-stimulation was also low in the vaccinated group, suggesting poor mycobacterial-specific responses in this setting, although the detection of IFNγ may not have been optimal. This may reflect changes to BCG SSI growth characteristics with recent batches[199] or genetic differences of Danish children with children from Guinea-Bissau.

Studies conducted in the UK have also used a panel of heterologous pathogens and TLR-ligands to interrogate the NSE of BCG vaccination on cytokine production. Smith *et al.* randomised UK infants to receive BCG SSI at 6 weeks of age or none and took blood samples 4 months later for 48-hour whole blood stimulation with LPS, Pam3CSK4, *C.albicans*, *S.aureus* and *E.coli*.[200] This showed increases in epidermal growth factor (EGF), eotaxin, IL-6, IL-7, IL-8, IL-10 and IL12p40, monocyte chemo-attractant protein-2 (MCP-2), macrophage inflammatory protein 1a (MIP-1a), CD40L and platelet derived growth factor-AB/BB (PDGF-AB/BB), and decreases in IL-2, IL-13, IL-17, granulocyte-macrophage colony stimulating factor (GMCSF), GRO and IFNγ-inducible protein 10, in various combinations for different non-specific stimuli. EGF, IL-6 and PDGF-AB/BB were commonly higher after Pam3Cys, *C.albicans* and *S.aureus.* Monocyte activation markers did not differ by vaccination status, but significant increases in NK cell activation markers were seen (CD69) in response to Pam3Cys, which correlated with the magnitude of its stimulated IL-12p40 and IL-10 response. The finding of altered cytokine production with prior BCG vaccination,

particularly in response to Pam3Cys, is similar to the findings of Jensen *et al.* in Guinea-Bissau, although the cytokines affected were different. Particularly, no effects of heterologous stimuli on TNFα or IL-1β production were seen in the Smith study. Again, the differing vaccination/blood sample timings and ethnicity of the participants may have affected results, and there may have been interactions with routine immunisations, which were received in the UK study, but not in Guinea-Bissau.

A recently conducted RCT in Australia is more comparable in design to Jensen *et al*'s study. This study randomised infants to BCG SSI at birth or nil and followed up for clinical allergic outcomes, with immunological sub-studies conducted to investigate the NSE of BCG. The first immunological results, from samples taken at 7 days post-randomisation and stimulated for 20 hours with heat-killed *E.coli, H.influenzae, S.aureus,* Group B Streptococcus (GBS), *S. pneumonia, L.monocytogenes, C.albicans*, peptidoglycan (TLR-2 agonist), Pam3CSK4, resiquimod (TLR-7/8 agonist) and RPMI have recently been published. These showed increased background IL-6 and IL-1ra in unstimulated samples, but decreased IL-1Ra, IL-6, IL-10, MIP-1α, MIP-1B and MCP-1 after TLR2 and TLR7/8 stimulation, and decreased MCP-1 with heterologous pathogen stimulation. Thus, the authors suggest that there is an overall decreased anti-inflammatory response to heterologous pathogens, on a background of increased pro-inflammatory cytokines. They suggest that this might produce a pro-inflammatory bias upon heterologous pathogen challenge with prior BCG vaccination. The overall conclusions of this study were similar to studies conducted in Guinea-Bissau and the UK, but the stimuli and cytokine/chemokine effects were different. The authors also reported a sex-differential effect on MIF with decreased levels in BCG vaccinated males and increased levels in BCG vaccinated female infants. Stronger effects were reported in infants receiving their BCG after 48 hours of age, which is comparable with the results from Nissen *et al.*, in Denmark.

Perhaps the most exciting mechanistic studies investigating the NSE of BCG have been those conducted by the Netea group in The Netherlands. These studies have suggested that BCG can 'train' the innate immune system to increase cytokine production from monocytes, and possibly natural killer (NK) cells, in response to heterologous pathogens, by inducing long-term epigenetic modifications at the promoter region of pro-inflammatory cytokines. This was first shown in humans in a longitudinal study of 29 Dutch adults, comparing stimulated cytokine responses before and at 2 weeks, 3

months and 1 year following BCG SSI vaccination. In this study PBMCs stimulated for 24 or 48 hours with heat-killed *S.aureus* or *C.albicans* showed significantly increased TNFα and IL-1β (~2 fold higher) at 3-months post-BCG compared to baseline.[168] Production was also increased at 2 weeks, though non-significantly. *E.coli* LPS showed the same trend, although this was only significant for IL-1β production. The increased cytokine production was associated with significantly increased histone-3 lyseine-4 trimethylation (H3K4me3 - a stimulatory epigenetic modification) at the promoter regions of TNFα and IL-6 ($p<0.05$, IL-1β data not reported), and corresponding increases in mRNA expression of TNFα and IL-1β (IL-6 data not reported), following *in vitro* stimulation with *S.aureus* and *C.albicans* at 2 weeks and 3 months post-BCG. Corresponding *in vitro* studies incubating PBMCs with BCG to 'train' them, showed that the increased cytokine production to heterologous pathogens, induced by BCG, was entirely abrogated by addition of a methylation inhibitor. Similar trends toward increased NK cell cytokine production following heterologous stimulation were seen at 2 weeks and 3 months post-BCG vaccination, being significant for IL-1β production, although epigenetic modification and mRNA expression data from NK cells were not reported.[201] Although no changes in monocyte or NK cell numbers were seen, monocyte activity markers CD14 and CD11b were significantly increased post-BCG vaccination. As a whole this evidence strongly suggests that the NSE of BCG are mediated by epigenetic modifications that train the innate immune system to respond in an up-regulated manner in response to heterologous invasive pathogens. In further support of these findings, the group showed that BCG was entirely protective against disseminated Candidiasis in mice without a functioning adaptive immune system (SCID mice), suggesting that the effects are mediated through alterations in innate immunity. Mice lacking both T/B cells and NK cells had intermediate protection from prior BCG vaccination, confirming that monocytes and NK cells are both likely to play a role in BCG-mediated heterologous protection, at least against Candida. Follow-up studies by the group showed that the cytokine potentiation in monocytes and NK cells had largely disappeared 1 year after BCG vaccination, but significant increases in Th17-derived IL-17 were still seen in response to *S.aureus* and *C.albicans* stimulation, with similar though non-significant responses in IL-22.[202] Th1 derived IFNγ was also significantly increased by *S.aureus,* though not *C.albicans*, at one year of follow-up. These findings may suggest that early NSE of BCG are mediated through influences on the innate immune system, with longer term effects mediated more through epigenetic modification of adaptive cells. In fact another study by the Netea group using γ-

irradiated BCG failed to show increases in IL-6 or IL-1β with heterologous stimulation, although significant increases in IFNγ and IL-22 were shown.[203] This suggests that live-replicating BCG is required to produce training effects on the innate immune system, with non-live BCG mediating effects mainly through adaptive immunity. This might, hypothetically, provide an explanation for the differential timing of effects seen post-BCG vaccination in the Kleinninjuis studies.[168, 202] Training of the innate immune system to respond in an up-regulated manner to heterologous pathogens might occur whilst live-BCG circulates post-vaccination. When BCG has been killed by the host immune system, effects mediated through the adaptive immune system take over. Although the persistence of viable BCG post-vaccination is not known, animal studies have reported circulating BCG vaccination for up to 16 months post vaccination.[204] The longitudinal nature of the original studies, and the fact that subjects were chosen from a travel clinic where they presented for BCG prior to overseas travel, means that potentiating effects of non-tuberculous mycobacterial exposure might confound the results. However, subsequent randomised controlled studies conducted by the same group have confirmed increased IL-1β, IL-6 and TNFα production in response to *in vitro* heterologous stimulation at 1-month post-randomisation, in BCG vaccinated compared to placebo vaccinated adult males. The increase in IL-1β was epigenetically mediated (H3K27me3 - this correlates with H3K4me3 but is a more dynamic mark) and strongly predicted subsequent reductions in *in vivo* yellow fever viraemia following live-vaccine challenge.[196] Interestingly, the BCG pre-treated group had lower levels of circulating pro-inflammatory cytokines following yellow fever vaccination, though the authors suggest that this may be due to lower circulating viral loads.

3.3.3 Summary: immunological mechanisms of the NSE of BCG

Immunological studies exploring mechanisms underlying BCG NSE are varied in their conclusions. A systematic review of studies published prior to January 2014 was undertaken at the request of the World Health Organization.[205] This concluded that the current evidence supported a heterologous effect of BCG, particularly with respect to increased IFNγ production. No strong sex-differential effects or interactions with other EPI vaccinations were found. The heterogeneity of study design and outcomes measured precluded meta-analysis, however, and no alterations to BCG vaccination guidelines were made.

Many of the studies designed purposely to investigate the NSE of BCG have been published subsequent to the above systematic review. Overall these studies support the hypothesis that BCG induced protection against heterologous pathogens occurs via upregulation of pro-inflammatory cytokines and chemokines (or alteration of the pro/anti-inflammatory cytokine balance). There are strong suggestions that this is mediated through epigenetic modification of monocytes +/- NK cells. However, the magnitude, direction and duration of cytokine responses to non-mycobacterial stimuli are extremely heterogeneous between studies. This may reflect differences in BCG strain used, participant ages, population genetics, blood sample timings, stimulants and blood cell types used for assays, assay durations and the impact of routine immunisations. Importantly, the epigenetic studies have all been carried out in adults from high-income settings, and whether similar effects occur in low-income neonatal populations has not been investigated.

Whether BCG produces heterologous effects on the adaptive immune system is less clear. Many of the studies investigating mycobacterial-specific responses of BCG that reported non-specific responses from positive and negative control stimuli used prolonged *in vitro* stimulation assays. Cytokine levels in these studies are likely to be more reflective of adaptive cell activation and tended to show no difference, or increases only in IFNγ in response to heterologous stimulation. Alterations in adaptive cell numbers, distribution or activity markers have generally not been described. Although alterations in antibody production to EPI vaccinations has been shown in some studies with prior BCG vaccination these have tended to be weak and inconsistent effects.

3.4 Possible effect modifiers of the NSE of BCG

As has been alluded to in the reviews of the clinical and immunological evidence surrounding the NSE of BCG, several studies have suggested that the magnitude and/or durability of effects may be influenced by a number of factors.

3.4.1 Interactions with other routine immunisations and age at immunisation

Although the evidence reviewed for this thesis concerns the NSE of BCG, the theory extends to other routine immunisations. The Aaby group has proposed that live vaccines (particularly BCG, measles and OPV, but also smallpox) have non-specific beneficial effects in terms of all-cause mortality, but that inactive vaccines (particularly the alum-containing DTP vaccines) have negative non-specific effects, increasing all-cause mortality, particularly in girls (see 'Effect modification by sex' section below). This highly controversial theory was first proposed following a trial of measles vaccine in Guinea-Bissau, where all-cause female mortality was shown to be two-fold higher in infants receiving high-titre measles vaccine at 5 months of age than in infants who received standard-titre measles at 10 months of age.[206] Subsequent analysis showed that altering the timing of measles vaccination in the study had resulted in many infants receiving DTP after measles vaccine, and the higher mortality was confined to these infants.[207] Longitudinal cohort studies from the group also suggest divergent mortality rates by vaccination status, with infants who have not received DTP vaccination having a lower mortality rate than DTP vaccinated infants of the same age.[13] This pattern reverses for BCG and measles vaccinations, with infants missing vaccinations having increased all-cause mortality rates. This finding appears consistent, if not always significantly so, in other studies.[38] The finding of altered patterns of mortality rates by vaccination type is one of the strongest arguments against there being unmeasured confounders that unduly affect the interpretation of epidemiological studies on the NSE of vaccinations, because a confounder such as frailty bias is unlikely to act in an opposing way for different immunisations. However, the evidence for routine immunisations other than BCG having non-specific effects is limited by the ethical difficulties in conducting RCTs of established immunisations, and the resultant reliance on epidemiological studies at high risk of bias.[208] No RCTs of DTP immunisations have been conducted to date. Meta-analysis of epidemiological studies of DTP showed a tendency toward a negative effect of DTP for all-cause mortality (RR 1.38 (0.92-2.08)),

which became significant when one study at very high risk of bias was excluded (RR 1.53 (1.02-2.30)).[38] The WHO has concluded that the observational evidence is currently insufficient to recommend a change of policy but recommends that further high quality trials are conducted.[208] Further studies are particularly important as the number of antigens received concurrently with DTP in the EPI-schedule has increased, all of which may have interacting NSE. For measles vaccine epidemiological studies overall suggest strong beneficial effects, particularly in girls. However, a meta-analysis of four randomised controlled trials was not statistically significant (RR 0.74 (0.51-1.07)), with low numbers of deaths and short follow-up limiting conclusions.[38] Potential NSE of live oral polio vaccine (OPV) may be of particular interest, as it is given concurrently with both BCG and DTP containing regimes in many areas of the world. The Aaby group has conducted a number of studies utilising the impact of national OPV catch-up days to investigate its NSE.[209-213] These studies tend to suggest a beneficial NSE of OPV for all-cause mortality particularly in children <6 months, although one study notably suggested increased male infant mortality with OPV given at birth.[212] A recent RCT from the group suggested that BCG and OPV at birth in normal birthweight infants produces increased beneficial NSE, with lower all-cause mortality prior to other EPI vaccine administration at 6-weeks of age (HR 0.65 (0.45-1.0)) compared to BCG alone.[214] These effects were particularly strong in boys.

If routine immunisations do have differing NSE, then the timing of vaccinations may influence the overall effect of mortality. The Aaby group have proposed that administration of a live vaccine, either with or shortly after inactive/DTP containing vaccines may abrogate their negative NSE. Higgins *et al*., in their meta-analysis comparing various vaccination regimes (BCG before DTP vs. BCG after DTP vs. BCG with DTP), showed a consistent trend to mortality benefit when BCG was given with or after DTP, compared to DTP after BCG.[38] These findings were corroborated in a recently published re-analysis of a study from Bangladesh.[15] Conversely, the three trials of early vs. delayed BCG vaccination in Guinea-Bissau suggest that early BCG (before DTP) is superior to BCG given with DTP, although the marked early effects on neonatal deaths may account for this. Revaccination with BCG at 19 months of age in Guinea-Bissau (theoretically after all routine-EPI vaccinations) did not show any non-specific beneficial effects, although the occurrence of a national DTP catch-up campaign during the study was thought to have confounded results.[197]

The rapid developmental changes that occur to the immune system in the first year of post-natal life mean that altering the age at which BCG is administered may theoretically modify its beneficial NSE quite apart from affecting its interactions with other routine immunisations. Although neonates produce robust Th1 responses to BCG when it is given at birth[215] a number of studies have investigated whether delaying administration might enhance anti-tuberculous protection. Trials delaying BCG from birth have tended to show larger scar formation,[55, 216] enhanced magnitude and duration of tuberculin/PPD reactivity[55, 216, 217] and enhanced Th1 cytokine and memory T-cell responses,[58, 183] although not exclusively.[178] However, as most deaths in infancy occur in the neonatal period, if BCG does reduce all-cause mortality non-specifically then neonatal administration may still have the greatest overall benefit, even if the actual immunological effect is lower. The trials of early BCG administration in LBW infants in Guinea-Bissau support this, with much of the protective effect of BCG being confined to the neonatal period, as do epidemiological studies.[38] Notably, trials comparing BCG vaccination with placebo at later time-points have tended not to report significant effects.[218]

3.4.2 Sex

Males and females differ in their immunological responses.[219] In general, adult females have stronger innate and adaptive immune responses compared to adult males. These result in greater protection against many infectious pathogens and a reduced incidence of malignant cancers but increased rates of autoimmune disorders, particularly during the reproductive years. Sex differences are mediated through a combination of hormonal, genetic and epigenetic mechanisms, although environmental factors such as health-seeking behaviour also alter the clinical *sequalae* of such differences.

Due to altering hormonal status throughout the life-course, the influence of sex on immune response also varies. Although this is less well studied, evidence from cord blood suggests that male infants may produce more robust immune responses than female infants, with increased numbers of CD8+ T-cells, monocytes, basophils and NK cells,[220, 221] increased innate cytokine production to LPS stimulation[222] and increased IgE levels. Cord blood from female infants shows increased CD4+ T-cells and a higher CD4+/CD8+ ratio than male. There is no current evidence of differences in T-reg cells by sex.[223]

Sex differences in the response to vaccines are well documented.[218] Antibody responses to childhood vaccines against hepatitis B, diphtheria, pertussis, rabies, pneumococci, human papilloma virus, and to the RTS,S malaria vaccine are greater in females.[224] Adverse reactions are also reported more frequently and have greater severity in females compared to males, suggesting stronger immune responses.[218] Whether these immunological differences translate to altered clinical protection against vaccine-specific diseases is harder to ascertain, although females have lower attack rates of influenza, hepatitis A and hepatitis B post-vaccination, and males have lower clinical disease post-pneumococcal vaccine.[218]

Evidence for a sex-differential effect of BCG in neonates, either specifically or non-specifically, is debatable. Many early studies either did not undertake, or did not mention, analysis by sex. Several large meta-analyses of TB-specific clinical protection afforded by BCG have not interrogated the impact of sex,[225, 226] neither has one investigating IFNγ specific immune responses.[225] Male infants in The Gambia have larger scars and TST responses than females,[59] though mycobacterial-specific Th1/Th2 responses have not been shown to differ between sexes in infants.[227] A recent analysis of the durability of PPD-induced IFNγ responses from childhood vaccination showed a weak trend toward higher long-term responses in adult males.[228]

That the NSE of vaccines may act differently in males and females was proposed by the Aaby group from the earliest days of the theory. In general, they proposed that both the beneficial effects of live vaccines, and the detrimental effects of inactive vaccines, occur to a greater extent in females than males. The WHO-sponsored analysis of NSE studies up to 2014 found evidence to suggest that beneficial effects of measles vaccine were stronger in females than males, but concluded that there was not enough evidence regarding BCG and DTP vaccinations.[38] Table 3.5 summarises the current evidence for BCG having sex-differential effects. The majority of studies investigating sex effects were conducted by the Aaby group and have varying results, most tending to suggest no effects. Epidemiological studies suggest that if sex-differential beneficial effects do exist for BCG, they act by modifying a negative effect of subsequent DTP in females. Conversely, clinical studies suggest that males have early non-specific benefits from BCG and that females have later benefits, but provide no clear evidence for a subsequent interaction with DTP vaccination. Immunological studies have produced varying sex-differential results.

Table 3.5. Evidence of sex-differential effects of BCG

Evidence for sex-differential effects of BCG	RCTs	Epidemiological studies	Immunological studies
NSE of BCG more in females	Meta-analysis of three Guinea-Bissau studies: BCG vaccinated female infants MRR 0.56 (0.31-1.0) vs. unvaccinated, in weeks 2-4 post vaccination. No significant differences at 1 week post-vaccination.[35]	No difference with BCG vaccination alone but DTP reduces beneficial effect in girls with F:M MRR increased post-DTP but equal pre-DTP. Shown in studies from India[11, 16, 23] Malawi[22] and Senegal.[31] ALRI hospitalisation more in unvaccinated females than vaccinated. No effect of BCG on male hospitalisations in Guinea-Bissau.[62]	Guinea-Bissau.[193] Increased total leucocytes, monocytes and basophils with BCG vaccination in females only. Tendency to increased pro-inflammatory cytokine responses in females (significant for IL-1β to Pam3Cys and IFNγ to PPD). Australia.[92] Higher MIF in BCG vaccinated females than unvaccinated.
NSE of BCG more in males	Meta-analysis of three Guinea-Bissau studies: Male infants MRR in first week of life 0.36 (0.20-0.67) in BCG vaccinated vs. unvaccinated. No significant differences after.[35]		Australia.[92] Lower MIF in BCG vaccinated males than females to intracellular heterologous stimuli
No sex-differential NSE of BCG	No significant effect reported in any individual trial of BCG: Guinea-Bissau[35, 51, 52, 54] Denmark[36]	Guinea-Bissau[8, 20, 21, 24, 65, 66] Burkino-fasso[9] Bangladesh[15] Papua New Guinea[12, 30] Uganda[32] Denmark[7] Greenland[67]	The Gambia.[218] Some sex-differential immunological changes were shown over time but minimally impacted by BCG and not persistent. In this study BCG Russia was used and infants were vaccinated at 6wks not birth. Denmark[194, 198] No sex-differential effects on pro-inflammatory cytokine production at 4d, 3mth or 13mth of age or antibody production at 13mth of age after BCG at birth vs. nil.

RCT, randomised controlled trial; NSE, non-specific effects; BCG, Bacille Calmette Guerin; MRR, mortality rate reduction; DTP, Diptheria-Tetanus-Pertussis; F:M, female:male; ALRI, acute lower respiratory tract infection; IFN, interferon; PPD, purified protein derivative; MIF, macrophage migration inhibitory factor; d, days; wks, weeks; mth, months;

3.4.3 Strain and batch differences

Calmette and Guérin first developed BCG by serial passage from a virulent *Mycobacterium bovis* strain in 1921. Since then it has been estimated to have been given more than 4 billion times.[229] When it was first used widely, lyophilisation techniques were not available. Live BCG was therefore distributed around the world to be grown and maintained for local use. With continual serial passage and genetic mutations, more than 14 sub-strains of BCG developed world-wide.[230] In the 1960s lyophilisation techniques became available and BCG seed-lots were produced, with a maximum of 12 serial passages from each seed-lot recommended, which reduced further deviation from the original BCG. Strains that have remained in use for routine immunisations today can be divided into groups, according to when they mutated away from the original strain, and therefore how genetically similar they are to it:

- Early (Group 1): BCG Moscow (Russia – also used in BCG India and BCG Bulgaria), BCG Tokyo (Japan)
- Mid (Groups 2 and 3): BCG Glaxo, BCG SSI (Danish)
- Late (Group 4): BCG Pasteur (Paris)

Of these, only BCG Danish, Japan and Russia are WHO-prequalified vaccines and hence provide much of the worldwide supply. Other strains include Moreau (Early), Sweden, Berkhaug, Jena (mid), Connaught (Toronto) and Tice (Chicago) (late). These are no longer in routine clinical use, apart from Connaught and Tice, which are used for bladder cancer immunotherapy only.[137]

The genetic variation of BCG strains has led to concerns that this may result in downstream variation in mycobacterial-specific, and non-specific, immunogenicity.[231] *In vitro* studies suggest that earlier strains are more immunostimulatory than later strains.[232] Murine studies suggest that protection against pulmonary TB and delayed-type hypersensitivity reactions are stronger from BCG Pasteur and BCG SSI strains, compared to BCG Glaxo and BCG Japan.[233] Comparison of cytokine production, lymphocyte proliferation and CD8+ T-cell cytotoxicity in mice has also suggested that protective activity is higher in BCG Pasteur compared to BCG Glaxo and BCG Russia, although BCG Russia showed the highest cytotoxicity.[234, 235] A more recent guinea-pig model comparing early with late strains suggested that TB-specific protection did not vary greatly by strain.[236] Studies conducted in infants in South Africa comparing BCG SSI with BCG Japan suggested higher lymphoproliferative and cytotoxic responses in

the later.[183, 237] A RCT comparing BCG strains in Australian neonates showed significantly higher mycobacterial-specific polyfunctional CD4-Tcells in infants immunized with BCG Denmark or BCG Japan than with BCG Russia (p=0.018 and p=0.003 respectively).[238] Infants immunised with BCG Japan in this study had the highest levels of soluble Th1-cytokine production. BCG Pasteur and BCG Danish strains have long been reported as more 'reactogenic' with increased reports of ulceration at vaccination sites, suppurative lymphadenitis and local lymphadenopathy compared to BCG Japan, BCG Glaxo or BCG Moreau strains.[239] Studies in Guinea-Bissau suggested that infants vaccinated with BCG Russia developed a scar less frequently than those with BCG Danish (87% vs. 97%).[240] Comparison of neonatal vaccination with BCG Danish, BCG Russia and BCG Bulgaria (a sub-strain of BCG Russia) was also made in a longitudinal cohort study of infants in Uganda.[42] Presence of a scar at one year of age was significantly increased in infants receiving BCG Danish ($p<0.0001$), as were BCG related adverse events (p=0.03). Specific IFNγ and IL-10 responses were higher in BCG Danish vaccinated infants, as were IFNγ, IL-10 and IL-13 responses to PHA stimulation. A large RCT in neonates in Hong Kong showed a 45% (22%-61%) reduction in TB incidence following BCG Pasteur, rather than BCG Glaxo administration.[241] A cohort study in Kazakhstan showed a 69% (61%-75%) reduced risk of TB diagnosis following BCG Japan, compared to 43% (31%-53% and 22% (7%-35%) following BCG Serbia and BCG Russia respectively.[242]

Thus, in general, *in vitro*, murine and human studies suggest that there are variations in BCG-induced immunogenicity by strain. On balance the evidence suggests greater immunogenicity and reactogenicity with BCG Japan, BCG Pasteur and BCG SSI compared to BCG Russia and BCG Glaxo. This may explain some of the differing results seen in clinical and immunological studies of the NSE of BCG. How important these differences are, however, remains to be seen. A meta-analysis of studies investigating human TB-specific protection from BCG has shown limited evidence of strain effects.[225] Neither of the two WHO-commissioned systematic reviews of the clinical[38] and immunological[205] NSE of vaccines investigated the effect modification of strains. Notably, the majority of studies reporting NSE of BCG have used BCG SSI or BCG Pasteur as the immunising strain, and many studies showing limited effects have used BCG Russia or BCG Glaxo (see Tables 3.1, 3.3 and 3.5). The most common strain in use worldwide at present is BCG Russia.

Aside from strain differences, batch variations amongst the same strain may also influence the specific and non-specific effects of BCG. This was highlighted in a report from the Aaby group.[199] They noted that known differences in the growth characteristics of batches of BCG SSI used in their clinical trials were associated with different specific and non-specific effects of vaccination. Infants immunised with slow-growing batches of BCG had larger scars and PPD reactions at one year, than those with normal growing batches. Monocytes primed *in vitro* with slow growing BCG SSI had increased IL-6 and TNFα ($p=0.03$) production following secondary heterologous stimulation, compared to monocytes primed with normal growing batches. This has led to concerns that the large beneficial NSE of BCG reported in their original low birthweight infant RCT may have been a batch specific effect, although the recent publication of confirmatory results from a subsequent trial (using BCG with normal/fast growth), suggests that this may not be a major issue.[53]

3.4.4 Maternal BCG vaccination and TB exposure

Interactions between maternal immunity and neonatal vaccine responses are well documented. The presence of maternal antibodies in early life may block the development of immunological memory and protective responses for vaccines such as measles and tetanus, which have primarily antibody mediated immunological protection.[243] This has previously been thought to be less of a concern for BCG vaccination, as antibody production has not been considered an important component of the specific immune response and strong Th1 mediated reactions are produced when BCG is given in the neonatal period.[244] However, there is increasing evidence to suggest that maternal BCG vaccination or latent TB infection (LTBI) may modify the subsequent specific and non-specific immune responses in infants to BCG. Work conducted by the Ugandan group has shown that maternal LTBI is associated with significantly reduced infant anti-mycobacterial T-cell responses at one-week post-BCG vaccination.[245] In contrast, pro-inflammatory cytokine production in response to both mycobacterial specific and non-specific antigens is increased in cord blood and samples taken 1 and 6 weeks post-BCG vaccination, in infants born to mothers with a BCG scar.[246] (Appendix 1. Mawa *et al.*[246]). Corresponding up-regulation of interferon and inflammatory pathway gene expression was also shown in these infants. Immunological results from the Australian RCT investigating the NSE of BCG on allergic outcomes

have similarly suggested that cytokine production in response to heterologous stimuli is altered by maternal BCG vaccination status, with a tendency toward higher pro-inflammatory cytokine production in BCG vaccinated infants born to vaccinated mothers.[92] Clinical evidence for an interaction between maternal and infant BCG vaccination was reported in The Danish Calmette study, which showed reduced illness presentations exclusively in BCG vaccinated infants of vaccinated mothers (IRR 0.62 (0.39-0.98)).[36] However, no effect of maternal BCG status on the allergic or immunological effects of BCG was described.

The mechanisms by which a potentiating effect of maternal BCG on NSE of infant BCG might occur are obscure, but may include *in utero* priming,[247] or trans-generational epigenetic modification.

3.4.5 Micronutrient supplementation

The possibility of interactions between routine immunisations and potentially immuno-modulatory vitamin supplementation (especially vitamin A), have been highlighted by the Aaby group, who have conducted a number of studies to investigate this.[248] No conclusive evidence for an interaction with BCG has been found,[38] and as neonatal vitamin A supplementation is no longer recommended worldwide[249] (Appendix 2, Frontiers article) this will not be further discussed.

3.4.6 Latitude

A comprehensive meta-analysis of studies investigating mycobacterial-specific immunogenicity of BCG has shown large variations in protection against pulmonary TB worldwide.[225] Protection tends to be highest in latitudes closer to the poles, with reduced efficacy toward the equator. The reasons for this are unclear but may include higher environmental mycobacterial exposure in equatorial/tropical countries, genetic differences and differences in maternal exposure/vaccination. It is possible that the NSE of BCG are similarly variable, although current evidence does not suggest that this is the case, with some beneficial effects described both clinically and immunologically in temperate and equatorial countries.

3.5 Summary, statement of existing problems with the evidence and rationale for conducting further investigations.

That BCG may have beneficial NSE against all-cause mortality has been suggested by animal studies, epidemiological studies and randomised controlled trials. However, there are a number of outstanding problems with the evidence, which need to be addressed before the theory can be accepted sufficiently to produce policy changes:

1. Are the NSE of BCG globally applicable?

One group, working in an area of extremely high infant mortality, has produced much of the data supporting the NSE of BCG and other vaccines. A trial from the same group in a high-income country has failed to show any benefit. In fact, in their three major, related trials showing benefit of early BCG, infants were low birthweight, and thus a particularly high-risk group. Whether these results translate to normal-weight infants, even in areas of high infant mortality, has yet to be confirmed.

2. What immunological mechanisms underlie the NSE of BCG in neonates?

Experiments in adults in high-income countries suggest that the NSE of BCG may be induced by epigenetic modification of monocytes and NK cells to produce long-term changes in innate cytokine production in response to heterologous stimuli. However, whether similar effects occur in the context of the rapid immunological development of neonates, and particularly in high-mortality settings where exposure to other pathogens may be theorised to produce similar, confounding effects, has never been studied.

3. What is the optimal timing for BCG for overall non-specific benefit?

High-quality, trial evidence regarding the best timing of BCG vaccination for overall benefit, given its possible interactions with other EPI vaccinations, is not available.

4. Are there sex-differential effects of neonatal BCG?

Most studies investigating the NSE of BCG have not investigated a possible sex-differential effect. Studies reporting sex-differential effects have produced contrasting results, and in many of these studies it was not clear whether the decision to analyse by sex was built into the study design, or was a post-hoc finding. If vaccinations have different effects on all-cause mortality by sex, this may argue for the need for different vaccine schedules for boys and girls.

It may be argued that as current WHO BCG immunisation guidelines recommend that infants in areas with high rates of tuberculosis are BCG vaccinated as soon as possible after birth,[250] further investigation of the NSE of neonatal BCG are futile as they are unlikely to change BCG vaccination policy. There are a number of reasons why continued investigations into the NSE of BCG are imperative:

- Although recommended at birth in high TB-incidence settings, BCG vaccination is often delayed well beyond the first day of life for logistical reasons. Deliveries outside of health-care settings remain commonplace in many areas of the world. In these cases, first contact with a trained health-care provider may be significantly delayed, often occurring at the 6-week health check. Combined estimates of vaccination timings in low-income countries suggest that BCG vaccination may be delayed past the neonatal period in nearly half of all infants.[251] Even with earlier presentations or delivery within a health-care setting, timely receipt of BCG vaccination is not guaranteed. UNICEF-procured BCG formulations supplied to the majority of high-burden settings come in 20-dose vials, which expire within 4 hours of opening. Vaccinators are often advised not to open a vial if only a small number of infants require BCG, but to ask them to return to clinic at a later date, again often at the 6-week immunisation visit. Recent global manufacturing issues for BCG have also led to prolonged durations of stock-outs within countries. If BCG does provide protection against all-cause mortality, even small delays to receipt may be important in the high-risk neonatal period.
- In low-burden areas (average annual risk of TB <0.1%), BCG vaccination of neonates is generally limited to groups at high risk of TB or omitted entirely in favour of intensified case detection and supervised early treatment.[250] If BCG does protect against heterologous invasive infectious disease, then neonatal administration for high-risk infants in these settings (e.g. low birthweight babies in neonatal intensive care settings) may also prove to be of benefit.
- Protection afforded by BCG against pulmonary TB is notoriously poor in high-incidence settings (although it does provide good protection against meningeal and miliary TB in childhood).[252] Alternative immunisation strategies against TB are therefore being sought. If a superior vaccination for TB-specific protection is discovered, BCG use might be phased out worldwide. However, if it does

provide substantial protection against all-cause mortality and invasive infectious disease, then its maintenance in immunisation regimes would be essential.

Thus, addressing some of the issues surrounding the evidence for a non-specific effect of BCG may influence policy decisions regarding vaccination, with resultant impacts on global child health.

4. Rationale for the studies conducted and their design

To investigate whether BCG vaccination in neonates can protect infants against heterologous infectious disease by stimulating the innate immune system, I conducted two trials:

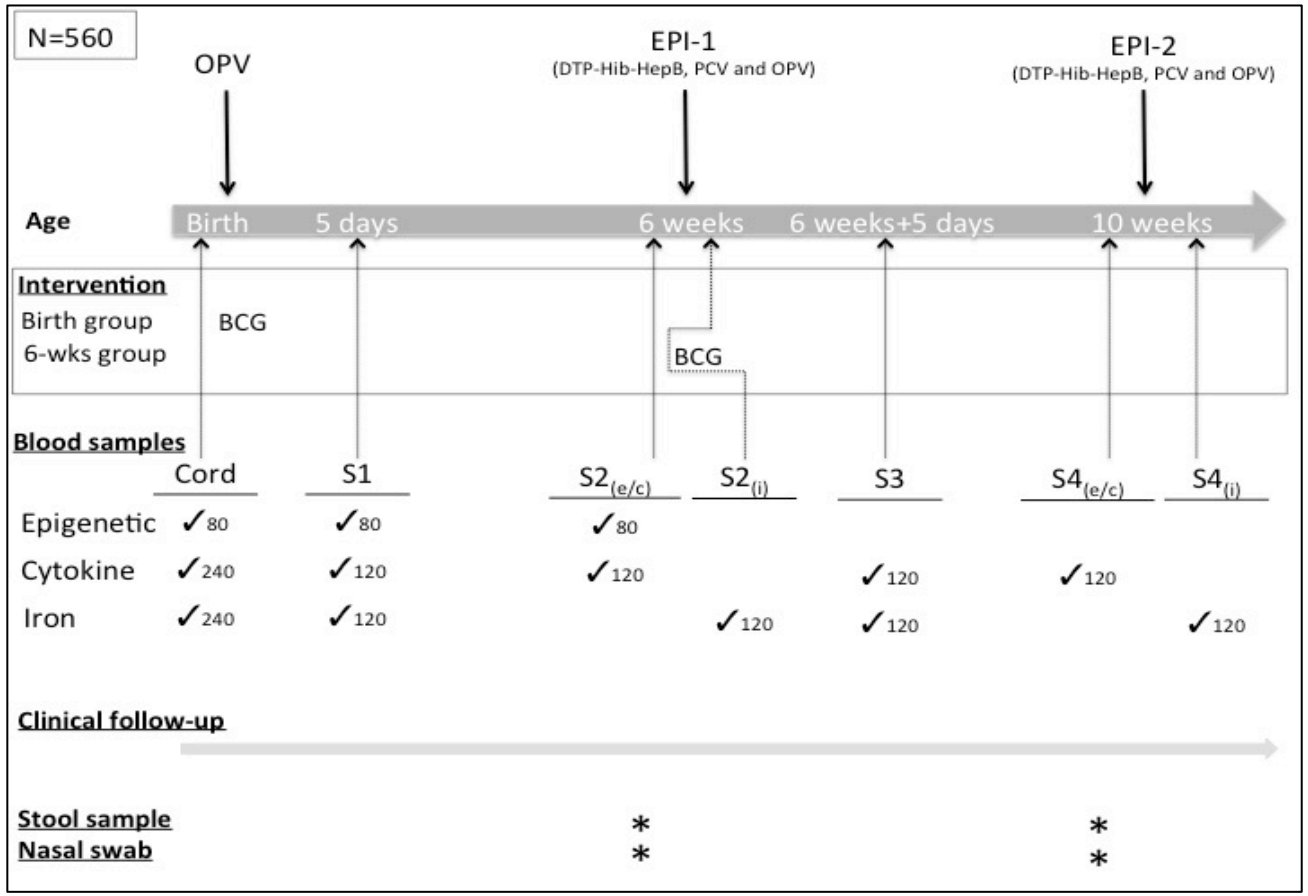
- A pilot study conducted in The Gambia to investigate the novel theory that BCG may ultimately mediate its non-specific protective effects by innate immune system-induced reductions in serum iron. The rationale for, and design of, this study is discussed in Chapter 5.
- A larger randomised controlled trial in Ugandan neonates. This trial forms the main part of the thesis. The rationale for, and design of, the main study is addressed below, with the detailed methods and results of the study described in Chapters 6 and 7.

These studies were specifically designed to address some of the outstanding issues with the evidence for the NSE of BCG in neonates, principally: its global applicability, identification of a putative biological mechanism for such effects, exploration of sex-differential effects and exploration of the impact of vaccination timings and interactions.

4.1 Study Design: Main trial

I conducted an investigator-blind randomised controlled trial comparing BCG vaccination at birth, with BCG vaccination at 6 weeks, in healthy Ugandan neonates. The schema for the trial is shown in Figure 4.1.

Figure 4.1. Study Schema; main trial



n, participant numbers; OPV, oral polio vaccine; EPI, expanded programme of immunisations; DTP, diphtheria-tetanus-pertussis; Hib, haemophilus influenza type B; HepB, hepatitis B; PCV, pneumococcal vaccine; BCG, Bacille Calmette Guerin; S1, blood sample time-point 1; S2(e/c), blood sample time-point 2 in the epigenetic/cytokine sub-studies; S2(i) – blood sample time-point 2 in the iron sub-study; S3, blood sample time-point 3; S4(c), blood sample time-point 4 in the cytokine sub-study; S4, blood sample time-point 4 in the iron sub-study

4.1.1 Study population

Healthy Ugandan neonates were recruited to this study. This provided a geographically distinct setting from the majority of previous studies, whilst still being an area with high infant mortality and infectious disease incidence. Any infant well enough to be discharged directly from hospital, without the need for medical intervention, was eligible for recruitment, regardless of their gestational age and birthweight. This was to provide 'real-world' estimates of BCG effects. Low birthweight and premature infants were not excluded, because previous studies had suggested that BCG may be particularly effective in these populations.[35] Other exclusion and inclusion criteria and their rationale are described in Table 4.1.

4.1.2 Primary outcomes

The primary outcomes for the study were immunological. The evidence available at the time of study design suggested that in adults, NSE of BCG were mediated through epigenetic modification of monocytes, leading to increased innate cytokine production in response to heterologous stimulation.[168, 201, 253] Whether this occurs similarly in neonates was not known and was therefore interrogated in this study, with additional investigations as to whether alterations in innate cytokine production might have down-stream effects on the inflammatory iron-axis. Ethical considerations limiting blood sample volumes in research studies conducted in neonates meant that it was not possible to investigate all immunological outcomes for every infant. Therefore infants were recruited to one of three sub-studies utilising the same overall study schema. A brief overview of these sub-studies and their rationale is provided below, with detailed description of the methods used found in Chapter 6.

Table 4.1. Exclusion criteria

Exclusion Criteria	Explanatory notes
Mother or father not interested in the study or withholding consent	
Expected residence outside Wakiso district study area during the 10-week study period	Previous studies conducted in the area had problems with retention of participants, as many mothers in urban areas travel to their parent's home for support during the immediate post-natal period.
Mother known to be HIV positive	Infants born to mothers with HIV have different immunological responses to routine vaccinations than infants born to mothers without HIV, even if they remain uninfected.[253]
TB risk: Mother known to have TB or Household contacts known to have TB or Mother or household contacts with clinical features suggestive of TB: • Cough>2 weeks • Recent haemoptysis • >3kg of unintended weight loss in past month • Recurrent fevers/chills or night sweats for the past 3 days or more.	As half of our study infants would receive BCG vaccination delayed to 6 weeks of age, they would be at greater risk of acquiring TB during this period. Previous studies in the study area showed that in children, the vast majority of TB infections were transmitted from household contacts.[254] This is likely to be particularly true of neonates, where the cultural norm is to keep them largely within the home for the first few months of life. A fuller discussion of the ethical arguments surrounding the delay of BCG vaccination are given in the supplementary sections of the published Trials article (paper 3).[249]
Complicated delivery (including C-section) or infant unwell at delivery/before randomisation or infant born with major congenital malformations	These exclusion criteria were for the following reasons: • The aim of the study was to investigate the impact of BCG on healthy infants. • BCG vaccination is often delayed until the child is well (often >24 hours), and thus the impact of early BCG would be more difficult to assess in these children. • Early immunological samples may be altered due to the reason for the infant to be unwell, confounding the ability to detect the impact of BCG.
Cord blood not collected	Cord blood was the baseline sample in this study, being the only pre-intervention sample. It was therefore deemed critical for the interpretation of the changes to infant immune responses over time by BCG vaccination status.

1. Epigenetic sub-study

Aims

To compare histone-3-lyseine-4 trimethylation (H3K4me3) and histone-3-lyseine-9 trimethylation (H3K9me3) at the promoter regions of pro-inflammatory cytokines (TNFα, IL-6 and IL-1β) in whole blood of BCG vaccinated and naïve infants.

Primary objectives

Cross-sectional between-group comparison of epigenetic modification in PBMCs collected at:

1. Cord blood (baseline)
2. 6 weeks of age (pre-routine immunisations) ($S2_e$)

Note: blood samples were also collected at 5 days of age, but were not analysed for this thesis due to funding and time constraints.

Secondary objectives

Comparison of within-infant changes to epigenetic modification over time by BCG vaccination timing

Rationale

This sub-study was designed to investigate whether the training effects of BCG on innate immunity previously shown in Dutch adults also occur in neonates in a high-mortality setting.[168]

The epigenetic marks were chosen as they provided a stimulatory (H3K4me3) and inhibitory (H3K9me3) mark. These marks had previously been shown to be important in BCG-induced trained immunity in adults, as had the pro-inflammatory cytokines chosen.[168]

PBMCs was used in these experiments, as opposed to the monocytes used in adult experiments, due to low neonatal sample volume.

The blood sampling points chosen allowed the longer-term influences of BCG on epigenetic modifications to be assessed, comparing BCG vaccinated with naïve infants,

prior to the potential confounding influence of EPI vaccinations. Further time-points were not collected due to funding constraints.

2. Cytokine sub-study

Aims

Comparison of TNFα, IL-1β, IL-6, IFNγ and IL-10 production in whole blood stimulated for 24 hours with *Staphylococcus aureus, Streptococcus pneumonia, Escherichia coli, Candida albicans*, polyinosinic:polycytidylic acid (Poly I:C) and CpG-oligodeoxynuclotides (CpG ODN), Purified Protein Derivative (PPD) and RPMI, between infants who were BCG vaccinated at birth and infants who were BCG vaccinated at 6 weeks.

Primary objectives

Cross-sectional between-group comparison of *in vitro* inflammatory cytokine production following heterologous pathogen stimulation in whole-blood collected at:

1. 5 days of age (S1).

   Comparison of BCG vaccinated vs. naïve, short-term effects post-vaccination

2. 6 weeks of age, pre-EPI-1 vaccinations ($S2_c$).

   Comparison of BCG vaccinated vs. naïve infants, longer-term effects post-vaccination

3. 6 weeks + 5 days of age (post-EPI-1 vaccinations and BCG in 6-week group) (S3)

   Comparison of early BCG vs. delayed BCG, short-term effects post-vaccination

4. 10 weeks of age, pre-EPI-2 vaccinations ($S4_c$)

   Comparison of early vs. delayed BCG, longer-term effects post-vaccination

Secondary objectives

Comparison of within-infant changes with inflammatory cytokine production following *in-vitro* heterologous stimulation over time, by BCG vaccination timing.

Rationale

Training experiments using BCG in Dutch adults suggest that epigenetic modification at the promoter region of pro-inflammatory cytokines leads to increased production of these cytokines in response to heterologous pathogens *in vitro.*[168] Investigation of both epigenetic modifications and resultant heterologous cytokine production in this study was not possible in the same infant, due to limitations in blood sample volumes. Therefore a separate cohort of infants was recruited to investigate this.

The heterologous stimuli used were chosen to represent a range of pathogen types (gram positive and gram negative bacteria, fungal and viral-type stimulants), and to provide consistency with the adult studies (which used *S.aureus* and *C.albicans*).

Whole pathogens were chosen in preference to specific Toll-like receptor ligands in an attempt to better mimic *in-vivo* conditions. A similar argument explains the use of whole blood, rather than separated PBMCs, along with the limitations in neonatal sample volume.

The chosen cytokines reflected those previously investigated in Dutch adults (monocyte derived cytokines TNFα, IL-6 and IL-1β) with IFNγ to assess Th1 T-cell responses, and IL-10 as more representative of Th2/anti-inflammatory responses. Evidence of non-specific effects in NK cells had not been published at the time of study design, so IL-17 and IL-22 were not measured.

The blood sample time-points were chosen to enable investigation of both the short (S1 and S3) and longer-term ($S2_c$ and $S4_c$) effects of BCG vaccination on heterologous cytokine production, and exploration of the impact of BCG timing and interactions with EPI vaccinations (see 'Rationale for blood sample timings' section below).

3. Iron sub-study

Aims

Comparison of the inflammatory-iron axis following *in-vivo* heterologous inflammatory stimulation between infants receiving BCG at birth vs. BCG at 6 weeks of age.

Primary objectives

Cross-sectional between-group comparison of transferrin saturation (TSAT), hepcidin, IL-6 and ferritin levels in whole blood at:

1. 5 days of age (S1).

   Comparison of BCG vaccinated vs. naïve, short-term effects. Unstimulated sample

2. 6 weeks of age, 1 day post-EPI-1 vaccination (but pre-BCG vaccination in the delayed group) ($S2_i$).

   Comparison of BCG vaccinated vs. naïve infants, longer-term effects. *In vivo* stimulated sample.

3. 6 weeks + 5 days of age (post-EPI-1 vaccinations and BCG in 6-week group) (S3)

   Comparison of early BCG vs. delayed BCG, short-term effects. Unstimulated sample.

4. 10 weeks of age, post-EPI-2 vaccinations ($S4_i$)

   Comparison of early vs. delayed BCG, longer-term effects. *In vivo* stimulated sample.

Secondary objectives

Comparisons of within-infant changes in TSAT, hepcidin, IL-6 and ferritin following *in-vitro* heterologous stimulation over time, by BCG vaccination timing.

Comparisons of cross-sectional and within-infant changes over time in other elements of the inflammatory-iron axis, red blood cell indices and leucocyte counts.

Rationale

Induction of hepcidin production in response to pro-inflammatory cytokines reduces serum iron, limiting its availability for pathogen growth and virulence.[255] This could be a downstream mechanism by which increased innate cytokine production from BCG-trained monocytes exerts a protective effect non-specifically against invasive heterologous pathogens (see Chapter 5: Pilot study section). This may be particularly important in the perinatal period due to high iron flux.

Hepcidin, the primary regulator of iron homeostasis, is produced in the liver. Alterations to the iron-inflammatory axis can therefore not be measured following *in vitro* non-specific stimulation. A safe *in vivo* heterologous stimulant was required to investigate the impacts of BCG on the inflammatory-iron axis. Although not ideal, because of potential interactions with the NSE of BCG, routine immunisations received at 6 weeks and 10 weeks of age were chosen as *in vivo* non-specific stimuli. Confirmation of their inflammatory effects on the immune system is described in Chapter 6. Other potential *in vivo* pathogen challenge models, such as live yellow fever vaccine, are not licensed for use in neonatal populations.

The blood sample time-points were chosen to enable investigation of both the short (S1 and S3) and longer-term ($S2_i$ and $S4_i$) effects of BCG vaccination on heterologous cytokine production, as well as comparison of unstimulated and *in vivo* stimulated samples. The time-points also allowed exploration of the impact of altered BCG vaccination timings on its NSE on the iron-inflammatory axis.

4.1.3 Secondary outcomes

Infants were actively followed-up for all-cause morbidity and mortality during the 10-week study duration by:

- Open access to physician review at the study clinic where investigations and medications were provided free of charge
- Weekly telephone follow-up, with recommendation to attend clinic if there were parental concerns about participant illness
- Interview at routine clinic visits regarding interim illnesses for which the participant was not reviewed in clinic
- Physician review at all routine clinic visits for current illness
- Close links to the neighbouring district general hospital allowed presentations of participants directly to hospital to be identified.

The study was not powered to look at clinical end-points, due to the funding and time constraints incumbent on PhD studies. However, the combined number of participants in the immunological studies provided 80% power to detect a ≥40% difference in all-cause illness events in this setting.

Nasal swab samples and stool samples were taken at the 6- and 10-week study visits (prior to routine-immunisations), to allow future investigations of the impact of neonatal BCG on the microbiome and mucosal immunity.

4.1.4 Rationale for the intervention and blinding strategy used

BCG SSI 1331 (BCG Danish) was used in this study. It was chosen to provide homology with other clinical trials investigating the NSE of BCG[35, 36, 175] and the adult studies in The Netherlands that suggested trained-immunity as the immunological mechanism underlying the NSE of BCG.[168]

A single batch was used throughout the study (113033c), to mitigate against possible between-batch differences in immunogenicity.[199]

A placebo vaccination was not used in this study for ethical reasons: a) to minimise unnecessary painful procedures in neonates and b) so that mothers were aware of the vaccination status of their child, meaning that any unvaccinated child lost to follow-up would be likely to receive BCG in the community. The latter argument also underlies why mothers were not blinded to vaccination status of the participant. All investigators were blinded to intervention status (see Chapter 6 for detailed methods). As the primary end-points were immunological, it was reasoned that maternal blinding should not unduly alter these outcomes. For the secondary, clinical outcomes, maternal knowledge of vaccination status might alter health-care seeking behaviour, and thus the number of attendances for clinician review. It was presumed that clinician blinding should ensure that the numbers of physician-diagnosed infectious illnesses was not majorly influenced by maternal knowledge of vaccination status.

4.1.5 Rationale for vaccination timings

A 6-week delay in BCG was used for the delayed group for several reasons:

- Infants who do not receive BCG at birth in low-income settings are most likely to receive it at 6 weeks of age when other routine immunisations are received. The comparison of BCG at birth with BCG at 6 weeks of age therefore had real-world significance. It was not considered ethical to delay it beyond when it might reasonably be given, for TB-specific protection.
- Other clinical trials of early vs. delayed BCG vaccination have tended to delay administration to 6 weeks, allowing direct comparison of results.
- Administration with EPI vaccinations allowed some exploration of their potential confounding influence.

Due to the need for EPI vaccinations to be used as *in vivo* non-specific stimulants in the iron sub-study, BCG was administered one day after EPI vaccinations in this sub-study, as opposed to their concurrent administration in the epigenetic and cytokine sub-studies.

4.1.6 Rationale for blood sample timings

Cord blood was collected in all infants to provide a baseline, pre-vaccination, blood sample for all infants in this study.

Post-natal blood collection time-points were at 5 days of age (S1) and 6 weeks of age (S2) to allow comparison of short and longer-term NSE of BCG, comparing BCG vaccinated vs. naïve infants. Similar time-points were conducted following BCG vaccination at 6 weeks (6 weeks + 5 days (S3) and 10 weeks (S4)). This allowed investigation of the short and longer-term NSE of BCG, by comparing early vs. delayed administration.

The timings of the longer-term blood samples (S2 and S4) were chosen because they were the longest durations available without the potentially confounding influences of routine immunisations. This explains the differences in weeks between BCG vaccination for S2 (6 weeks) and S4 (10 weeks). Timings of these samples varied between sub-studies. In the epigenetic and cytokine sub-studies blood was taken pre-EPI immunisations (and pre-BCG in the delayed group at S2), to avoid potential confounding effects. As EPI immunisations were used as *in-vivo* non-specific stimuli in the iron sub-study, $S2_i$ and $S4_i$ were taken one day after EPI (but pre-BCG in the delayed group at S2).

For ethical reasons each infant was only sampled at two out of the possible four post-natal time-points. The blood sampling time-points that an infant was assigned to were randomly allocated (see Chapter 6, Randomisation section) allowing within-infant changes over time to be compared.

4.1.7 Rationale for study numbers

Sample size calculations were made for each individual sub-study, based on evidence available at the time of study design (in 2013). The total study numbers, and therefore the numbers available for investigation of clinical illness outcomes, was the summation of those required for the three sub-studies.

Epigenetic sub-study: n=80

The only previous study available to base sample size calculations on was the Kleinnijenhuis study in Dutch adults,[168] which required 20 subjects followed longitudinally. 40 subjects were recruited to each intervention arm in this study to allow for attrition and due to the requirement of 2ml blood for epigenetic analysis, which was unlikely to be obtained for all subjects.

Cytokine sub-study: n=240

Due to paucity of published data in this area, an approach based on standard deviation (SD) change in average population cytokine levels was used. 48 subjects per intervention group were needed at each blood sampling time-point to show a 0.66 SD change in average population cytokine levels at 90% power and 5% significance. 60 infants per intervention group per time-point were recruited to allow for attrition. As each child was bled at a maximum of two post-natal time-points, double the required infants were recruited to provide samples at all four post-natal time-points. Thus, 240 infants in total were recruited:

$$\frac{\text{60 infants x 2 intervention groups x 4 time-points}}{\text{Each child bled at 2 time-points}} = \text{240 infants}$$

Iron sub-study: n=240

Sample size determination was performed using TSAT, as it is the only primary outcome parameter of clinical relevance. Average neonatal TSAT in low-income settings is 55%.[256] Substantial responses in this end-point would be required to support its role on the causal pathway of the NSE of BCG. 50 infants per group at each time-point were needed to show a 30% reduction in TSAT (reduction to average adult levels in low income settings) at 90% power and 5% significance. 60 subjects were recruited to each intervention group at each time-point to allow for attrition. As with the cytokine sub-study, each child was bled at a maximum of two post-natal time-points. Therefore double the number of required infants were

recruited to provide samples at all four post-natal time-points. Thus, 240 infants in total were recruited:

$$\frac{\text{60 infants x 2 intervention groups x 4 time-points}}{\text{Each child bled at 2 time-points}} = \text{240 infants}$$

Overall sample size: n=560

Based on data from a previous study in Entebbe[257] 560 infants would provide 80% power to detect a ≥40% reduction in physician diagnosed invasive infections with $p<0.05$. The effect of BCG was felt unlikely to be this pronounced, but it was reasoned that these preliminary data should provide sufficient evidence to determine whether there were indications of differences by group, which may support further investigation in a larger study.

Recruitment to the study was stratified by sex, to allow for analysis of any sex-differential effects of BCG.

5. Preliminary study: the inflammatory-iron axis in neonates and the effect of vaccinations

The possibility that some of the NSE of BCG might be mediated via impacts on the inflammatory-iron axis has never previously been considered. As discussed in the introductory sections of the following two papers, iron is an essential element for the growth and virulence of the majority of human pathogens.[255] In adults and older children, regulatory mechanisms reduce serum iron during infections, limiting its availability to pathogens. Increases in innate cytokines (particularly IL-6, but also IL-22, IL-1 and IFNα)[258] induce production of the hormone hepcidin, which decreases serum iron by reducing uptake in duodenal enterocytes, and locking circulating iron in macrophages until the inflammatory challenge has receded. As studies in adults have suggested that epigenetic modification of the promoter region of pro-inflammatory cytokines such as IL-6 and IL-1β in monocytes, and IL-22 in NK cells, can lead to long-term up-regulation of their production in response to heterologous stimuli,[168, 201] it was theorised that effects on the inflammatory-iron pathway might be one of the effector mechanisms by which BCG might mediate its NSE. This might be particularly important in the early neonatal period (when the beneficial NSE of BCG appear to be most concentrated), as it is a period of high iron-flux resulting from the breakdown of the excess red cells that occurs during transition from fetal to neonatal life.

As this had never been studied before, I conducted a small trial in The Gambia to investigate:

1) Whether the inflammatory-iron axis was intact in early neonatal life (previous studies had been conducted only in cord blood, and had not shown strong correlations between hepcidin and iron parameters) (Paper 1[259])
2) Whether the inflammatory-iron axis was affected by BCG, OPV and Hepatitis B in the first 4 days of life (Paper 1)
3) Whether alterations in serum iron might play an important role in innate immune protection against common neonatal pathogens (Paper 2. Prentice *et al.,* Submitted to JAMA Paediatrics)

5.1 Paper 1: The effect of BCG on iron metabolism in the early neonatal period: a controlled trial in Gambian neonates.

London School of Hygiene & Tropical Medicine
Keppel Street, London WC1E 7HT
www.lshtm.ac.uk

Registry
T: +44(0)20 7299 4646
F: +44(0)20 7299 4656
E: registry@lshtm.ac.uk

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SECTION A – Student Details

Student	Sarah Prentice
Principal Supervisor	Stephen Cose
Thesis Title	Investigating the Non-Specific Effects of BCG in Neonates

If the Research Paper has previously been published please complete Section B, if not please move to Section C

SECTION B – Paper already published

Where was the work published?	Vaccine		
When was the work published?	June 2015		
If the work was published prior to registration for your research degree, give a brief rationale for its inclusion	Not applicable		
Have you retained the copyright for the work?*	Yes	Was the work subject to academic peer review?	Yes

e

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SECTION C – Prepared for publication, but not yet published

Where is the work intended to be published?	
Please list the paper's authors in the intended authorship order:	
Stage of publication	

SECTION D – Multi-authored work

For multi-authored work, give full details of your role in the research included in the paper and in the preparation of the paper. (Attach a further sheet if necessary)	Please see attached sheet

Student Signature: Prentice **Date:** 25/03/2018

Supervisor Signature: **Date:** 25/03/2018

I was responsible for the study design, statistical analysis and manuscript preparation in collaboration with AMP. I conducted the participant recruitment and clinical follow-up with assistance from ES. I conducted the inflammatory-iron parameter laboratory analysis with MJW.

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Brief report

The effect of BCG on iron metabolism in the early neonatal period: A controlled trial in Gambian neonates

Sarah Prentice[a,*], Momodou W. Jallow[b], Andrew M. Prentice[b], MRC-International Nutrition Group[b]

[a] Department of Clinical Research London School of Hygiene and Tropical Medicine, Keppel Street, London WC1E 7HT, UK
[b] MRC International Nutrition Group, MRC Keneba, The Gambia

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ABSTRACT

Bacillus Calmette-Guerin (BCG) vaccination has been reported to protect neonates from non-tuberculous pathogens, but no biological mechanism to explain such effects is known. We hypothesised that BCG produces broad-spectrum anti-microbial protection via a hepcidin-mediated hypoferraemia, limiting iron availability for pathogens.

To test this we conducted a trial in 120 Gambian neonates comparing iron status in the first 5-days of life after allocation to: (1) All routine vaccinations at birth (BCG/Oral Polio Vaccine (OPV)/Hepatitis B Vaccine (HBV)); (2) BCG delayed until after the study period (at day 5); and (3) All routine vaccinations delayed until after the study period.

Vaccine regime at birth did not significantly impact on any measured parameter of iron metabolism. However, the ability to detect an effect of BCG on iron metabolism may have been limited by short follow-up time and high activation of the inflammatory-iron axis in the study population.

1. Background

The possibility that BCG vaccination might protect neonates against non-tuberculous infections has been suggested by two randomised controlled trials [1,2] and numerous epidemiological studies [3–7]. However, the theory has failed to gain acceptance, partly due to the lack of a putative biological mechanism to explain such effects. The randomised trials indicated that protection was strongest within 3 days post-vaccination thus implicating an effect on innate immunity [2]. We theorised that BCG might mediate its heterologous effects by stimulating an iron-withholding response, as part of an acute phase reaction to vaccination.

Iron is critical for the growth and virulence of the majority of human pathogens [8]. The acute phase response produces a rapid reduction in serum iron limiting its availability for pathogens. This hypoferraemia is thought to be primarily orchestrated by IL-6 (and possibly other inflammatory cytokines) up-regulating hepcidin in the liver. The iron-regulatory hormone hepcidin acts on macrophages and enterocytes to internalise the transmembrane iron-transporter protein ferroportin. This sequesters circulating iron within macrophages and reduces enteric absorption of dietary iron.

The kinetics of iron metabolism in the early neonatal period are poorly described, but it is believed to be a period of high iron flux. Fetal red cell mass is higher than post-natally [9], with excess erythrocytes broken down in the first few days following birth. Difficulties metabolising the haem component of haemoglobin are commonly seen in neonates, in the form of jaundice. High iron loads may contribute to the enhanced risk of infections that occur during the neonatal period, exemplified by the 20-fold increased risk of *Escherichia coli* sepsis that occurred in Polynesian infants following provision of iron dextran at birth [10]. Thus, reduction of serum iron as an innate immune strategy to limit the growth of pathogens may be particularly beneficial in the neonatal period.

The effects of BCG, and other vaccines, on the inflammatory-iron pathway in humans are unknown. Several lines of evidence, however, suggest that impacts on this pathway do occur: (1) BCG is a strong inducer of IL-6 [11] and other innate cytokines [12] *in-vivo*; (2) live-vaccinations similar to BCG produce strong up-regulation of hepcidin in fish [13]; and (3) BCG in guinea-pigs leads to a rapid bacteriostatic hypoferraemia [14].

Abbreviations: BCG, Bacillus Calmette-Guerin; EDTA, ethylenediaminetetraacetic acid; ELISA, enzyme linked immunosorbant assay; HBV, Hepatitis B Vaccine; IL-6, interleukin 6; OPV, oral polio vaccine; TSAT, transferrin saturation.

* Corresponding author. Tel.: +44 207 958 8125.
E-mail addresses: sarah.prentice@lshtm.ac.uk (S. Prentice), mwjallow@mrc.gm (M.W. Jallow), Andrew.prentice@lshtm.ac.uk (A.M. Prentice).

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We therefore conducted a proof-of-principal controlled trial in Gambian neonates to investigate the impact of BCG, and other vaccines received at birth, on iron metabolism in the first five days of life.

2. Methods

80 healthy Gambian neonates were randomly allocated to receive BCG (Danish Strain 1331, Batch 11023B, 0.05 ml intradermally into the left deltoid) either at birth, or after completion of study procedures at five days old. All other routine immunisations (Oral Polio Vaccine (OPV)) and Hepatitis B Vaccine (HBV) were given at birth as normal. A data manager not directly involved in the study, conducted randomisation using Microsoft Access, upon delivery of an eligible infant. Blocked randomisation using blocks of six with a 1:1 allocation ratio was used. Due to concerns regarding the potential confounding influence of OPV and HBV at birth, a third non-randomised group of 40 infants was subsequently recruited and received all vaccinations after completion of study procedures at five days of age. Recruitment ran from May 2013 until February 2014, with the first two, randomised groups, recruited during both rainy and dry seasons, and the third non-randomised group recruited during the dry season.

All participants had a 2 ml baseline venous blood sample taken within 24 h of delivery, prior to receipt of any vaccinations, and a further 2 ml venous blood sample taken either 24–48 or 72–96 h post-intervention. Blood was collected directly into microtainers (Becton–Dickson: 0.5 ml collected into EDTA containing tubes, 1.5 ml into lithium–heparin containing tubes) from the dorsum of the hand. Full blood counts were assessed from EDTA blood using the automated Medonic analyser. Lithium–heparinised blood was centrifuged for 4 min at 3600 g within 4 h of collection and the plasma stored at −70 °C until analysis. Iron parameters were measured using the automated Cobas Integra 400 plus (Roche Diagnostics). Plasma hepcidin was measured in duplicate, using a 1:20 dilution by competitive ELISA (Bachem-25, USA) with detection range 0.02–25 ng/ml. Plasma IL-6 was measured in duplicate using a 1:2 dilution by competitive ELISA (BD OptEIA, Oxford, UK), with detection range 0.49–250 pg/ml. Samples with readings outside the linear portion of the curve were re-run at alternative dilutions. Values below the limit of detection were imputed using limit of detection/$\sqrt{2}$. Any samples with an intra-assay co-efficient of variance >15% were re-analysed.

Demographic, birth details and anthropometry were collected at enrolment. Due to the rural nature of the study site, all births were vaginal. Deliveries and follow-up visits were conducted at the participant's home.

Full informed consent was obtained from mothers antenatally by a trained midwife. Inclusion criteria were (1) Consenting mother (2) Residence within the study area. Exclusion criteria were (1) Infant weighing <2000 g (2) Maternal HIV or TB (3) TB contact in the home (4) complicated delivery (5) major congenital anomaly (6) infant unwell as judged by a doctor or a midwife. The Consort flow diagram for the study can be found as supplementary material.

Clinical investigators and mothers were not blinded to intervention allocation due to lack of feasibility (BCG produces a visible reaction) and for safety, so that any mothers would be aware of the vaccination status of the child. Laboratory investigators were blinded to intervention allocation, with assays conducted by anonymous study number. Data were analysed using Stata Version 11.0. Categorical variables were compared using the chi-squared test and continuous variables by one-way ANOVA. Hepcidin and IL-6 results were not normally distributed and were log-transformed prior to comparison. Intervention allocation code was not broken until the data were cleaned and locked.

As this study was a small proof-of-principal trial, with short follow-up and no clinical endpoints, no data safety monitoring board was appointed. Safety data were monitored in real time by clinical investigators who were not blinded to intervention allocation. There was no significant difference in incidence of serious adverse events by intervention allocation group (see Table 1).

Ethical approval was obtained from the joint Gambia Government/MRC Unit The Gambia ethics committee (Ref: SCC1325) and the London School of Hygiene and Tropical Medicine ethics committee (Ref: 012-045). This trial was conducted according to the principles of the Declaration of Helsinki.

3. Results

Baseline demographic variables were balanced amongst the three intervention groups (Table 1), suggesting that adequate randomisation occurred and that the third, non-randomised arm, was comparable.

As shown in Fig. 1, there was no significant impact of BCG or other routine immunisations received at birth on any measured parameters of the inflammatory-iron axis at either 24–48 h or 72–96 h post-intervention. No significant differences were found when comparing (1) intervention groups at each blood sampling point (Table 2), (2) within-infant changes to parameters over time by intervention group and (3) infants receiving any vaccines at birth (groups 1 and (2) with vaccination naïve infants (group 3) (data not shown, all p-values > 0.05). The hepcidin levels in group 3 (recruited separately in the dry season) showed a trend toward being lower at all time-points. However this finding was not significant and was

Table 1
Population characteristics by intervention group.

	Group 1	Group 2	Group 3	p-Value[c]
	BCG/OPV/HBV	OPV/HBV	No vaccines	
	$n = 40$	$n = 40$	$n = 40$	
Gender (male, %)	51.2	48.7	47.5	0.94
Gestational age (weeks)	38.2	38.0	38.1	0.89
Birth weight (g)	3065	3069	3045	0.71
Length (cm)	50.8	50.5	50.7	0.91
Head circumference (cm)	34.4	34.1	34.1	0.48
Parity	3.2	3.6	4.3	0.48
Maternal iron supplementation	95.1%	100%	97.5%	0.38
Timing of pre-intervention blood sample (hours)	6.85	5.92	7.69	0.29
Admissions to hospital during study period[a]	1	2	1	1.0
Deaths during study period[b]	0	0	1	0.33

[a] All admissions were for presumed neonatal sepsis. All infants received antibiotics and improved within 48 h. They were discharged when blood cultures were negative.
[b] One study participant died at home between the first and second study visits, cause of death unknown.
[c] Categorical variables were compared using the Chi-squared test. Continuous variables were compared using one-way ANOVA.

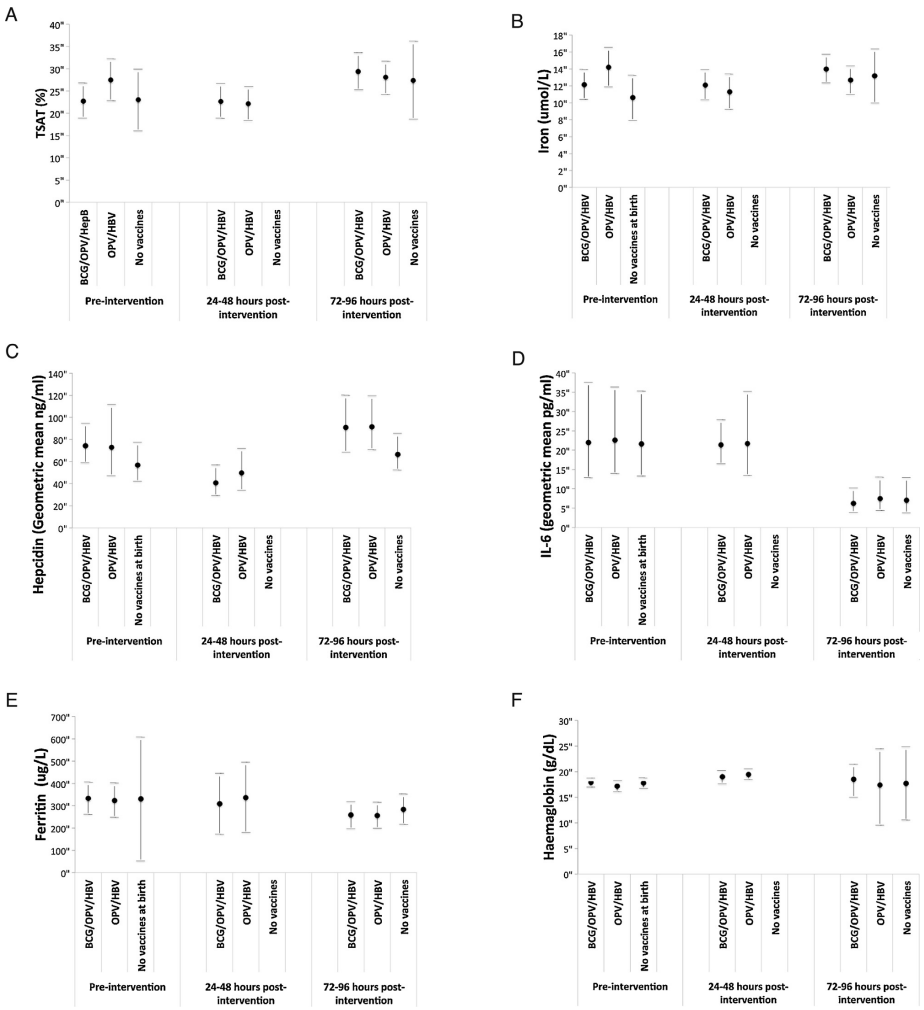

Fig. 1. Iron parameters (means ± 95% confidence intervals) by intervention group and time post-intervention.

not reflected by higher iron or TSAT levels. It is thus unlikely to represent a true difference.

As previous trials reported more significant effects of BCG in male infants results were also analysed by gender (Table 2). In general no differences in the impact of vaccine timing on parameters by gender was found. However, IL-6 was significantly higher in male infants receiving BCG at birth than delayed ($p = 0.02$), and hepcidin which was significantly lower in girls who had received all vaccines delayed ($p = 0.004$). As these findings were not reflected in changes to any other parameters of the inflammatory-iron axis, they may reflect multiple testing artefacts.

4. Discussion

This study found no evidence that BCG or other routine immunisations at birth impact significantly on iron metabolism. However, we may have failed to identify an inherent ability for vaccinations to stimulate the inflammatory-iron pathway for a number of reasons:

First, BCG is a slowly replicating live-organism and may take time to reach a level in the body able to stimulate a systemic response. The later time-point of 72–96 h post-vaccination may have been too early to identify any impact of BCG on iron metabolism.

Table 2
Comparison of mean iron metabolism pathway parameters by intervention group and time post-intervention.

	Pre-intervention (<24 h of age)				24–48 h post-intervention				72–96 h post-intervention			
	Group 1	Group 2	Group 3	*p*-Value[b]	Group 1	Group 2	Group 3	*p*-Value	Group 1	Group 2	Group 3	*p*-Value
	n = 39[a]	*n* = 37	*n* = 35		*n* = 17	*n* = 15	*n* = 0[c]		*n* = 20	*n* = 20	*n* = 25	
Iron (μmol/L)	12.2	14.2	10.6	0.08	12.0	11.3	–	0.65	14.0	12.7	13.2	0.72
Male	*11.5*	*13.4*	*11.4*	*0.55*	*11.3*	*10.5*		*0.49*	*14.0*	*12.0*	*12.5*	*0.55*
Female	*12.9*	*15.3*	*9.6*	*0.08*	*12.7*	*12.8*		*0.98*	*14.0*	*13.1*	*13.9*	*0.97*
TSAT (%)	22.8	27.5	23.1	0.37	22.5	22.2	–	0.89	29.4	28.1	27.4	0.88
Male	*21.1*	*26.4*	*22.9*	*0.46*	*22.8*	*21.2*		*0.72*	*30.5*	*27.2*	*24.3*	*0.30*
Female	*25.3*	*28.9*	*23.4*	*0.71*	*22.0*	*24.1*		*0.79*	*28.2*	*28.9*	*30.9*	*0.93*
Hepcidin (ng/ml)[d]	74.5	72.9	56.9	0.52	40.9	49.8	–	0.41	91.0	91.7	66.5	0.32
Male	*76.7*	*100.2*	*63.1*	*0.74*	*35.1*	*49.4*		*0.51*	*80.9*	*85.1*	*89.2*	*0.87*
Female	*72.0*	*54.0*	*51.6*	*0.33*	*49.7*	*50.3*		*0.86*	*101.3*	*98.8*	*48.4*	*0.004*
IL-6 (pg/ml)[d]	22.0	22.6	21.6	0.71	21.4	21.7	–	0.12	6.3	7.5	7.1	0.90
Male	*30.6*	*28.1*	*21.3*	*0.62*	*24.5*	*18.1*		*0.02*	*5.8*	*10.3*	*7.6*	*0.54*
Female	*15.8*	*17.4*	*22.0*	*0.44*	*16.7*	*26.1*		*0.39*	*6.8*	*5.3*	*6.6*	*0.94*
Ferritin (μg/L)	333.6	324.2	330.9	0.99	308.7	337.3	–	0.43	259.1	256.3	283.5	0.75
Male	*287.0*	*282.8*	*367.0*	*0.85*	*250.4*	*293.9*		*0.54*	*227.9*	*235.1*	*285.0*	*0.53*
Female	*393.6*	*371.5*	*240.6*	*0.27*	*396.2*	*380.7*		*0.92*	*304.3*	*268.2*	*281.6*	*0.86*
Haemoglobin (g/dL)	17.9	17.2	17.8	0.47	18.8	19.5	–	0.45	18.5	17.4	17.7	0.65
Male	*17.4*	*16.8*	*17.2*	*0.72*	*18.0*	*19.5*		*0.10*	*18.3*	*15.7*	*17.5*	*0.15*
Female	*18.4*	*17.7*	*18.5*	*0.70*	*20.0*	*19.4*		*0.60*	*18.6*	*19.5*	*17.9*	*0.68*

[a] Number for each group is the maximum number of blood samples available. Not all parameters were available for all samples due to volume constraints.
[b] One-way ANOVA.
[c] Infants in group 3 were only sampled at the 72–96 h sampling time-point.
[d] Geometric means.

Second, mean IL-6, hepcidin and ferritin levels in these neonates were high, with IL-6 initially 10–20 fold higher [15], hepcidin 1.5–2 fold higher [16] and ferritin 5–10 fold higher [17] than reported circulating levels in older children. Correspondingly TSAT and iron levels were at the lower end of the normal range, approximately 50% lower than previously reported ranges from cord blood [18]. This suggests that the inflammatory-iron axis, whether mediated by hepcidin-dependent or independent pathways [19] was already stimulated in all of our study participants, perhaps due to acute inflammation precipitated by the birth process [20]. If the axis is already maximally stimulated in these infants any additional impact of BCG or other vaccines would not have been detectable. The non-specific effects of BCG are reportedly highest in low birth-weight/premature infants. It may be that stimulation of the inflammatory-iron axis at birth is blunted in this population and is enhanced by immunisations. Thus, impacts on the iron-inflammatory axis cannot be ruled out as a potential biological mechanism to explain the non-specific effects of BCG in such babies.

To fully understand whether BCG and other routine immunisations have an impact on iron metabolism, similar studies in premature neonates and older infants, from different geographical regions and with longer blood sampling time points, are necessary.

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Conflict of interest statement
None.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2015.04.087

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5.2 Paper 2: Iron metabolism in the immediate post-natal period and its effect on pathogen growth: identification of a novel therapeutic target not vulnerable to anti-microbial resistance (submitted to JAMA Pediatrics).

London School of Hygiene & Tropical Medicine
Keppel Street, London WC1E 7HT
www.lshtm.ac.uk

Registry
T: +44(0)20 7299 4646
F: +44(0)20 7299 4656
E: registry@lshtm.ac.uk

LONDON SCHOOL of HYGIENE &TROPICAL MEDICINE

RESEARCH PAPER COVER SHEET

PLEASE NOTE THAT A COVER SHEET MUST BE COMPLETED FOR EACH RESEARCH PAPER INCLUDED IN A THESIS.

SECTION A – Student Details

Student	Sarah Prentice
Principal Supervisor	Stephen Cose
Thesis Title	Investigating the Non-Specific Effects of BCG in Neonates

If the Research Paper has previously been published please complete Section B, if not please move to Section C

SECTION B – Paper already published

Where was the work published?			
When was the work published?			
If the work was published prior to registration for your research degree, give a brief rationale for its inclusion			
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SECTION C – Prepared for publication, but not yet published

Where is the work intended to be published?	Journal of the American Medical Association Paediatrics
Please list the paper's authors in the intended authorship order:	Prentice S, Jallow AT, Sinjanka E, Jallow MW, Sise E, Kessler N, Wegmuller R, Cerami C, Prentice AM
Stage of publication	Submitted

SECTION D – Multi-authored work

For multi-authored work, give full details of your role in the research included in the paper and in the preparation of the paper. (Attach a further sheet if necessary)	I was responsible for the study design, statistical analysis and manuscript preparation in collaboration with CC and AMP. I conducted the study procedures in collaboration with ES. I conducted the inflammatory-iron parameter analysis with MWJ. The bacterial growth assays were conducted by AJT

Student Signature: [redacted] **Date:** 25/03/2018

Supervisor Signature: [redacted] **Date:** 25/03/2018

Title: Iron metabolism in the immediate post-natal period and its effect on pathogen growth: identification of a novel therapeutic target not vulnerable to anti-microbial resistance

Authors: Sarah Prentice, MBBS, MSc [1,2] *, Amadou T. Jallow, BSc [2], Edrissa Sinjanka, RN[2], Momodou W. Jallow, BSc[2], Ebrima Sise, BSc[2], Noah Kessler, BSc[2,3], Rita Wegmuller, PhD[2], Carla Cerami MD, PhD[2], Andrew M. Prentice PhD [2,3]

[1] Clinical Research Department, London School of Hygiene and Tropical Medicine, Keppel Street, London, WC1E 7HT

[2] Nutrition Theme, MRC Unit The Gambia, Atlantic Road, Fajara, The Gambia and MRC Keneba.

[3] MRC International Nutrition Group, LSHTM, Keppel Street, London, WC1E 7HT

*Corresponding author. Sarah.prentice@lshtm.ac.uk

Word Count [2970]

Key Points

Question

Do changes to neonatal iron parameters in the first four post-natal days influence neonatal susceptibility to bacterial pathogens?

Findings

Infants undergo a rapid post-natal hypoferraemia during the first 12 post-natal hours, which is sustained to at least four days of age. This reduction in serum iron correlates with inhibition of ex-vivo growth of common neonatal pathogens.

Meaning

Augmentation of the rapid post-natal reduction of serum iron in neonates may represent a novel therapeutic target to aid prevention or treatment of infections that is not susceptible to anti-microbial resistance.

Abstract:

Importance: Septicemia is a leading cause of death among neonates in low-income settings, a situation that is deteriorating due to high levels of antimicrobial resistance. Novel interventions are urgently needed. Iron stimulates the growth of most bacteria and the hypoferraemia of the acute phase response is a key element of innate immunity. Cord blood, which has high levels of hemoglobin, iron and transferrin saturation, has hitherto been used as a proxy for the iron status of neonates. We investigated whether iron metabolism in the immediate post-natal period might influence pathogen susceptibility and represent a therapeutic target for neonatal sepsis.

Objective: To describe iron metabolism in the first four post-natal days and investigate its effects on ex-vivo growth of common neonatal pathogens.

Design: Nested cohort study within a randomized control trial. Cord blood and two further blood samples up to 96 hours of age were analysed for parameters of iron metabolism. Samples pooled by transferrin saturation were used to conduct ex-vivo growth assays with *Staphylococcus aureus, Streptococcus agalactiae, Escherichia coli* and *Klebsiella pneumonia.*

Setting: Single-Centre, rural Gambia.

Participants: 120 healthy, vaginally-delivered neonates.

Main outcome and measures: Primary outcomes were 1) transferrin saturation at birth, 24h, 48h and 96h of age. 2) 6hr ex-vivo bacterial growth.

Results: A profound reduction in transferrin saturation occurred within the first 12h of life, from high mean levels in cord blood (47.6% (95% CI 43.7-51.5%)) to levels at the lower end of the normal reference range by 24h of age (24.4% (21.2-27.6%)). These levels remained suppressed to 48h of age with some recovery by 96h. Reductions in serum iron were associated with high hepcidin and IL-6 levels. Ex-vivo growth of all studied neonatal pathogens was strongly associated with serum transferrin saturation.

Conclusions and relevance: Human neonates elicit a rapid post-natal hypoferremia that supports lower rates of bacterial replication than cord serum for some common causes of neonatal bacteremia. Early post-natal iron and transferrin saturation levels were inversely associated with IL-6 and hepcidin suggesting the possibility that the hypoferremia could be

augmented (e.g. by mini-hepcidins) as a novel therapeutic option that would not be vulnerable to antimicrobial resistance.

Trial registration: The original trial in which this study was nested is registered at ISRCTN, number 93854442

Introduction

The recent *Lancet* Every Newborn Series estimated that 2.9 million neonates die each year from largely preventable causes; 600,000 of these from neonatal infections.[260] With the rapid spread of antimicrobial resistance (AMR), these statistics are likely to worsen.[261] AMR frequently contributes to neonatal septicemia in low-income countries (*Klebsiella* spp, *E. coli* and *S. aureus*), and is almost certainly rising.[262] Poor susceptibility to almost all commonly-used antibiotics has been reported for *Klebsiella* species and *S. aureus* in neonatal settings.[263] AMR is especially devastating for neonatal care units because babies succumb rapidly and often before it is possible to screen for AMR or try alternative antibiotics. For the very reasons that AMR has already emerged (rapid microbial mutation/selection enhanced by drug pressure, horizontal transmission of resistance plasmids from non-human pathogens, indiscriminate antibiotic usage), it is likely to remain a problem with new generations of antibiotics.[261] Against this background, there is a pressing need to better understand why neonates are so susceptible to blood-borne infections and to develop adjunctive therapies that could aid their protection perhaps by augmenting first-line innate responses.

The growth and virulence of most human pathogens is contingent on their ability to assimilate iron from their human host. High host iron states can lead to increased susceptibility to many infectious diseases.[255] As a result, systemic iron homeostasis in humans is tightly controlled; a process mediated primarily by hepcidin,[255] and possibly also by hepcidin-independent pathways in response to infectious threat.[264] In the acute phase response hepcidin is rapidly up-regulated by inflammatory cytokines (primarily IL-6). This leads to internalization of the transmembrane protein, ferroportin, in enterocytes and macrophages, which reduces serum iron by blocking enteric absorption of dietary iron and sequestering transferrin-bound iron in macrophages.[255] The reduction in serum iron with inflammation is believed to be an evolutionary mechanism designed to withhold iron from microbes and thus limit their growth and virulence. This has now been clearly demonstrated in mouse models.[265-267]

Neonates are born with high levels of fetal hemoglobin, ferritin, serum iron and transferrin saturation (TSAT) as evidenced by cord blood levels.[268] The physiological challenge of dealing with high heme levels at birth is illustrated by the fact that around half of all

neonates show transient jaundice.[269] We therefore hypothesized that these elevated iron levels and fluxes might contribute to the high susceptibility of neonates to septicemia, especially preterm and low birth-weight babies, and may partially explain the characteristic spectrum of causal organisms. Here we report that healthy vaginally-delivered African babies display a very rapid post-natal hypoferremia that is correlated with changes in IL6 and hepcidin. We suggest that this represents an evolved protective mechanism that could potentially be augmented to provide a broad-spectrum innate protection against neonatal septicemia.

Methods

Participants and study procedures

Blood samples for this study were collected during a trial investigating the impact of different vaccination strategies at birth on the iron status of neonates. A detailed description of the study methods can be found elsewhere.[259] In summary, 120 healthy Gambian neonates were recruited on the first day of life and randomly allocated to receive either 1) routine immunisations at birth (Bacillus Calmette Guerin (BCG), Hepatitis B and Oral Polio Vaccine (OPV)) 2) Hepatitis B and OPV at birth, BCG vaccination delayed to after study completion (>72h of age) or 3) all immunisations delayed until after study completion (BCG, Hepatitis B and OPV at >72h of age). All infants had a placental cord blood sample, a neonatal blood sample taken within 24h of birth (S1) and were then randomly assigned to have one further blood sample taken at either 24-48 (S2) or 72-96 (S3) hours of age. As none of the different vaccination strategies had a significant impact on neonatal iron metabolism,[259] the results from all groups were combined in this study to investigate the physiological changes in iron metabolism within the first 4 post-natal days.

Full informed consent for infant involvement in the study was obtained from pregnant mothers antenatally and eligible infants were enrolled on the day of birth. Any healthy infant born to a consenting mother within the West Kiang region of The Gambia was eligible for inclusion, providing that they were not already enrolled in another research study. No gestational age limit was set, however infants weighing <2000g (more than 2 standard deviations from the average Gambian birth-weight) were excluded (one exclusion). Other criteria for infant exclusion were; severe birth complications (six

exclusions), major congenital malformations (no exclusions), unwell at birth (two exclusions), mother with known HIV or TB (no exclusions), and infants with a known case of active TB within the same compound of residence (no exclusions). Most mothers received supplementary iron and folic acid as part of their routine antenatal care, as per WHO guidelines.

Ethical approvals

The study was approved by The Gambia Government/MRC Joint Ethics Committee (SCC1325) and the London School of Hygiene and Tropical Medicine ethics committee (012-045). The study was conducted according to the principles of the Declaration of Helsinki.

Laboratory methods

Blood collection and iron parameter analysis

Whole blood was drawn from the umbilical vein at birth or from the dorsum of the hand at the indicated time points after birth, into Becton Dickson microtainer SST II Advanced collection tubes. Red cell parameters were measured on fresh whole blood drawn into EDTA microtainers (Becton Dickson, Oxford, UK) using a Medonic M-series haematology analyser (Boule Diagnostics Int AB, Stockholm, Sweden). Iron parameters were analysed using plasma collected into lithium-heparin anti-coagulant using the automated Cobas Integra 400 plus (Roche Diagnostics, Basel, Switzerland). Plasma hepcidin and IL6 were measured in duplicate by ELISA, Bachem-25, USA and BD OptEIA, Oxford, UK respectively, as per manufacturers' instructions as previously described.[259]

Due to low volume of residual blood, bacterial growth assays were performed on plasma samples that were pooled according to time of collection (Cord, S1 (6-24h after birth), S2 (25-48h after birth), and S3 (72-96h after birth)) and then according to TSAT. The following sample pools were made and run in triplicate through the bacterial growth assays: Cord 70-100% (n=6, pools=4); Cord 60-69% (n=12, pools=6); Cord 50-59% (n=15, pools=5); Cord 40-49% (n=20, pools=9); Cord 30-39% (n=14, pools=2); Cord 20-29% (n=10, pools=3); Cord 10-29% (n=4, pools=1); S1 30-60% (n=12, pools= 2); S1 20-30%

(n=26, pools= 2); S1 0-20% (n=30, pools= 2); S2 20-30% (n=12, pools= 2); S2 10-20% (n=9, pools= 2); S3 30-40% (n=15, pools= 2); S3 20-30% (n=21, pools= 3); and S3 0-20% (n=13, pools= 2).

Bacterial growth assays

Staphylococcus aureus (strain NCTC8325), *Escherichia coli* (strain *Crooks,* ATCC8739), *Streptococcus agalactiae* Lehmann and Neumann (ATCC 13813, Lancefield's group B) and *Klebsiella pneumoniae* (ATCC13883, strain NCTC96633) were grown overnight for 18h at 37°C in 5mls iron-free minimal growth media, Iscove's Modified Dulbecco's Medium (IMDM, Invitrogen) with continuous shaking (250 rpm). All growth assays were run in triplicate in IMDM containing 50% heat-inactivated human neonatal serum. Bacterial growth was monitored by measuring the optical density at 620 (OD_{620}) hourly for 12h using a Multiscan FC ELISA plate reader (Thermo Scientific).

Statistical analysis

Statistical analysis and preparation of graphs was conducted using STATA v14.1 (Stat-Corp LP, College Station, TX, USA), DataDesk version 7.0.2 (Data Description Inc), GraphPad Prism (GraphPad Software INC, CA 92037, USA) and R (r-project, Lucent Technologies, New Jersey, USA). Non-normally distributed parameters (hepcidin and IL-6) were log-transformed prior to any analysis. Bacterial growth rates were compared using repeated measures ANOVA with pooled sample (discrete variable), cord/neonatal sampling period (discrete variable) and growth rate incubation time (continuous variable) as independent variables. Pearson product-moment correlation was used to obtain pair-wise correlations between parameters. Graphs of changes in parameters overtime were generated using local polynomial regression fitting.

Results

Neonatal characteristics

Baseline demographics for the 120 study participants are shown in **Table 1**. Children in this cohort were healthy term infants, with median anthropometric measurements falling between the 25th and 50th centile on the WHO growth charts for gestational age. Nearly all

(97.5%) of mothers received iron and folic acid antenatally, as per WHO guidelines. Six infants in the cohort became unwell during the study period (five with suspected sepsis, one with suspected meconium aspiration) and were excluded from analysis.

Alterations to iron metabolism in the acute post-natal period

Iron metabolism parameters in the first 96h of life are shown in **Table 2** and **Figure 1**. Mean TSAT was high in cord blood (47.6%, 95% confidence interval (CI) 43.7-51.5%) with levels higher than the reported reference range for older children. TSAT levels had halved by 12h post-partum (24.4%, CI 21.2-27.6%) and remained low until 72-96h when levels began to rise again (30.9%, CI 26.9-34.8%). TSAT alterations were largely driven by alterations in serum iron rather than by changes to the chaperone protein transferrin, as total iron binding capacity (TIBC) remained relatively constant, though showing a slight fall by 72-96h of age. Geometric mean hepcidin levels in cord blood (43.8ng/ml, CI 36.8-52.3ng/ml) were within the expected reference range for healthy older children,[270-272] and had almost doubled by the first post-natal blood draw at a median time of 6h post-partum (79.4, CI 68.1-92.4; $p<0.0001$). This was followed by a decline at the subsequent sampling point at 24-48h ($p<0.0002$)) and a rise again by 72-96h ($p<0.0001$). Geometric mean IL-6 levels were moderately raised in cord blood, remained raised until 24-48h of age, but had halved by 72-96h. Cord blood hemoglobin levels (14.4g/dl, CI 13.8-14.9g/dl) were within previously reported reference ranges.[268] Levels then rose until 24-48h of age (19.2g/dl, CI 18.3-20.0g/dl) and began to fall subsequently (17.9g/dl, CI 17.0-18.7g/dl) at 72-96h of age as expected for this age group.

Likely effectors of changes in iron metabolism in the acute post-natal period

Pearson pairwise correlation coefficients between the iron parameters (serum iron, TIBC, TSAT and Hb) and the putative regulators of these parameters (IL-6 and hepcidin) are shown in **Supplementary Table 1**. We focus the discussion here on the possible mediators of the acute post-natal hypoferremia. Day 1 hepcidin and IL-6 values were correlated with their respective cord levels (+0.66; $p<0.001$ and +0.37; $p<0.05$ respectively) and Day 1 hepcidin was correlated with Day 1 IL-6 (+0.38; $p<0.01$). Day 1 TSAT was correlated with cord TSAT (+0.54; $p<0.0001$) and there were similar correlations between cord and Day 1

serum iron (+0.55; $p<0.0001$) and TIBC (+0.64; $p<0.0001$). Day 1 TSAT was inversely correlated with Day 1 hepcidin (-0.47; $p<0.001$) and IL-6 (-0.40; $p<0.05$) and similarly for serum iron which was the major determinant of TSAT. At the later sampling points TSAT levels were not significantly associated with hepcidin but showed a strong inverse association with IL-6 in the 72-96h interval (-0.70; $p<0.0001$). Hemoglobin levels were strongly correlated across time within babies but did not appear to influence any of the iron parameters, hepcidin or IL-6.

Ex vivo assays of growth of sentinel organisms

The *ex vivo* growth patterns of standard lab strains of *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella pneumoniae* and *Streptococcus agalactiae* were assayed in iron-free medium supplemented with cord and neonatal serum pooled according to time and TSAT (**Figure 2, Table 3**). Post-natal sera clearly supported lower growth levels of all organisms and this was especially true of the Day 1 sera. The effect was least pronounced for *S. aureus*. Repeated measures ANOVA including incubation time and cord/neonatal sampling time confirmed that growth rates of all four organisms were significantly associated with TSAT ($p<0.001$) (**Table 3**).

Discussion

We demonstrate that normal healthy term newborns display a rapid and profound suppression of serum iron and TSAT within the first 6-24h post-partum. This reduction in extracellular iron persisted until 2-3d of age, with a slight increase subsequently. The correlation of suppressed iron and TSAT levels with raised hepcidin levels, particularly in the first 24h of life, suggests that hepcidin regulation of iron homeostasis is intact in the human neonate and that this is likely to be the key mediator of the hypoferremia through redistribution of iron to macrophages. Similar correlations with raised IL-6 levels (10-20 fold higher than adult normal levels), suggest that inflammatory stimulation of hepcidin also occurs in early life, and that the inflammatory conditions induced by the birth process may be at least partly driving the hypoferremia of early post-natal life. However, correlations between iron parameters and IL-6 were weak, and have not been observed in previous studies looking at cord blood parameters of iron metabolism.[273] This could suggest

that other unmeasured inflammatory mediators, such as IL-22[274] may also be up-regulating hepcidin in response to the birth process.

Two previous reports have similarly reported low iron levels in post-natal blood draws.[275,276] The data from Szabo were based on 10 infants who were sampled due to clinical indications (jaundice or infection) at a mean post-natal age of 48±4h.[275] Serum iron decreased from 23.2umol/l in cord to 7.2umol/l post-natally (arithmetic means). The data from Sturgeon based on 72 infants sampled by 12h post-partum showed a decrease from 193ug/100cc (34.5umol/L) to 46ug/100cc (8.2umol/L) (arithmetic means). These compare favourably with our values of 24.7 vs. 13.6umol/L (geometric means) by 6h (IQR 2-11h).[276] Thus, there can be no doubt that normal neonates elicit a rapid and profound (2-4 fold) hepcidin-mediated decrease in extracellular iron in the early post-natal period. Since newborns have a negligible iron intake from colostrum the hypoferremia must be achieved by redistribution of iron; presumably in macrophages where iron egress is blocked by hepcidin's inactivation of the transmembrane iron exporter, ferroportin.

A previous study linked a fall in serum iron with an increase in the anti-oxidant capacity of post-natal serum, suggesting that this may protect new-born infants against free-radical damage during the transition from fetal to post-natal life.[275] In this study we hypothesized that the hypoferremia may be a protective mechanism to withhold iron from bacteria and other human pathogens. Early post-natal life is characterized by massive colonization of the skin and gastrointestinal tract with a variety of commensal organisms.[277] A reduction in the availability of serum iron may be an evolved innate mechanism to help prevent these organisms overwhelming the immature adaptive immune responses of neonates. To test this we devised micro culture methods based on lab isolates of four organisms that frequently cause neonatal sepsis in sub-Saharan Africa. The growth rates of *E. coli, S. aureus, S. pneumoniae* and *S. agalactiae* were highly significantly lower in neonatal serum than in cord serum and for each organism growth rates were significantly associated with TSAT. *S. aureus,* which favours heme iron as a source[278] was least responsive though still clearly influenced by TSAT. *E. coli* was most responsive which is consistent with the findings of the infamous studies of Polynesian neonates given intramuscular iron, where the intervention caused an increase in neonatal septicemia and a major shift towards *E. coli* as the most frequently identified causal organism.[279] Our *ex vivo* assays need to be interpreted with caution and will certainly not replicate conditions *in vivo*, but have been validated by

titrating with differing concentrations of exogenous iron and are coherent with the known dependence of bacterial growth on iron supply.

The wider applicability of these findings may be limited because the study population was restricted to vaginally delivered, healthy neonates above 2000g from one area of West Africa. Nearly all (97.5%) of infants were born to mothers receiving antenatal iron and folic acid supplementation, which may have altered levels of iron parameters at birth. TSAT levels in our study were lower than those reported in a recent systematic review of cord blood iron parameters (weighted mean TSAT 61.2%), although fell within the reported 2.5^{th}-97.5^{th} centile range.[268] Cord blood hepcidin in our study was also lower than has been previously reported,[268, 273, 280, 281] although the lack of a standardized immunoassay for hepcidin detection makes comparing levels between studies difficult. However, these results could suggest that despite almost universal iron supplementation, our study infants' iron stores remained relatively lower at term than in other populations. This may indicate low adherence rates to supplementation or might reflect physiological differences in this population, for instance reduced gut absorption of elemental iron. It would therefore be interesting to see whether neonatal hypoferremia is even more exaggerated in different, iron replete, settings as hinted by the previous studies of Szabo[275] and Sturgeon.[276] A recent prospective study characterizing hepcidin levels in cord blood also showed lower levels in premature infants, those born small-for-gestational-age and those delivered by elective caesarean section.[273] We have now initiated a study to test whether a blunting of the physiological hypoferremia of early neonatal life occurs in these situations, putatively increasing the potential for iron-induced free-radical damage and bacterial pathogenicity from low virulence organisms, such as is noted particularly in premature infant populations.[282]

Low plasma iron is bacteriostatic rather than bacteriocidal, but nonetheless could tilt the balance towards host survival by slowing the multiplication of pathogens that might otherwise overwhelm the immature adaptive defenses of newborns. If it were possible to artificially augment such responses this could form the basis of a novel intervention. Small molecule orally-administered mini-hepcidins are currently under development and first-in-human testing as hepcidin agonists.[283] These molecules would not affect the neonate's longer-term iron status because they would only cause a transient redistribution of iron away from the circulation where it is most available to pathogens. Although it presently

remains a distant prospect, hepcidin analogues might prove to be useful adjuvants in the face of the rapidly growing levels of antimicrobial resistance.

Conclusions

Healthy term neonates undergo a rapid and profound reduction in serum iron levels during the first 12 hours of life, at least partly mediated by the hormone hepcidin. This hypoferraemia is likely to produce protection against common bacterial pathogens and may be an evolved innate immune strategy to protect the infant during the first few days of microbial colonisation. Identification of situations where this physiological hypoferraemia is blunted should be a research goal. Mechanisms to enhance this hypoferraemia, such as hepcidin agonists, represent an exciting novel therapeutic target that would not be susceptible to the threat of anti-microbial resistance.

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Author contributions: SP, CC and AP were responsible for study design, statistical analysis and manuscript preparation. SP and EB carried out participant recruitment and study procedures. SP, ES, EC, AJ and MJ conducted laboratory analysis. NK provided statistical support and produced the graphs used in the manuscript. RW provided in-country support and advice. All authors approved the final manuscript. **Competing interests:** The authors have no competing interests to declare.

Tables:

Table 4. Participant characteristics

Characteristic	Median (IQR)
Gestational Age (weeks)	38 (37-40)
Birth weight (g)	3085 (2858-3325)
Head circumference (cm)	34 (33-35)
Length (cm)	51 (49-52)
Maternal parity	3 (1-6)
Percentage male (%)	49%
Percentage of mothers on antenatal iron/folic acid supplementation at recruitment	97.5%
Age at post-natal blood sampling (hours)	
<24 hour sample (S1)	6 (2-11)
24-48 hour sample (S2)	29 (26-34)
72-96 hour sample (S3)	77 (74-82)

Table 5. Parameters of iron metabolism by post-natal age

	Cord blood N>81***	Age <24 hours (S1) N>53	p-value****	Age 24-48 hours (S2) N>21	p-value	Age 72-96 hours (S3) N>33	p-values
TSAT* (%)	47.6 (43.7-51.5)	24.4 (21.2–27.6)	<0.0001[1]	21.8 (18.8-24.7)	<0.0001[1] 0.86[2]	30.9 (26.9-34.8)	<0.0001[1] 0.04[2] 0.003[3]
Iron*(µmol/L)	24.7 (22.5-26.9)	13.6 (12.0-15.2)	<0.0001[1]	11.6 (10.1-13.1)	<0.0001[1] 0.29[2]	14.5 (13.1-16.0)	<0.0001[1] 0.24[2] 0.11[3]
TIBC*(µmol/L)	52.2 (49.0-55.4)	54.0 (51.4-56.6)	0.43[1]	51.0 (47.3-54.7)	0.51[1] 0.09[2]	47.9 (45.3-50.4)	0.10[1] 0.01[2] 0.03[3]
Hepcidin (ng/ml)**	43.8 (36.8-52.3)	79.4 (68.1-92.4)	<0.0001[1]	45.9 (36.5-57.8)	0.7[1] 0.0002[2]	87.1 (73.8-102.7)	<0.0001[1] 0.23[2] <0.0001[3]
IL-6 (pg/ml)**	23.7 (14.7-38.1)	26.9 (18.9-38.2)	0.67[1]	24.4 (18.0-33.0)	0.39[1] 0.09[2]	10.7 (7.3-15.6)	0.10[1] 0.03[2] 0.002[3]
Hb (g/dl)*	14.4 (13.8 – 14.9)	17.6 (17.1-18.2)	<0.0001[1]	19.2 (18.3-20.0)	<0.0001[1] 0.38[2]	17.9 (17.0-18.7)	<0.0001[1] 0.20[2] 0.01[3]

* = mean and 95% CI, **= geometric mean and 95% CI, ***= number of available results differs by each parameter, due to limitations in blood sample volume for some participants., ****= p-values for significance of difference between values at different time points: [1]Cord blood, [2]Age<24 hours [3]Age 24-48 hours

Table 3. Summary statistics for the ex vivo bacterial growth curves in cord and post-natal serum samples

Comparison	*Escherichia coli*	*Staphylococcus aureus*	*Klebsiella pneumoniae*	*Streptococcus agalactiae*
Sampling time				
Trend[1]	<0.0001	<0.0001	<0.0001	<0.0025
Cord vs S1	<0.0001	<0.01	<0.0001	<0.005
Cord vs S2	<0.0001	<0.001	NS	NS
Cord vs S3	<0.001	<0.001	<0.005	NS
TSAT				
Correlation[2]	<0.0001	<0.0001	<0.0001	<0.0001

[1] ANOVA for trend across all time points.

[2] Correlation between TSAT and bacterial growth rates using all data points for the sampling periods combined.

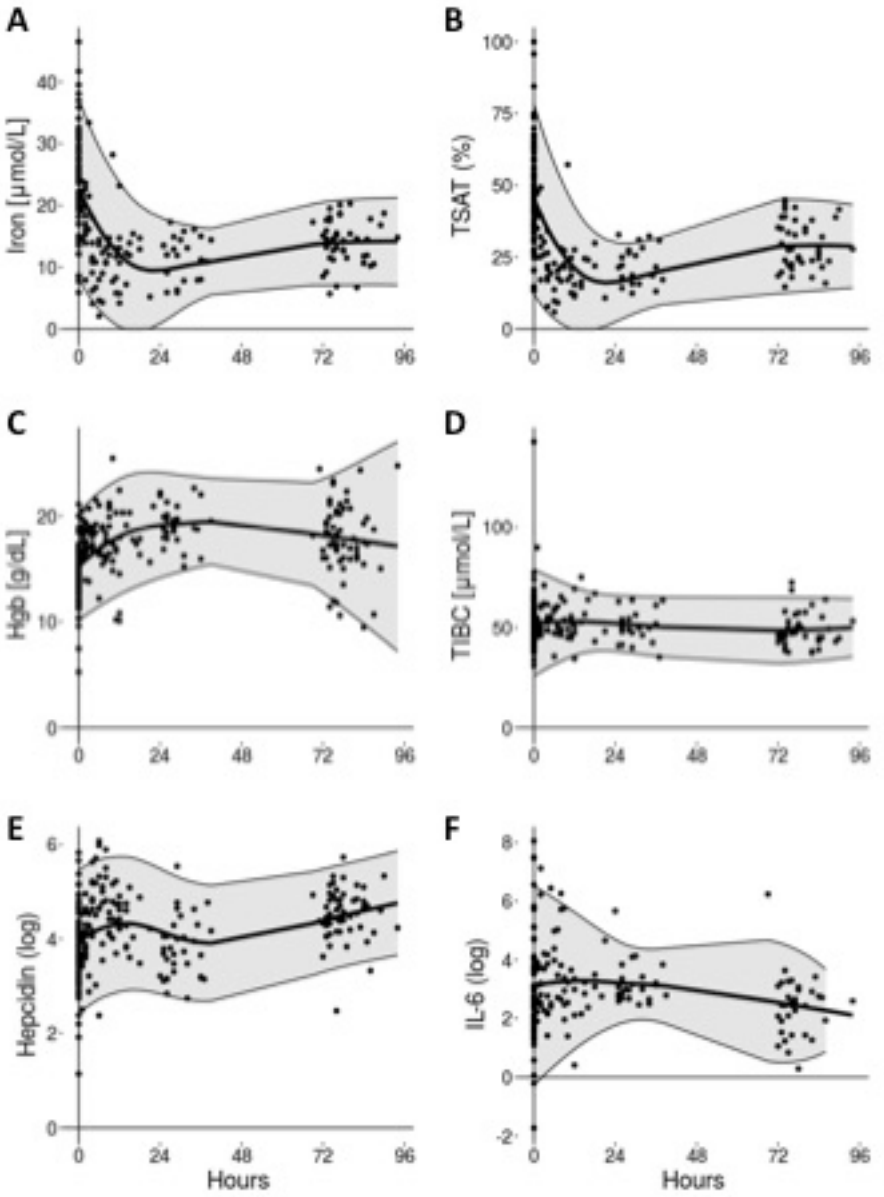


Figure 1: Changes to iron parameters during the first 96 hours of life.

Levels of iron (**A**) transferrin saturation (**B)** haemoglobin (**C**) total iron binding capacity (**D**) hepcidin (**E**) and IL-6 (**F**) in blood drawn from either the umbilical cord at birth or from the dorsum of the hand at the indicated times post-natal. Dots represent individual measurements. The bold line is a Loess fit curve with 95% Confidence Intervals shaded in grey.

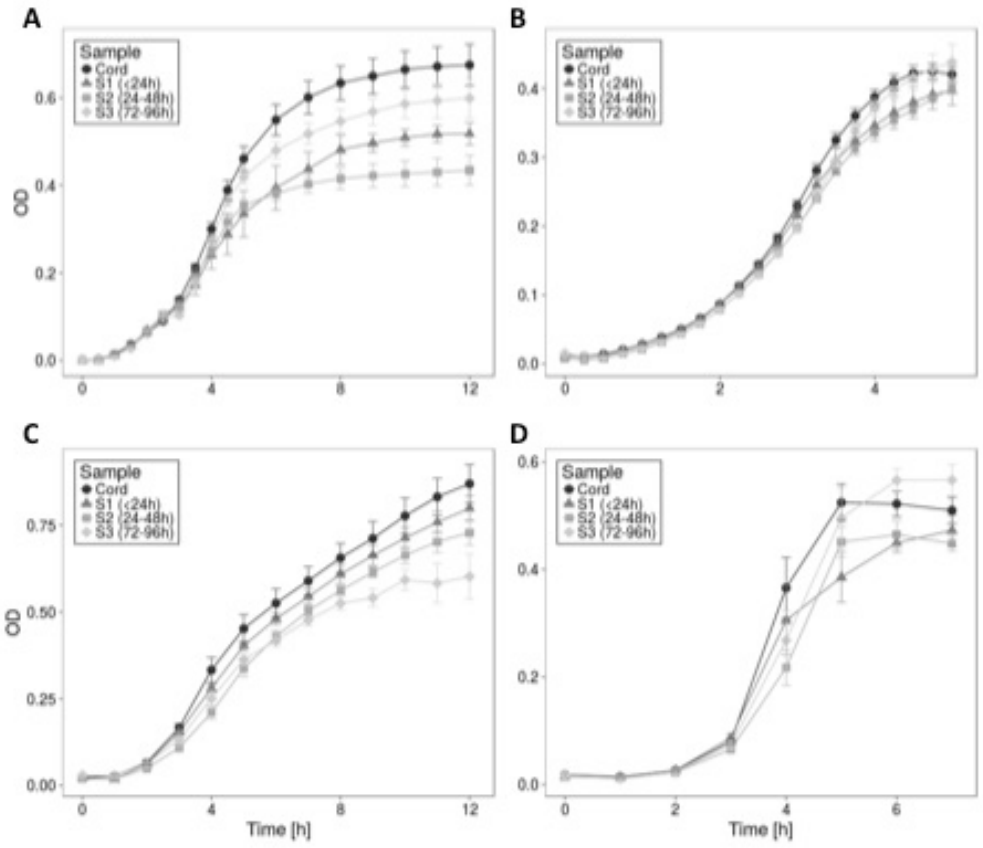


Figure 2: Ex-vivo bacterial growth assays

Growth of *E.coli* (**A**), *K. pneumoniae* (**B**), *S. aureus* (**C**) *and S. agalactiae* (**D**) in subject serum drawn from the umbilical cord or from the dorsum of the hand at the following time points after birth, S1 (<24 hours), S2 (24-48 hours) and S3 (72-96 hours). Dots represent the mean at each time point, error bars represent the SE.

5.3 Summary

The preliminary study confirmed that the inflammatory-iron axis was intact and highly activated in the first 4 days of post-natal life. It also showed that growth of common neonatal pathogens *in vitro* was highly correlated with alterations in serum iron, suggesting that it may play an important role in non-specific innate immune responses.

Although no effect of BCG on the inflammatory-iron axis was shown, this may have been influenced by a number of factors:

1. The inflammatory-iron axis appeared to be highly constitutively activated in all infants in this study, presumably as an evolved mechanism to protect against pathogenicity during microbial colonisation in the early post-natal period. It is possible that serum iron was maximally suppressed in all of our study infants, meaning that a theoretical effect of BCG would not be seen even if it did exist. It is possible that some populations of high-risk infants may have reduced activation of the inflammatory-iron axis at birth, for which BCG may provide beneficial effects. Small studies such as this one would have limited power to detect such an effect.
2. Responses measured in this study were baseline responses following vaccinations, rather than upon secondary heterologous stimulant challenge.
3. Follow-up was only to four days of age meaning medium/longer-term influences of BCG could not be investigated.

Thus, although the preliminary study did not provide good evidence for an early NSE of BCG being mediated through alterations in the inflammatory-iron axis, it provided sufficient grounds for a further interrogation in the larger Ugandan RCT that forms the major part of this thesis.

6. Methods

An overview of the methods used in the Ugandan study is found in the published study protocol paper.[284] This paper can be found at the end of this chapter. More detailed methods for the main study are outlined below. The methods for the preliminary study conducted in The Gambia are found in the papers in Chapter 5.

6.1 Detailed methods: main study

6.1.1 Regulatory approvals

The study was approved by the LSHTM research ethics committee, MRC/UVRI Research Ethics Committee, the Uganda National Council for Science and Technology and the Office of the President of Uganda. Approval letters can be found in Appendix 8. A thorough discussion of the ethical considerations of delaying BCG from birth to six weeks of age in half our study infants can be found in the attached Trials paper.

As the BCG strain used in the study was not used in Uganda during the study period, and the timing of its administration was altered, approval for the trial was also obtained from the National Drugs Authority of Uganda (Appendix 8d).

The study was conducted according to the principals of the Declaration of Helsinki.

6.1.2 Participant recruitment, consent and randomisation

Mothers presenting in early labour to Entebbe Grade B district general hospital were approached by study nurses and asked if they would like to participate in the study. Each mother approached was assigned a maternal study number, in chronological order. Information was provided and, for interested mothers, the first eligibility form was completed (maternal criteria, Appendix 3a). Consent forms were signed pre-delivery, enabling cord blood collection (Appendix 4b). Consent forms included provision for long-term storage and further studies using any excess blood samples. As far as possible the father was involved in the consent process, as there had been problems with participant withdrawal from previous studies when a mother had consented in the absence of the father. An independent person, either another family member or a midwife not involved in the study, witnessed information giving and consent.

After delivery, consent was verbally reconfirmed from the mother, and further information provided about the study if required. The study nurse completed the second

eligibility form (infant criteria, Appendix 3b). Detailed explanation of the exclusion criteria for the study is found in Chapter 4, Table 4.1. Of note, no gestational age limit or lower weight limit was specified for the study, preferring to use the clinical state of the child as a guide to inclusion. This was for two reasons: 1) to replicate the real life scenario in Uganda, where in general any infant well enough to be discharged from hospital will receive BCG regardless of gestational age or weight and 2) previous studies have suggested that the NSE of BCG may be of most importance in premature/LBW infants,[51] and thus it was important to include them in the study

Following confirmation of full eligibility and consent for the study, participants were randomised, stratified by sex. Randomisation occurred according to a) BCG timing (birth vs. 6 weeks) and b) blood sampling time-point. In this study infant blood sampling was limited to two out of the possible four time-points within the first 10 weeks, for both ethical and parental acceptability reasons. This resulted in 12 different possible combinations of BCG and blood sampling time-points, for each sex. The possible blood sampling time-points are shown in Table 2, page 7 of the published study protocol, at the end of this chapter. Note, the time-points vary slightly by immunological sub-study, due to the need to use primary immunisations as an *in-vivo* non-specific stimulant for the iron sub-study, and therefore randomisation was conducted separately for each sub-study. Randomisation lists were created using STATA in blocks of 24, stratified by sex, by an MRC/UVRI statistician who was not directly involved in the study.

Study files were prepared according to study group. File contents are shown in Box 6.1. The study cards and the number of study visit packs required per study file varied according to the group. Prepared study files of the correct group were placed within a large opaque, brown envelope with the corresponding study ID labelled on the outside of the envelope. The files were placed in order, in separate piles according to sex. This was carried out by two members of the host research institute, not directly involved in this study, who cross-checked each other's work. The master list correlating study ID to study group was held by the preparing, non-study, statistician and only accessed once the study was completed and the data cleaned and locked.

Box 6.1 Study file contents

Maternal and infant demographic details form (Appendix 3c)

Study visit packs

Routine visit form + phlebotomy/vaccination details form + brown envelope (for concealment of study card during visits) (Appendix 3f and 3g).

Study appointment cards

One copy for mother, one copy to remain in the study file. Cards were colour coded according to BCG vaccination timing (blue for early BCG, yellow for BCG at 6 weeks) to provide a visual aide memoir for vaccination nurses (Appendix 3f).

Final status form (Appendix 3j)

Colour coded ribbon (blue for BCG at birth, yellow for BCG at 6 weeks)

Tied around the infant's arm as an aide memoir for vaccination staff.

To randomise eligible infants, study nurses took the next two brown envelopes in ascending study ID order, according to the baby's sex. The mother was then asked to choose between the envelopes. The envelope not chosen was added back to the top of the sex-appropriate pile, for use during the next randomisation. This process was done to give a visual reinforcement of the randomisation process to mothers, to provide them with a sense of autonomy and to reassure them that the study team did not select the timing of BCG administration. Sufficient extra study files were prepared so that this process could still be carried out for the final participants in the study. This process of randomisation was extremely well received by the study mothers.

Upon opening the selected envelope, the colour-coded ribbon contained within was tied around the right-hand upper arm of the study infant (the location of BCG vaccination). This was to provide a clear visual reference for vaccination nurses as to whether the baby was to receive BCG before departure from the ward, or not. BCG at birth is the current standard of care in Uganda. At Entebbe Grade B hospital, vaccination of all infants born in the preceding 24 hours occurs every morning by teams of vaccination nurses not directly attached to the study. The period of time available for vaccination is often very short, due to pressures on beds and staff time, and therefore a quick method of identifying infants who were not to receive BCG at birth was required. Each day, the vaccination nurses called the women whose babies had yellow ribbons for vaccinations first. The infants in the delayed BCG arm received OPV only, and their names and dates of birth were documented in a log-book for cross-checking at the end of the study.

Infants with blue ribbons were called at the same time as the non-study infants and received both OPV and study BCG. The use of ribbons of different colours for this purpose was instituted after the initial piloting of study procedures, where concerns had been expressed by the study nurses about ensuring correct vaccination timing during busy periods. Ribbons were requested to remain on the infant until they were discharged from hospital.

The correct administration of the at-birth vaccines was re-confirmed by the study nurses prior to discharge. Study nurses then completed the date of vaccination and the expected clinic visit dates on both copies of the infant's study cards. If clinic visit dates fell on a weekend, those falling on a Saturday were assigned to the previous Friday and those on a Sunday to the following Monday.

Prior to discharge from hospital, maternal demographic details were collected (Appendix 3c) and routine anthropometry was conducted on all infants. Mothers were provided with information sheets about the study (Appendix 4a) and simple instructions about recognising signs of clinical illness in newborns (Appendix 4c).

Mothers, infants and their families were then driven home by a fieldworker, after being shown the location of the research clinic. This allowed the fieldworkers to confirm the participant's address and GPS co-ordinates to help with follow-up. Unfortunately, during very busy periods, some mothers preferred to leave without being driven home, as there were long waits.

6.1.3 Blinding

This study was single-blind. Mothers were not blind to infant BCG status a) for practical reasons (as BCG vaccination produces a visible scar) and b) so that mothers knew the vaccine status of their child and BCG might be given in the community in cases of loss-to-follow-up in the delayed BCG arm.

Investigators involved in clinical follow-up of the child were blinded to BCG vaccination status. This included clinicians, field-workers, study PI (myself) and laboratory technicians. The members of the study team not blinded are shown in Table 6.2. All non-blinded team members were aware of the need not to disclose the vaccination status of any study participant to other members of the study team.

Table 6.2. Study team members not blinded to BCG vaccination status

Study team members not blinded to BCG vaccination status	Reason
Nurses/midwives recruiting on labour ward	Responsible for randomisation and correct filling out of study cards, therefore they must know BCG vaccination timings.
Clinic receptionist	Responsible for placing plaster over expected/actual BCG scar site on right deltoid.
Clinic nurses carrying out immunisations and blood sampling	These nurses required access to the study cards, which detailed the timing of BCG administration. They also administered the BCG vaccination in the delayed BCG group and were therefore necessarily not blinded. These nurses carried out routine anthropometry but had no involvement in the clinical assessment of the child.

Investigator blinding was accomplished in the following ways:

- The receptionist placed a plaster over the right deltoid (at the actual or expected BCG scar site) of all participants immediately upon entry into the clinic waiting area. The paediatric team did the same for any study child who presented unwell directly at Entebbe Grade B hospital.
- Maternal and file study cards (containing details of immunisation and blood sampling timings) were placed in a sealed brown opaque envelope within the study file, immediately upon presentation of participants at the clinic.
- Immunisations and blood sampling (necessitating accessing of study cards by non-blinded nurses) were conducted as the final procedure at any clinic attendance in a separate room from any blinded investigator.
- Mothers were reminded not to tell investigators of the BCG status of their infant. Clinicians were asked not to enquire about vaccine status unless the mothers specifically expressed concerns, in which case this un-blinding of vaccination status was documented.
- Laboratory investigations were conducted by anonymous laboratory ID number, with linkage to study ID and vaccination status occurring only when data were cleaned and locked.

Blinding was largely successful, with eleven cases of un-blinding: three accidental and eight because of maternal concerns about the vaccination site.

6.1.4 Intervention

BCG Staten Serum Institut (SSI) strain 1331 batch 113033c expiry date September 2015 (product information sheet, Appendix 5) was used throughout this study. BCG was given at a dose of 0.05ml, administered intradermally to the right deltoid at either <24 hours of age or 6 weeks of age depending on study arm. Once reconstituted with diluent, BCG multi-dose vials were used for a maximum of 4 hours before being discarded, as per manufacturer's guidelines. Study BCG was used for all infants born at Entebbe Grade B hospital during the study period, on permission of the National Drug Authority of Uganda. This ensured that there would be no chance of administration of a non-study strain of BCG to study infants, and reduced wastage of doses of vaccine from the multi-dose vials.

BCG SSI 1331 was used because studies have suggested that it produces greater specific and non-specific immune responses compared to other BCG strains.[42] It was also the strain used in other studies investigating the potential NSE of BCG[35, 51, 91, 168, 175, 201] and therefore would allow for more direct comparisons.

In the early BCG arm, the aim was for participants to receive BCG at <24 hours of age. This was achieved in 98% of infants. For six infants, BCG was received at 24-90 hours of age. This occurred due to an inability of nurses to access the study vaccine during a bank-holiday weekend when the storage room at the hospital was locked. This delay in vaccination was noted on study records. The participants remained in the study, but the vaccination delay was accounted for during analysis, which was conducted as both 'per-protocol' and 'intention-to-treat'.

In the delayed arm, BCG timing in relation to the first dose of primary immunisations (EPI-1 – see below) varied according to sub-study. Due to the use of EPI-1 vaccines as *in-vivo* non-specific stimuli in the iron sub-study, BCG was administered one day after EPI-1. This allowed for a blood sample to be taken immediately prior to BCG administration, to compare acute responses to non-specific stimuli in BCG vaccinated and unvaccinated participants. In the cytokine and epigenetic sub-studies, BCG was administered at the same time as EPI-1 vaccinations. Participants presenting late for their six-week vaccinations or blood sample collection were still retained in the study, providing that they had not received immunisations elsewhere in the community. Although not optimal, it was felt that slight alterations in vaccination timing reflected

the real world scenario of vaccinations, and that it was therefore important to take this into account when assessing the likely impact of BCG on outcomes. Any delay was noted, allowing for adjustment during final analysis as necessary. Ten infants received non-study BCG in the community and were discontinued from further study procedures.

6.1.5 Other routine vaccinations

Study infants received all vaccinations according to the current expanded programme of immunisations (EPI) during the 10-week study period (see Table 6.3).

Table 6.3. Routine immunisations received by all study participants

At birth	OPV
At 6 weeks of age (EPI-1)	DTwP-Hib-HepB PCV10 OPV
At 10 weeks of age (EPI-2)	DTwP-Hib-HepB PCV10 OPV

OPV, Oral Polio Vaccine; DTwP-Hib-HepB, Diptheria Tetanus whole cell Pertusis *Haemophilus influenza type B,* Hepatitis B (5-in-1); PCV10, 10-valent Pneumococcal Conjugate Vaccine; EPI, Expanded Programme of Immunisations.

Of note, rotavirus vaccination roll-out in Uganda occurred after completion of the study and therefore was not received by any participant.

6.1.5.1 Storage of vaccines

All vaccines were stored in UNICEF/WHO approved ice-lined refrigerators. These maintain storage temperatures for at least 24 hours in the event of a loss of power supply. The main storage of vaccines was at the Maternal and Child Health Clinic (MCHC) adjacent to the study clinic, with boxes of BCG vials and diluent taken to Entebbe Grade B MCHC at regular intervals (transported in cool boxes with ice packs; journey time was approximately 10 minutes). Refrigerators were maintained at 4˚C (optimal range 2˚C – 8˚C) and a twice-daily log of temperatures was kept, reviewed on

a daily basis by study staff. Temperature monitoring was installed which alarmed when out of range, in which case the study PI was informed and corrective action taken.

6.1.6 Anthropometry

Weight

Infant weight was measured using Seca electronic scales, accurate to within 10g. Weight was measured with the infant fully unclothed and without a nappy. The scales were calibrated weekly. During the study, the scales at the clinic broke and could not be used for a period of 8 weeks. Weight at routine and illness visits could not be documented during this time.

Length

Infant length was measured using a length board and a two-person technique.

Head circumference

At birth, the occipito-frontal circumference (OFC) was measured using a tape measure. Measurements were taken three times, and the largest measurement recorded.

6.1.7 Vital sign measurement

Heart rate

Heart rate was assessed by auscultation over the precordium, with rate measured for 30 seconds and multiplied by 2, or for a full 60 seconds, depending on nurse preference.

Respiratory rate

Respiratory rate was assessed by auscultation over the chest in combination with observations of chest wall movement. Measurement took place for a full 60 seconds to allow for periodic breathing, which may occur in young infants.

Temperature

Temperature was measured using a digital axillary thermometer, as per the current WHO recommendations.

6.1.8 Blood sampling and handling

6.1.8.1 Cord blood sampling

Cord blood was obtained by direct venepuncture through the outside of the cord into the visible umbilical veins using a 10ml syringe and 21-gauge needle. This was accomplished within 5 minutes of delivery (and mainly immediately upon delivery of the placenta). Up to 10ml of cord blood was collected and transferred to one 5ml heparinised and one 5ml EDTA container.

Blood samples remained at room temperature prior to transfer to the laboratory for processing. Samples collected during the day were transferred to the laboratory within 4 hours of collection. Samples collected at night were transferred the following morning, resulting in a maximum of 16 hours delay in processing. Time from collection to laboratory processing was documented in all cases allowing for adjustment during the final analysis.

6.1.8.2 Infant blood sampling

Blood from infants was collected by venepuncture from the dorsum of either the hands or feet by study nurses. Blood collection was accomplished using 24-gauge cannulas, allowing blood to drop into microtainers under gravity. This procedure was determined to be the most successful following piloting of procedures, in comparison to 23-gauge butterfly needles and syringe collection. Both techniques were shown to lead to equivalent sterility of samples after culturing, when performed using aseptic procedures. Up to three attempts at venepuncture were allowed, providing the mother consented and the child was not overtly distressed. After two failed attempts, a senior clinician was requested to attempt venepuncture for the final time. More invasive techniques, such as femoral stab, were not conducted for routine visits but were used if the participant presented to the clinic unwell and it was indicated by their clinical condition.

Up to 2ml of infant blood was collected into microtainers (Becton-Dickinson, UK) with preservative varying due to the requirements of each sub-study:

Iron sub-study 1.5ml lithium heparin, 0.5ml EDTA

Cytokine and epigenetic sub-studies 2ml sodium heparin

As lithium heparin and sodium heparin tubes both have green caps, the sodium heparin tubes (cytokine and epigenetic sub-studies) were labelled with an additional orange sticker. This allowed rapid identification of tubes for both the phlebotomy nurses and the laboratory technicians.

Infant blood samples were stored at room temperature for up to four hours prior to transfer to the laboratory for processing. Time from collection to processing was documented for all samples. Blood sample tubes were labelled with study ID, date and time of collection, and blood collection forms were completed for transfer to the laboratory with the samples.

Participants presenting to the clinic unwell had additional blood samples (and other clinical investigations such as blood culture, urine culture, stool culture and lumbar puncture) carried out as indicated by their clinical condition and under the direction of the attending clinician and myself.

6.1.9 Stool sampling and handling

Mothers were requested to bring stool samples from their children at the 6-week study visit (prior to EPI-1 +/- BCG receipt) and at the 10-week study visit (prior to EPI-2 receipt). Stool pots (plain storage tubes with spoons integrated on the underside of the lid) were provided on discharge from the labour ward and verbal instructions on stool collection given. Mothers were reminded to bring stool samples on clinic attendance, during telephone follow-up. Mothers were requested to collect the stool sample on the morning of the clinic visit, or the night before and for the sample to be refrigerated if available.

On receipt at the clinic stool samples were processed and frozen for future microbiome analysis.

Despite regular reminders and requests for stool samples, compliance with this aspect of the study was low. A total of 358 samples were collected (out of an intended 1120), with only 93 participants providing samples at both the 6- and 10-week time-points. Anecdotally, this was due to a mixture of maternal forgetfulness, misplacing pots and lack of stool passage from the infant on the morning of attendance.

6.1.10 Nasal swab sampling and handling

Nasal swab collection at the 6- and 10-weeks (pre-EPI-1 and 2) was added to the study protocol mid-way through. This was added due to the publication of a study suggesting impacts of routine immunisation on nasal pathogen carriage.[285] It also provided an additional mucosal surface microbiome on which to study the impact of BCG, and was also a more reliable technique than stool sample collection, being carried out during routine visits rather than by mothers at home.

Box 6.2 STGG culture medium

Skimmed milk powder 2g
Tryptone soya broth 3g
Glucose 0.5g
Glycerol 10ml
Distilled water 100ml

This study followed a similar protocol for nasal swab collection as the published study investigating the impacts of routine immunisation on pathogen carriage in the nasopharynx,[285] to make the two studies as comparable as possible. Nasal swab culture medium (skimmed-milk, tryptone, glucose, glycerol - STGG) was prepared in advance, 1ml aliquoted into 1.5 ml tubes and frozen at -20˚C until use. The STGG culture medium recipe is found in Box 6.2.

Immediately prior to EPI vaccinations at the 6- and 10-week time-points, nurses inserted a paediatric calcium-alginate nasopharyngeal swab (Medical Wire) into the left naso-pharynx of the participant, extending until resistance was met. The swab was held in place for 5 seconds if possible, and rotated during removal. The swab was then placed in the culture medium and the wire cut off sufficiently to allow the cap to be replaced. The vial was shaken for 5 seconds and placed in a cool box with ice packs for transfer to -80˚C storage within 4 hours. In total 437 nasal swabs were collected, with 178 participants having paired samples at the 6-week and 10-week time-points.

6.1.11 Assessment of clinical outcomes

All participants were followed-up clinically for the duration of the 10-week study period, to determine the number, type and severity of illness episodes. This was accomplished in a number of ways, to ensure all illness episodes had been captured:

- At each routine visit clinicians questioned mothers and examined the participant for any current illness that the participant might have (Routine Visit CRF. Appendix 3d).

- At each routine visit, clinicians also questioned mothers about the type, duration and outcome of any illness that the participant had suffered since their last clinic visit (Routine Visit CRF. Appendix 3d).
- Mothers were strongly encouraged to bring their child to the clinic if they had any concerns about illness. At these illness visits, participants were assessed by a clinician, treated and followed-up as necessary, free of charge (Illness Visit CRF. Appendix 3g and 3h).
- The paediatric ward at Entebbe Grade B hospital was provided with a phone and credit to enable them to alert the study team to any attendances of study participants. These participants were then reviewed by a member of the study team on the ward (usually the PI) with Illness visit and Follow-up forms completed as appropriate (Illness Visit and Follow-up CRFs, Appendix 3g and 3h). Thrice-weekly routine attendance by the study PI on the wards further ensured that all participant admissions were captured.
- Mothers were asked about the clinical status of their child during weekly telephone follow-up. Mothers who reported that their child was unwell were requested to bring them to the clinic for review by a study clinician.

Deaths were recorded and the possible cause of death was investigated as far as possible in each case. The majority of deaths of participants (eight in total) were discovered upon routine telephone follow-up, and had not presented to the clinic or a hospital prior to the event. The one exception to this was a participant presenting to the clinic in the first week of life with symptoms of congenital bowel atresia. This participant was immediately transferred to the surgical referral centre but died en-route. For deaths discovered during telephone follow-up, a field-worker was dispatched to the family home to convey the study team's condolences and to carry-out a brief verbal autopsy where possible.

Using these multiple methods to capture clinical outcomes reduced the chance that an illness would be missed, but risked capturing the same illness event a number of times.

Records of illness episodes for each participant were therefore reviewed after data-entry to identify any duplicated documentation.

6.1.12 Data collection

Data collection occurred using paper CRFs (Appendix 3). These were double data entered into a Microsoft Access database by the MRC/UVRI data entry team. Initial attempts at designing a direct computerised data entry system for data capture suffered delays and it was not usable in time for participant recruitment. Paper forms for each participant were linked using a unique participant identifier. Study files were stored in locked filing cabinets in the study clinic whilst in use, prior to being transferred to MRC/UVRI for data entry and long term storage following completion of study procedures.

6.1.13 Serious adverse event reporting

Serious adverse events (SAEs) were reported as per the LSHTM protocol (Appendix 6) with reporting of serious adverse events to the local Uganda Virus Research Institute/Medical Research Council (UVRI/MRC) research ethics committee within 24 hours and to the LSHTM Ethics Committee in the annual report. Suspected unexpected serious adverse reactions (SUSARs) required expedited reporting to LSHTM.

There were 22 SAEs during the study and no SUSARs (see Table 6.4). All were reported as per requirements.

Table 6.4. Adverse events

Adverse event type	Number of this type of adverse event
Hospital admissions	14
Deaths	8 (+ 1 from a participant recently withdrawn from the study)
BCG vaccination site abscess	2

6.1.14 Study monitoring

The study was monitored on a day-to-day basis by the PI (myself) with oversight from Dr Stephen Cose, Professor Hazel Dockrell and Professor Alison Elliott. An internal study monitor, Miriam Akello, conducted regular monitoring assessments, including

prior to study commencement, throughout the study and upon completion. An independent Data Safety Monitoring Board (DSMB) met prior to the trial commencement and at its mid-point. The board comprised Professor Andrew Nunn (MRC Clinical Trials Unit, chair), Professor Elly Katabiri (Professor of Pediatrics, Makerere University) and Dr Phillipa Musoke (Lecturer in Pediatrics, Makerere University). The DSMB reviewed the conduct of the trial at two points during the study and assessed whether the trial needed to be terminated early for safety, futility or clear benefit in one arm. No major concerns were raised by the DSMB.

6.1.15 Routine appointment procedures

Participants attended routinely for blood samples and/or immunisations (routine EPI, and BCG at 6 weeks in the delayed arm) during the 10-week follow-up period.

Routine appointment procedures occurred as follows:

- Participant presented to clinic.
- Plaster applied over expected BCG vaccination site on the right deltoid by clinic receptionist.
- Participant's attendance at clinic logged in reception book.
- Study card obtained from mother and study file retrieved from locked filing cabinet.
- Both copies of study card placed into opaque brown envelope within the study file and sealed.
- Participant reviewed by a nurse who conducted anthropometry and measured vital signs.
- Participant reviewed by a clinician who enquired about any current and inter-appointment illness episodes, and performed a physical examination of the child (Routine Clinical Review Form. Appendix 3d). If the child was currently unwell, or abnormalities were found on examination, an illness episode form was also completed (Appendix 3g).
- Participant reviewed by nurses in the phlebotomy/vaccination room. The brown envelope concealing the study cards was opened and the procedures due for that visit were identified and completed. Depending on the visit this may have included one or more of: venous blood sampling, EPI vaccine administration, BCG vaccination, stool sample collection, nasal swab collection. On visits

where blood sampling and vaccination both occurred, blood samples were taken first. Nurses completed the routine clinical review form (Appendix 3d) and the accompanying laboratory forms for any samples (Appendix 3i). Both copies of the study cards were updated and the participant returned to reception.

- Participant's caregiver provided with transport reimbursement (10,000 Ugandan Shillings – equivalent to approximately £2.50 at the time), their copy of the study card and reminded about the date of the next clinic visit.
- The file copy of the study card was returned to the opaque brown envelope and the file returned to the locked filing cabinet.

6.1.16 Illness visit procedures

Participant's mothers were encouraged to bring their child for review at the clinic whenever they felt the child was unwell. Review and any treatments were provided free of charge, but transport reimbursements were not provided.

Illness episode procedures occurred as follows:

- Participant presented to clinic.
- Plaster applied over expected site of BCG vaccination on the right deltoid by clinic receptionist.
- Participant's attendance at the clinic logged in the reception book.
- Study card obtained from mother and the study file retrieved from locked filing cabinet.
- Both copies of study card placed into opaque brown envelope within the study file and sealed.
- Participant reviewed by a nurse who conducted anthropometry and measured vital signs.
- Participant reviewed by a clinician who performed a history and physical examination of the child.
- Investigations conducted by the clinician or a nurse, as per the clinicians instructions. Investigations available at the study clinic included haematology and blood biochemistry, culture of CSF, urine, swabs, stool and aspirate fluid, blood glucose and malaria parasite screen (microscopy and rapid diagnostic tests). Imaging and more invasive tests were available through private firms or at the tertiary referral hospital (Mulago) if required.

- Any medications prescribed were dispensed by the head nurse of the clinic. If not available at the clinic, medications were bought from a local pharmacy using study funds.
- The participant's caregiver was provided with their copy of the study card and reminded about the date of the next clinic visit (including follow-up for this illness visit, if required).
- The file copy of the study card returned to the opaque brown envelope and the file returned to the locked filing cabinet.

6.1.17 Telephone follow-up

Mothers were contacted on a weekly basis for the duration of the study. This was to check the clinical status of the child and to remind mothers about any routine visits that were due. Extra telephone contacts were also carried out in cases of missed routine appointments.

Contact was attempted by telephone in the first instance. If telephone contact was not successful on two consecutive days, a field worker was dispatched to the documented address to review the child and make any alterations to contact details as necessary. Occasionally, mothers and infant were not found at the documented address. In these cases, neighbours or family were asked for new addresses/telephone numbers.

If contact was not made, it was re-attempted weekly using the provided telephone details. With repeated non-contact, the study file remained open until the end of the 10-week study period, in case the participant presented directly to clinic. In cases of no contact, non-attendance and/or incorrect contact details having been provided the participant was assumed to have withdrawn from the study (20 participants).

6.1.18 Delays or non-attendance at routine appointments

Occasionally the mother had travelled out of the study area with the infant (33 participants). Mothers were encouraged to return for their infant's routine visits and immunisation. Participants that returned within the study period, but delayed, were retained in the study, provided that they had documented evidence of receipt of routine immunisations at the correct time in the community. Participants in the delayed BCG arm could not be kept in the study if they received BCG in the community, because of

likely strain differences in the vaccine used (BCG India was used routinely in Uganda during the study period, as opposed to the SSI 1331 strain used in the study).

6.2 Piloting of main study procedures

To test the proposed study protocols, a small pilot study was conducted from September to December 2014. This had the following aims:

1. To test study procedures - particularly to ensure that randomisation and blinding could be carried out effectively, but also to test that the CRFs were user friendly.
2. To confirm that EPI vaccines can act as *in-vivo* non-specific stimulants to the innate immune system and iron metabolism (blood samples 2 and 4), for the iron sub-study.
3. To identify the duration of the effects of EPI vaccines on the innate immune system, and thus identify when might be the optimal time to collect the blood sample post-BCG vaccination at 6 weeks to avoid confounding from EPI vaccinations (blood sample 3).
4. To identify the best time post-BCG vaccination to measure changes in the innate immune system (blood samples 1 and 3).

Ten neonates were recruited to the pilot study and randomised to receive BCG at birth or at 6 weeks of age. All infants had blood taken at 6 weeks, prior to receipt of EPI-1 vaccinations. Infants in the BCG-at-birth group were then randomly allocated to have their second blood sample 1-5 days post EPI-1 (one participant per day). Infants in the delayed-BCG group received BCG vaccination 6 days after receipt of EPI-1. This was the largest delay following EPI-1 allowed within the ethics approval for the study. The aim was to exclude any possible confounding caused by stimulation of the innate immune response by EPI-1 vaccination. Infants in the delayed BCG group were then randomly allocated to have their second blood sample collected 1-5 days post-BCG vaccination (7-11 days post-EPI-1). Figure 6.1 shows the vaccination and blood sampling schedules for the pilot study infants. IL-6 and hepcidin ELISAs were run on all blood samples, to analyse the impact of the vaccinations on the innate immune response and iron metabolism.

Figure 6.1. Pilot study blood sampling and vaccination timings

Participant	Birth		6 weeks + 0 days		EPI1 + 1d	EPI1 + 2 d	EPI1 + 3d	EPI1 + 4d	EPI1 + 5d	EPI1 + 6d	BCG + 1 d	BCG + 2d	BCG + 3d	BCG + 4d	BCG + 5d
1	**Cord blood**	BCG + OPV	**Blood sample**	EPI1	Blood sample										
2	**Cord blood**	BCG + OPV	**Blood sample**	EPI1		Blood sample									
3	**Cord blood**	BCG + OPV	**Blood sample**	EPI1			Blood sample								
4	**Cord blood**	BCG + OPV	**Blood sample**	EPI1				Blood sample							
5	**Cord blood**	BCG + OPV	**Blood sample**	EPI1					Blood sample						
6	**Cord blood**	OPV only	**Blood sample**	EPI1						**BCG**	Blood sample				
7	**Cord blood**	OPV only	**Blood sample**	EPI1						**BCG**		Blood sample			
8	**Cord blood**	OPV only	**Blood sample**	EPI1						**BCG**			Blood sample		
9	**Cord blood**	OPV only	**Blood sample**	EPI1						**BCG**				Blood sample	
10	**Cord blood**	OPV only	**Blood sample**	EPI1						**BCG**					Blood sample

BCG, Bacille Calmette Guerin; OPV, Oral Polio Vaccine; EPI1=Expanded Programme of Immunisations-1, d=days

6.2.1 Testing of study procedures

The pilot study confirmed that the study procedures in the study protocol of the main study were effective. Changes instituted as a result of the pilot study are listed below:

- Use of coloured ribbons on the labour ward to distinguish participants requiring BCG at birth easily for the government vaccination teams.
- Documentation of infants who did not receive BCG at birth in a log-book by government vaccination teams (stored in a locked filing cabinet and not accessed by the study team until the data were cleaned and locked). This was to provide an extra check at the end of the study that randomisation had occurred correctly.
- Use of paper CRFs as opposed to direct electronic data entry. These were tested alongside each other in the pilot study. However the electronic database was found to have many errors and was not easily and quickly useable by the study team. The decision to remain with paper CRFs was made on the recommendation of the study team.
- Use of cannulas with blood dropping into collection tubes under gravity for blood sampling in infants, rather than butterfly needle and syringe. This technique was found to be the easiest blood collection technique, and led to no increased contamination of samples (confirmed by culturing excess blood samples using the two techniques).

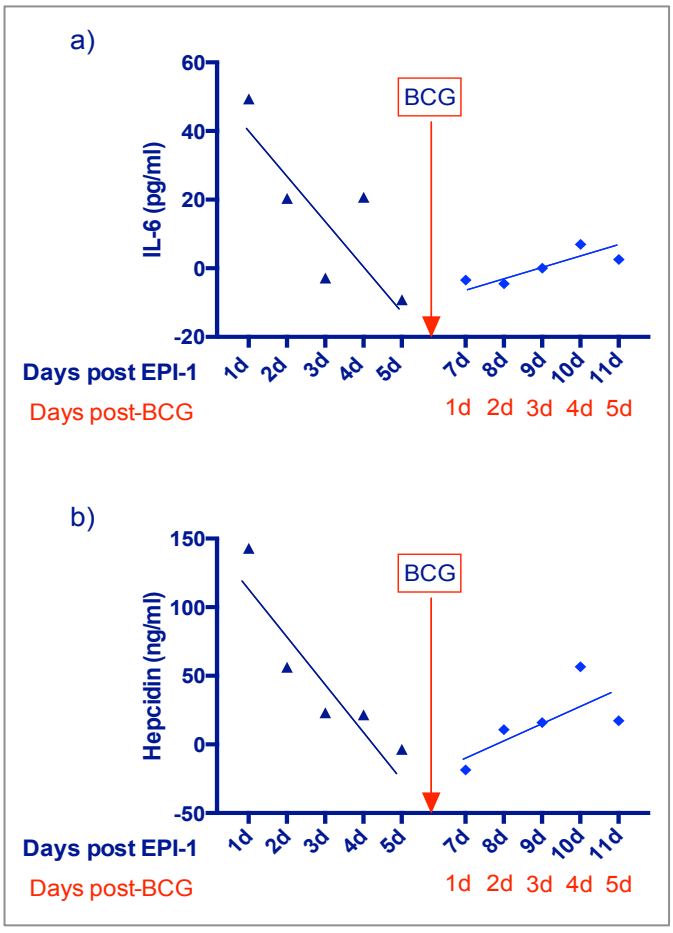
6.2.2 EPI vaccination effects on the innate immune system and the timing/duration of such changes

Figure 6.2 shows the change in IL-6 and hepcidin levels following EPI-1 vaccinations. As can be seen, both IL-6 and hepcidin appear to be highest at 1-day post-EPI-1. Levels then tended to decrease, and were lower than pre-vaccination levels by five days post-EPI-1.

6.2.3 BCG vaccination effects on the innate immune system and the timing of such changes

Figure 6.2 shows the change in IL-6 and hepcidin levels following BCG vaccination at 6 weeks + 6 days of age. A trend toward a slow increase of IL-6 and hepcidin levels at 4 and 5 days post-BCG was shown. In these infants, no changes from pre-vaccination levels of IL-6 or hepcidin were shown at 1-3 days post-BCG vaccination.

Figure 6.2. Innate immune system and iron metabolism responses following EPI-1 and BCG vaccination – pilot study



Levels of a) IL-6 and b) hepcidin in blood samples from individual participants following EPI-1 and BCG vaccinations, with lines of best fit for responses shown. n=10. EPI, expanded programme of immunisations; BCG, Bacille Calmette Guerin; d, day.

The evidence from the pilot study, therefore, suggested that EPI-1 vaccinations could be used as an *in-vivo* non-specific stimulant and that the best time to sample to show these changes would be at 24 hours post-EPI administration. Stimulation appeared to last for less than 5 days, whilst changes to innate responses following BCG vaccination did not

appear to be measurable until 4 or 5 days post-vaccination. Therefore the decision was made to vaccinate individuals in the delayed BCG group at 6 weeks + 1 day (in the iron sub-study) and sample for post-vaccination levels at 6 weeks + 5 days. Although interaction between BCG and EPI-1 vaccinations given this close together could not be ruled out, this timing was considered to produce a low chance of residual confounding from EPI-1 innate immune stimulation by the time the post-BCG vaccination sample was taken. It was also a time schedule that worked well logistically (meaning the 1 day post-EPI-1 blood sample and BCG vaccination time-points could be combined in the iron sub-study).

6.3 Detailed laboratory methods

6.3.1 Sample reception and initial processing – all sub-studies

Blood was received in the laboratory within 4 hours (infant samples) or 16 hours (cord blood samples) of being collected. Samples were recorded in the sample reception book and an anonymous laboratory ID number assigned. Sample reception forms were completed (Appendix 3i) which linked study ID number, sample date and type, and laboratory ID number.

6.3.1.1 Iron sub-study

Initial processing

Upon receipt in the laboratory, lithium-heparinised blood was centrifuged at 400g for 10 minutes at room temperature. The supernatant was divided into two aliquots, which were stored at -80˚C in separate freezers. This was to protect samples in case of freezer failure, and to reduce the number of freeze-thaw cycles that individual aliquots were subjected to. Remaining cell pellets were transferred to RNAase/DNAase free microtubes and stored at -80˚C. EDTA samples were transferred immediately to the clinical laboratory for full blood count analysis. Excess EDTA samples were stored in RNAlater at -80˚C. Time of processing and storage was documented in all cases.

Iron indices

Iron indices (TSAT, iron, ferritin, UIBC, TIBC, transferrin and sTFR) were measured using an automated COBAS Integra 400 plus (Roche Diagnostics, USA). This was conducted in batches on stored plasma after all recruitment and follow-up for iron sub-study participants had been completed. Samples were allocated into batches for analysis using their anonymous laboratory ID number and a random number generator in Microsoft Excel. The plasma samples had been subjected to one freeze-thaw cycle prior to iron analysis unless any analysis had needed to be repeated. Iron parameters are stable to multiple freeze-thaw cycles, so this should not have affected results.[286, 287] The COBAS Integra machine was calibrated daily prior to use, as per the manufacturer's instructions.

Hepcidin measurement

Hepcidin was measured by competitive ELISA kit (Bachem-25, California, USA), as per the manufacturer's guidelines. In essence:

- Hepcidin standards were created from stock standard:
  - Lyophilised standard was reconstituted in standard diluent to a concentration of 25ng/ml.
  - Two-fold serial dilutions were conducted to provide ten standard concentrations ranging from 0.049ng/ml to 25ng/ml.
  - Standard diluent alone was used for blank wells.
- Plasma samples were diluted using pooled peptide-free human serum as diluent, to an initial dilution of 1:20.
- 25µl of anti-hepcidin anti-serum was added to Bachem immunoplates pre-coated with antibody (except in the blank wells where buffer was added) and plates were incubated for 1 hour on a plate shaker at room temperature.
- 50µl of standards or diluted plasma samples were added to wells in duplicate. Diluent alone was added to the blank wells. Plates were incubated for 2 hours on a plate shaker at room temperature.
- 25µl of biotinylated-tracer (synthetic hepcidin-protein with biotin attached) was added to each well and plates were incubated for 18 hours on a shaker at 4˚C.
- Plates were equilibrated to room temperature on a plate shaker for 1 hour and then washed with buffer five times.
- 100µl Streptavidin-HRP (horseradish peroxidase) was added per well to bind to biotin and the plate incubated for 1 hour at room temperature.
- Plates were washed with buffer five times.
- 100µl of TMB (3,3',5,5'-tetramethylbenzadine) solution was added to all wells, inducing a blue colour change reaction with horseradish peroxidase, and plates incubated for 45 minutes at room temperature in the dark (placed in a drawer).
- 100µl of 2N HCL was added per well to stop the colour change reaction.
- Absorbance was read by an ELISA reader at 450nm within 10 minutes of 2N HCL addition, and analysed using 4-parameter logistic curve fitting software with blanks subtracted.

Samples were run in duplicate. Aliquots had been subject to one freeze-thaw cycle unless re-runs were required. Hepcidin has been shown to be stable for up to 4 freeze-

thaw cycles.[287] The detection range for the assay was 0.049-25ng/ml. Samples with concentrations outside the linear portion of the curve were re-run using alternative dilutions. Initial dilutions used were 1:20, as this level was previously shown to be appropriate in neonatal samples from the Gambian study. Samples with concentrations below the lower limit of detection were imputed using (limit of detection/√2). Samples with an intra-assay coefficient of variance (CV) >15% were re-run. Approximately 7% of samples needed to be re-run due to high CV.

IL-6 measurement

IL-6 was measured by competitive ELISA (BD-OptEIA sets, Oxford, UK). Half the manufacturer's recommended volume of sample and reagent were used throughout. Work conducted in the Co-Infection Studies Programme laboratory in Uganda has previously shown that this produces results comparable with using the full recommended volumes and reduces the volume of sample required per test. The ELISA methods used are outlined below:

- 96-well microplates (Immunolon 4HBX, Thermoscientific, UK) were coated with 50µl capture antibody diluted in coating buffer, incubated overnight at 4˚C, then washed three times with wash buffer.
- Plates were blocked with 100µl assay diluent, incubated for 1 hour at room temperature, then washed three times with wash buffer.
- IL-6 standards were created from stock standard:
  - Lyophilised stock standard was reconstituted in deionized water to 27ng/ml and used to prepare a 300pg/ml solution using assay diluent.
  - Two-fold serial dilutions were conducted to provide seven standard dilutions ranging from 0.49-300pg/ml
  - Standard diluent alone was used for blank wells.
- 50µl of standards, samples, control or diluent (blanks) were added to appropriate wells and incubated for 2 hours at room temperature, then washed five times with wash buffer.
- 50µl of working detector (biotinylated Anti-human IL-6 and streptavidin-HRP) was added to each well, incubated for 1 hour at room temperature and then washed seven times in wash buffer, with 1 minute soaks between washes.

- 50µl of substrate solution (TMB and hydrogen peroxide) was added to each well and the plates incubated for 30 minutes in the dark (placed in a drawer).
- 25µl of stop solution (2N H_2SO_4) added to each well.
- Absorbance read with an ELISA reader at 450nm, with wavelength correction at 570nm, within 10 minutes of stopping the reaction and analysed using 4-parameter logistic curve fitting software with blanks subtracted.

Samples were run in duplicate. The detection range for the assay was 0.49-250pg/ml. Samples were initially run undiluted. Samples with readings outside the linear portion of the curve were re-run using alternative dilutions. Samples with concentrations below the lower limit of detection were imputed using (limit of detection/√2). Samples with an intra-assay coefficient of variance (CV) >15% were re-run. Approximately 7% of samples needed to be re-run due to high CV. Plasma from a cord blood sample stimulated with polyinosinic:polycytidylic acid (poly I:C) was used as a positive control. Plates that had poly I:C values >2 SD from the average concentration were re-run (1 plate).

Haematology indices

EDTA whole blood samples were transferred to the clinical laboratory for automated analysis using a Coulter A^CT 5 Diff CP haematology analyser. Analysis occurred within a maximum of 16 hours of cord blood sample receipt, and 4 hours of infant sample receipt, with time from collection to analysis documented.

Results were reviewed by a laboratory technician during analysis. Samples with indices outside of the reference range for age were re-run, if volume permitted, and the PI informed. This occurred on only one occasion during the study, when extremely low white blood cell counts were found in one sample. The participant was recalled, reviewed and sampling repeated. The child was well and the white cell counts in a second blood sample had normalised. All other results were transferred to the PI for review within one-week.

6.3.1.2 Cytokine sub-study

Initial processing

On arrival at the laboratory for processing 1.3ml sodium-heparinised blood (or closest amount in the case of small sample volumes) was retained for use in the whole blood stimulation assay. 150µl of the remaining blood was placed in 800µl of RNAlater and stored as two aliquots at -20˚C for future analysis. Any remaining blood was centrifuged at 1000g for 10 minutes at room temperature, the plasma and cell pellet separated and stored at -80˚C for future use.

Whole-blood stimulation assay

Sodium-heparinised blood was incubated with six pathogenic stimulants, as well as a positive and a negative control stimulant as follows:

- Sodium-heparinised blood was diluted 1:2 with RPMI.
- A plate pre-coated with antigenic stimulants (see below) was thawed.
- 100µl of diluted blood was added per antigen stimulant well, giving a final blood dilution of 1:4.
- The plate was incubated for 24 hours at 37˚C in 5% CO_2.
- 120-150µl of supernatant was harvested per well and transferred to microtubes. Supernatants from duplicate/triplicate stimulation wells were pooled and divided into two aliquots.
- Aliquots were stored at -80˚C in different freezers, until cytokine ELISA analysis.

Stimulants used in the whole blood assay, their concentrations and their main toll-like receptor (TLR) targets are shown in Table 6.5.

Stimulants were selected a) to allow comparison with other studies investigating the non-specific effects of BCG,[92, 168, 193, 198] b) due to their importance as aetiologies of neonatal morbidity and mortality, and c) because they represented a range of pathogen types and TLR agonists. Whole pathogens were, largely, chosen over specific TLR agonists, in order to mimic *in vivo* neonatal infections as much as possible.

Table 6.5. Whole blood stimulation assay stimulants and their properties

Stimulant	Pathogen type	Main TLR recognition	Concentration (diluted from stock in RPMI)	Stock origin
Streptococcus pneumonia **(heat killed)**	Gram positive bacterium	2(+/- 1/6) and 9	$1x10^6$/ml	Radboud University Nijmegen
Staphylococcus aureus **(heat killed)**	Gram positive bacterium	2 (+/- 1/6) and 9	$1x10^6$/ml	Radboud University Nijmegen
Escherichia coli **(heat killed)**	Gram negative bacterium	4, 2(+/- 1/6)	$1x10^6$/ml	Radboud University Nijmegen
Candida albicans **(heat killed)**	Fungus	2(+/-1/6) and 4	$1x10^6$/ml	Radboud University Nijmegen
Poly I:C (1530/0913)	Virus-like stimulant (double stranded RNA)	3	5µg/ml	Sigma Aldrich, UK
CpG ODN (2395)	Virus-like stimulant (DNA)	9	5µg/ml	Invivogen, UK
Purified peptide derivative	Positive control for BCG vaccination/mycobacteria exposure	6, 2, 4, 1	10µg/ml	Statens Serum Institut, Denmark
RPMI	Negative control			Invitrogen, UK

Poly I:C, polyinosinic:polycytidylic acid, CpG ODN, CpG oligodeoxynucleotides; RPMI, Roswell Park Memorial Institute medium

Assay plates were pre-coated with stimulant (100µl per well) in batches, prior to study commencement. These were covered with sterile acetate films, stored at -20˚C and thawed upon receipt of a blood sample. The plate layout for stimulations is shown in Figure 6.3. Stimulations were conducted in triplicate to provide greater volumes of supernatant for harvesting, except for *C.albicans* (conducted in duplicate) and CpG ODN (conducted once only) due to reduced availability of stimulant.

Figure 6.3 Antigenic stimulation plate layout

		RPMI	PPD	Poly I:C	*S. pneumoniae*	*S. aureus*	*E.coli*	*C.albicans*	CpG ODN		
		RPMI	PPD	Poly I:C	*S. pneumoniae*	*S. aureus*	*E.coli*	*C.albicans*			
		RPMI	PPD	Poly I:C	*S. pneumoniae*	*S. aureus*	*E.coli*				

100µl of stimulant per well. 100µl of blood, diluted 1:2 in RMPI was added, for a final dilution of 1:4. Grey wells were filled with PBS to prevent drying out. RPMI, Roswell Park Memorial Institute medium; PPD, Purified Protein Derivative; Poly I:C, polyinosinic:polycytidylic acid, *S.pneumoniae, Streptococcus pneumoniae; S.aureus, Staphylococcus aureus; E.coli, Escherichia coli; C.albicans, Candida albicans;* CpG ODN, CpG oligodeoxynucleotides.

Optimisation experiments were conducted prior to commencement of the cytokine study, to determine the most appropriate dilution of blood, concentration of stimulants, and incubation time for innate cytokine recovery in neonatal blood samples. When determining the most appropriate methods to use, the degree of cytokine response at each condition was the main concern. However, consideration was also given to:

1. The methods used in previous and on-going studies into the non-specific effects of BCG, to allow for direct comparisons if possible
2. The limited volumes of blood available
3. The cost and availability of stimulants
4. The logistical impacts of different harvesting times.

Optimisation experiments were conducted using excess cord blood from another study. Cord blood from two infants was used per stimulant and tested under all variable conditions as shown in Table 6.5. ELISAs for TNFα and IL-10 were used to define the optimal responses, to represent pro- and anti-inflammatory cytokine reactions respectively.

Blood dilution

There was minimal difference in cytokine production for any stimulant tested comparing blood diluted 1:2, with blood diluted 1:4 (see Figure 6.4). Both dilutions largely produced TNFα and IL-10 cytokine responses within or above the measurable portion of the ELISA standard curve. The notable exceptions were at either dilution for TNFα at 48 hours of incubation, and for IL-10 at 6 hours of incubation. A dilution of 1:4 was chosen for the main experiments, as it provided greater volumes to work with, given the small starting blood volumes.

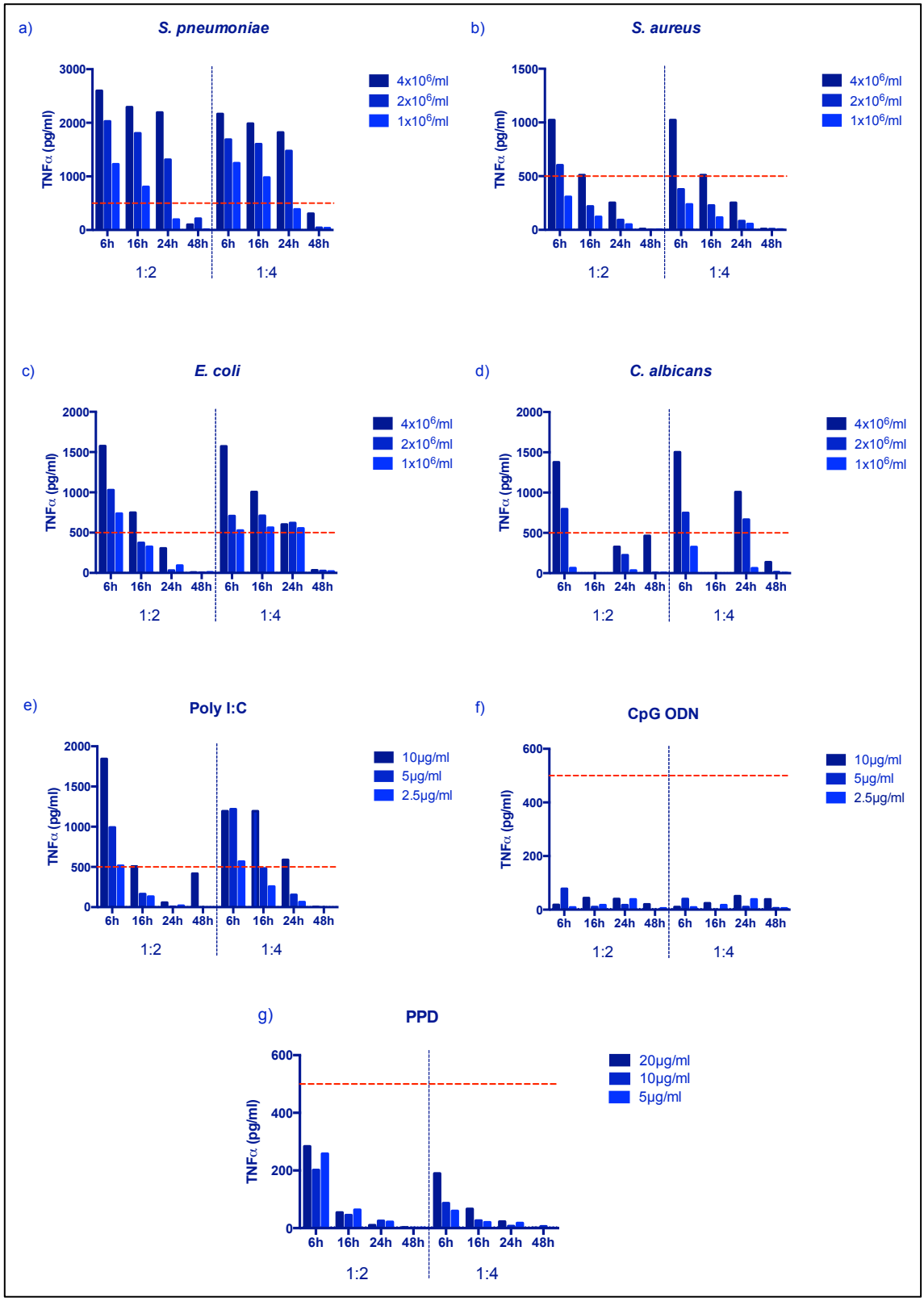
Incubation time

As shown in Figure 6.4, TNFα levels for all stimulants other than CpG ODN declined with incubation time, being maximal at 6 hours and low/unreadable at 48 hours. The reverse was seen with IL-10 (Figure 6.5), with stimulants mainly producing higher cytokine levels at 48 hours and minimal levels at 6 hours. At 16 and 24 hours of incubation, all stimulants produced readings within or above the measurable portion of the ELISA standard curves. An incubation time of 24 hours was, therefore, chosen for the main experiment, for logistical ease and for consistency with other studies.

Stimulant concentrations

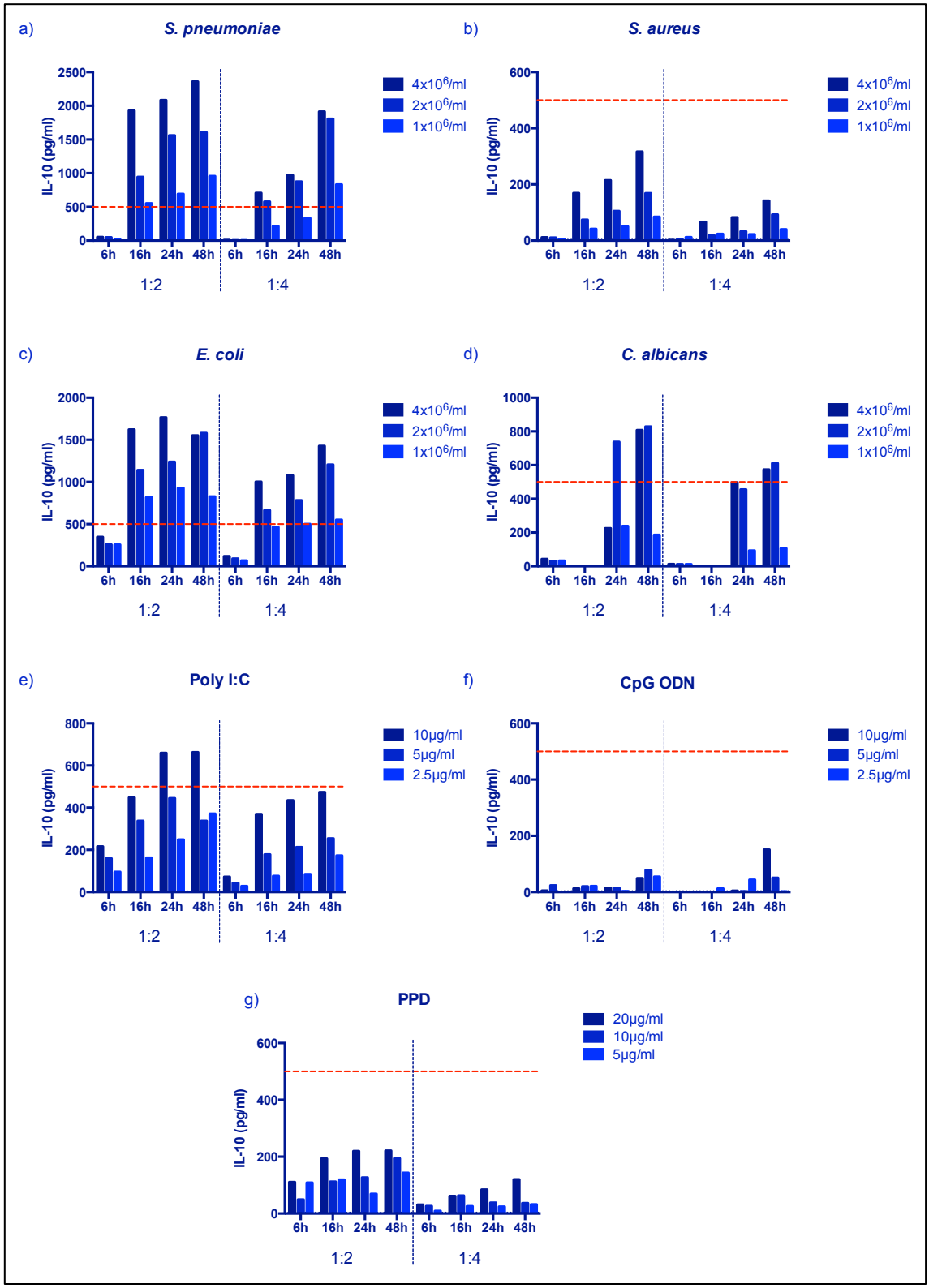
As expected, higher stimulant concentrations resulted in larger cytokine responses (Figures 6.4 and 6.5). When run with 1:4 blood dilution (1 part blood: 4 part RPMI) and 24 hours incubation time, all stimulants, at all concentrations (other than CpG ODN), gave average readings that could be measured by ELISA. However, when the lowest stimulant concentrations were used, some readings were at the very lower end of the standard curve. Therefore, to allow for the possibility of low cytokine responses in some infants, the middle concentration of those tested was chosen for the main experiment i.e. $2x10^6$/ml for bacterial and fungal stimulants and 5µg/ml for poly I:C and 10µg/ml for PPD. These bacterial and fungal concentrations chosen were also the same as previously used in adult studies of the NSE of BCG,[168] potentially allowing for direct comparisons. In general CpG ODN produced extremely low levels of both TNFα and IL-10 across the range of concentrations and incubation timings. A concentration of 5µg/ml was, therefore, chosen as the most likely to be optimal as per the manufacturer's guidelines.

Figure 6.4. TNFα concentrations by blood dilution, stimulant concentrations and incubation time



a) *Streptococcus pneumoniae* b) *Staphylococcus aureus* c) *Escherichia coli* d) *Candida albicans* e) Polyinosinic:polycytidylic acid f) CpG oligodeoxynucleotides g) Purified Protein Derivative. The red dotted line shows the upper limit of the detection range of the ELISA. Cord blood from 2 infants was used per stimulant and tested under all conditions.

Figure 6.5. IL-10 levels by blood dilution, stimulant concentrations and incubation time



a) *Streptococcus pneumoniae* b) *Staphylococcus aureus* c) *Escherichia coli* d) *Candida albicans* e) Polyinosinic:polycytidylic acid f) CpG oligodeoxynucleotides g) Purified Protein Derivative. The red dotted line shows the upper limit of the detection range of the ELISA. Cord blood from 2 infants was used per stimulant and tested under all conditions.

Cytokine ELISAs

ELISAs to quantify the levels of TNFα, IL-6, IL-1β, IL-10 and IFNγ in stimulated supernatants were conducted in batches from frozen supernatants as previously described for IL-6 (BD-OptEIA, Becton-Dickinson, UK, using half the manufacturer's recommended volume of sample and reagent). Assignment of samples to plates within each batch occurred randomly, using a list of lab ID numbers and a random number generator in Microsoft Excel. Due to limits on available supernatant volume, analysis was conducted in singlecate and transference of supernatants between ELISA plates occurred a maximum of one time, with samples used for TNFα transferred to IL-10 plates and samples used for IL-6 transferred to IL-1β plates. Cytokines were analysed in the order of sensitivity to freeze-thaw cycles: TNFα/IL-10 followed by IL-6/IL-1β and finally IFNγ. Supernatants were allowed to undergo a maximum of six freeze-thaw cycles, though this number only occurred in rare cases where multiple re-runs were required. As previously described, samples with readings outside the linear portion of the curve were re-run using alternative dilutions. Samples with concentrations below the lower limit of detection were imputed using (limit of detection/√2). Samples with an intra-assay coefficient of variance (CV) >15% were re-run.

Experiments were conducted prior to the commencement of cytokine ELISAs to confirm:

- that sample transference between ELISA plates could be conducted without alteration of cytokine concentrations
- the number of freeze-thaw cycles that cytokines were stable for (and thus the order of cytokine analysis)
- that cytokine analysis in singlecate as opposed to duplicate gave reliable results

These experiments used one spare cord blood sample diluted 1:2 with RPMI and stimulated overnight with poly I:C as per the whole blood assay methods previously described. The collected supernatant was then divided into aliquots, which were subjected to either 0, 2, 4 or 6 freeze-thaw cycles (cycling between room temperature and -80˚C). ELISA plates were prepared for each cytokine (TNFα, IL-6, IL-1β, IL-10 and IFNγ) as previously described, and four replicates per freeze-thaw condition were conducted with supernatant used a) directly from the stimulated sample and b) used after transference from a different ELISA plate. As shown in Table 6.6 variability was less than 10% for all cytokines tested, except for IFNγ, which showed higher variability,

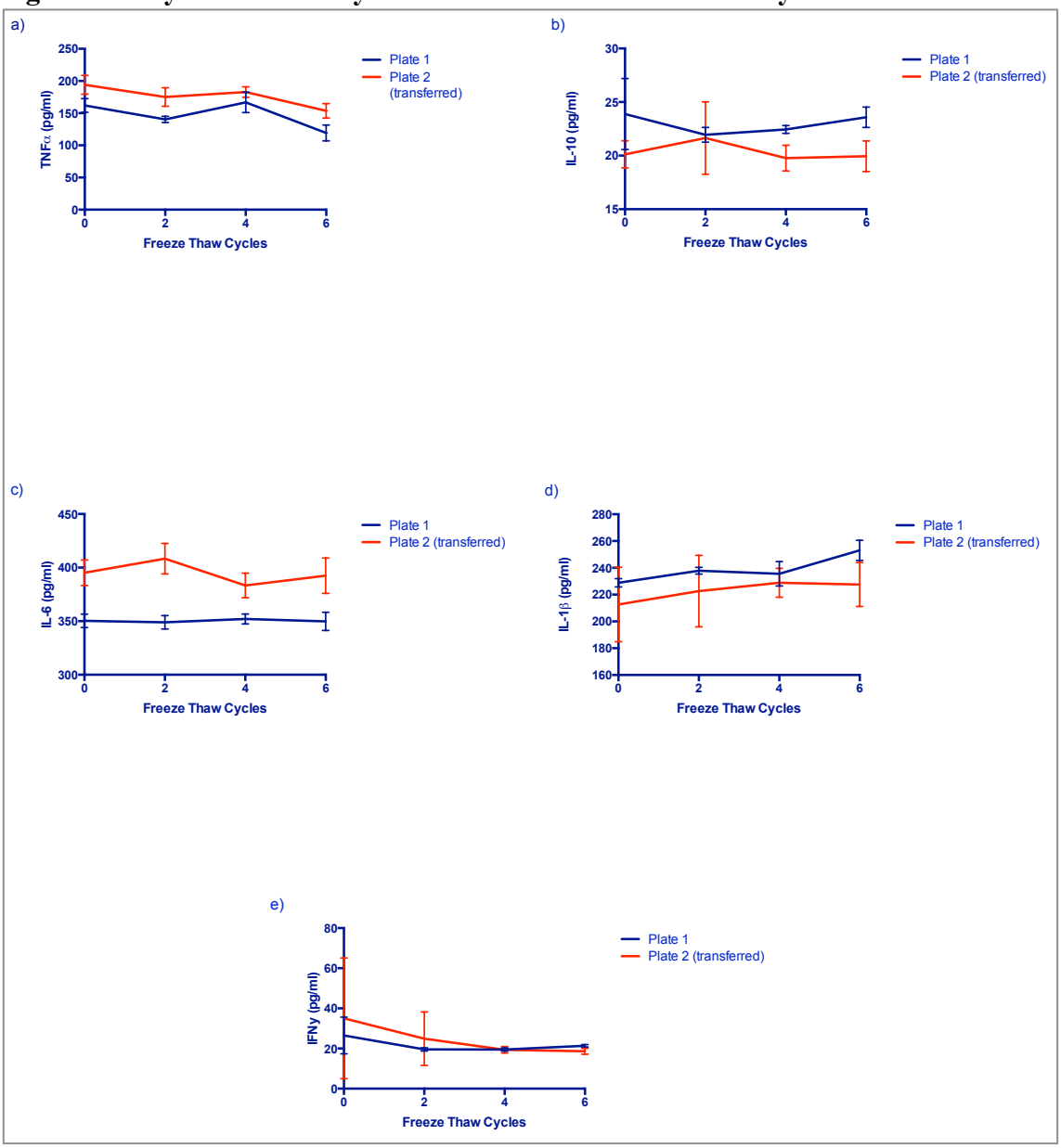
particularly if supernatants were transferred. Variability for the other cytokines tended to be similar across freeze-thaw cycles and whether the samples were from supernatants used directly or following transfer. Transferred IL-1β, IL-10 and IFNγ levels were not significantly different from supernatants used directly (Table 6.6) although in all cases there was a trend toward lower cytokine levels following transfer. TNFα and IL-6 levels were significantly higher following transfer (over all freeze-thaw conditions) than when supernatants were used directly. TNFα was the only cytokine that showed significantly different levels upon freeze-thawing, decreasing after 6 cycles. Therefore the decision was made to analyse the samples as follows: TNFα transferred to IL-10 plates (first freeze-thaw cycle), IL-6 transferred to IL-1β (second freeze-thaw cycle) and IFNγ alone (third freeze-thaw cycle). Re-runs were conducted at the same time (fourth freeze-thaw cycle) where possible. All were conducted in singlecate, as low variability was shown, but initial IFNγ samples were tested in duplicate to provide reassurance of low variability. Although transferring samples produced a non-significant trend toward lower cytokine responses, the decision was made to use transferred supernatants for IL-10 and IL-1β due to small supernatant volumes. As the use of transferring for IL-10 and IL-1β occurred consistently with all samples the ability to compare *in vitro* cytokine production between the two arms of the study should not have been affected.

Table 6.6. Variability of cytokine levels

Cytokine tested	Direct or transferred supernatant plate	Coefficient of Variance (%) (average of four replicates)				Average CV (%) across freeze-thaw cycles
		Freeze-thaw cycles				
		0	2	4	6	
TNFα	Direct	6.6	3.4	9.5	10.4	7.5
	Transferred	7.5	8.3	4.5	7.4	6.9
IL-10	Direct	13.9	3.1	1.7	4.0	5.7
	Transferred	6.3	14.9	6.1	7.2	8.6
IL-6	Direct	1.8	1.8	1.3	2.4	1.8
	Transferred	3.0	3.5	3.0	4.2	3.4
IL-1β	Direct	1.4	1.1	3.9	3.0	2.4
	Transferred	13.1	12.0	4.7	7.2	9.3
IFNγ	Direct	34.6	4.1	4.1	2.8	11.4
	Transferred	85.9	53.6	8.4	7.9	**39.0**

Four replicates per condition were tested for cytokine levels measured by ELISA and the results compared for consistency (coefficient of variance).

Figure 6.6. Cytokine stability after transfer and freeze-thaw cycles.



Mean and standard deviations are shown of cytokine levels a) TNFα b) IL-10 c) IL-6 d) IL-1β e) IFNγ. Cord blood from one infant stimulated with Poly I:C was used for these experiments. Four replicates were tested for each condition. Plates 1 were ELISAs conducted on supernatants tested directly, Plates 2 were supernatants tested following transfer after use on a different ELISA plate.

6.3.1.3 Epigenetic sub-study

Processing of sodium-heparinised blood for medium-term storage and transport to Radboud University Medical Centre, Nijmegen occurred in Uganda as follows:

PBMC separation

PBMCs were separated from whole blood using double density centrifugation on Ficoll-paque (Sigma-Aldrich). In short:

1. Heparinised whole blood was transferred to a 15ml falcon tube.
2. Blood was diluted 1:1 with RPMI 1640 (Invitrogen, with 4mM L-glutamine, 50,000 U/ml penicillin/streptomycin and 20mM HEPES buffer added) pre-warmed to 37˚C.
3. The solution was under-laid with Ficoll-paque at a ratio of 3 volumes of diluted blood to 1 volume of Ficoll-paque.
4. The Ficoll-paque/blood mixture was then centrifuged at 1000g for 22 minutes at room temperature and slowed with the brakes off.
5. PBMCs were removed from the interface and transferred to a sterile 50ml centrifuge tube with a Pasteur pipette.
6. The volume was made up to 40ml with RPMI 1640 and centrifuged at 650g for 7 minutes at room temperature.
7. Steps 1-6 were repeated once, to ensure removal of nucleated red blood cells.
8. The supernatant was removed and the pellet re-suspended in 5ml RPMI 1640.
9. 1:1 suspension of cell pellet and 0.4% trypan blue was prepared and viable mononuclear cells counted by microscopy.
10. The number of PBMC/ml was documented for future reference.

Comparison of different PBMC separation techniques was conducted prior to commencement of the epigenetic sub-study to ensure maximal recovery of viable PBMCs (with minimal nucleated red blood cell contamination). This was done using excess blood from cord blood samples. Comparison of techniques and recovery rates of PBMCs are shown in Table 6.7. Double layering over Ficoll-paque was chosen as the optimal technique to recover the most viable cells, with the least nucleated red blood cell contamination.

Table 6.7. PBMC recovery by separation technique

PBMC separation technique	Average PBMC recovery/ml
Single separation over Ficoll-paque	11 x 10^6 but many nucleated red blood cells
Single separation over Ficoll-paque with red cell lysis buffer added	8 x 10^6 but many dead cells
Double separation over Ficoll-paque	5 x 10^6

PBMC, peripheral blood mononuclear cell.

Previous epigenetic studies carried out in Nijmegen, which this aspect of the study was based on, used monocytes rather than PBMCs. Piloting of monocyte adherence methods of cell separation was conducted as outlined in Table 6.8. However, recovery rates for all methods were too low to allow for further epigenetic study and therefore it was decided to conduct the work using unseparated PBMCs, rather than monocytes. The current optimal separation technique, using magnetic bead selection, was not available for this study.

Table 6.8 Monocyte recovery by separation technique

Monocyte separation technique	Average monocyte recovery (% and count/ml)
Incubation at 37˚C on 6-well plate for 2 hours	31% recovery from PBMC sample 55,000/ml
Incubation at 37˚C on petri dish for 2 hours	16% recovery from PBMC sample 80,000/ml
Incubate at 37˚C on 6-well plate for 18 hours	10% recovery from PBMC sample 75,000/ml

PBMC, peripheral blood mononuclear cell.

PBMC fixation

The isolated PBMCs were fixed in formaldehyde, to ensure stability of epigenetic marks during medium-term storage prior to further processing, as follows:

- 1% formaldehyde was prepared in batches (10g paraformaldehyde powder dissolved in 1 litre of warmed phosphate buffered saline (PBS) at pH 7.4 then filtered and aliquoted), and stored at -20˚C until use, wrapped in aluminium foil to protect it from light.
- Formaldehyde allowed to reach room temperature.
- PBMC cell suspension centrifuged at 350g for 5 minutes at room temperature.
- Supernatant removed and discarded, leaving PBMC cell pellet.
- Cell pellet re-suspended in 4ml 1% formaldehyde.
- Incubated for 10 minutes at room temperature in a shaker (microplate incubator shaker set at minimal speed).
- 400µl 1.25M glycine (freshly prepared per day: 0.94g glycine dissolved in 10ml pure molecular biology grade water) added and incubated for 3 minutes at room temperature.
- 6ml PBS (calcium and magnesium free) added. Mixture centrifuged at 1600rpm for 5 minutes at 4˚C.
- Supernatant discarded and pellet re-suspended in 2 ml PBS (calcium and magnesium free).
- Divided into 1ml aliquots in labelled Eppendorf tubes and stored at +4˚C prior to refrigerated transfer to Radboud University Medical Centre, Nijmegen.

PBMC lysis and chromatin sonication

PBMC lysis, chromatin sonication and storage at -80˚C was carried out at Radboud University Medical Centre by Dr Rob Arts. This process occurred as follows:

- Cell suspension centrifuged at 1600rpm for 5 minutes at 4˚C and supernatant discarded
- Lysis buffer (40µl 20mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, (HEPES, ThermoFisher Scientific), 100µl 1% Sodium Dodecyl Sulfate (SDS – ThermoFisher Scientific), 143µl Protein Inhibitor Complex (PIC – Roche),

717µl sterile water) added at a ratio of 1ml per $15x10^6$ PBMCs immediately prior to sonication

- Sonicated in 100µl aliquots for 10 minutes at 4˚C (30 seconds on:30seconds off)
- Aliquots centrifuged at 13000rpm at room temperature for 5 minutes.
- Supernatant (chromatin) transferred to new Eppendorf tubes.
- Snap-frozen in liquid nitrogen.
- Stored at -80˚C until further use.

Chromatin immunoprecipitation and qPCR analysis

Chromatin immunoprecipitation and qPCR analysis was conducted in batches, after completion of the study in Uganda. The process occurred as follows:

Immunoprecipitation bead blocking

- Santa Cruz Protein A/G Plus-Agarose beads spun at 3000g for 5 minutes at 4˚C.
- Supernatant discarded and 1ml incubation buffer added (200µl (50mM Tris pH 8.0, 0.75M NaCl, 5mM ethylene diamine tetraacetic acid (EDTA), 2.5mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), 0.75% SDS, 5% Triton x-100) 20µl 5% bovine serum albumin (BSA), 780µl water).
- Spin repeated, supernatant discarded and incubation buffer added twice more. On final time only 500µl of incubation buffer was added.
- Rotated at 4˚C overnight.
- Stored at 4˚C.

Input samples prepared (total chromatin)

- 33.33µl of chromatin sample de-crosslinked by incubating for 1 hour at 65˚C (shaken at 1000rpm).
- Sample purified using MinElute PCR purification columns (Qiagen) under negative pressure, as per the manufacturer's instructions.

ChIP samples prepared (chromatin bound to specific antibody)

- Antibodies (1µl) corresponding to the studied mark (H3K4me3 or H3K9me3, Diagenode) added to 33.33µl chromatin sample, 20µl beads and 245.67µl dilution buffer (1m = 200µl (83.5mM Tris, 835mM NaCl, 6mM EDTA, 0.05% SDS, 5% Triton-X 100) with 20µl 5% BSA, 143µl PIC and 637µl water).
- Rotated overnight at 4˚C
- Centrifuged up to 5600rpm (stopped immediately when centrifuged reached 5600rpm) with brakes off. Tubes rotated 180˚ in the centrifuge and process repeated. Supernatant discarded.
- Beads washed six times.
- Supernatant discarded.
- Chromatin eluted using 200ul elution buffer (20µl 1% SDS, 40µl 0.1M $NaHCO_3$, 140µl water) and rotated for 20 minutes at room temperature on a rollerbank.
- Centrifuged at 7000rpm for 3 minutes at room temperature
- Supernatant transferred to new tube. 8µl 5M NaCl, 2µl proteinase K (Qiagen) added.
- Chromatin de-crosslinked by incubating for 4 hours at 65˚C (shaking at 1000rpm).
- DNA purified for PCR using MinElute purification columns (Qiagen) under negative pressure as per manufacturers guidelines.

qPCR run

- Input sample diluted 25 times with water.
- ChIP sample diluted 3 times with water.
- qPCR plates prepared with 2µl DNA sample, 5µl sybr-green, 0.8µl forward and reverse primers (100µm) and 2.2µl water per well. Primers used corresponded to IL-6, TNFα and IL-1ß. Positive and negative controls were used: myoglobin and histone 2B for H3K4me3, zinc fingers and glycerol-3-phosphate dehydrogenase for H3K9me3.
- qPCR performed.
- Samples calculated as the percentage of total chromatin (input) that is antibody bound (ChIP).

6.4 Detailed statistical methods

Baseline group characteristics were compared using Pearson's Chi-squared test for categorical variables and the t-test for continuous variables.

Cross-sectional between-group comparisons at each time-point were conducted using the t-test for significant difference of means, with logarithmic transformation ($log_{e)}$ of non-normally distributed data. Mann-Whitney two-tailed test was used for persistently skewed data. The following distributions were found:

Normally distributed

- Within-infant changes to erythrocyte parameters.

Normally distributed after logarithmic transformation

- Cross-sectional stimulated cytokine responses, inflammatory-iron parameters, erythrocyte parameters and leucocyte parameters.
- Within-infant changes to leucocyte parameters.

Non-normally distributed data after logarithmic transformation

- Cross-sectional H3K4me3 and H3K9me3 percentage recovery, unstimulated cytokine responses (negative control).
- Within-infant changes to H3K4me3 and H3K9me3 percentage recovery, inflammatory-iron parameters and erythrocyte parameters.

Between-group comparisons of the concentrations of cytokines produced following heterologous stimulation were conducted using both the raw data, and following subtraction of the unstimulated cytokine response. There was little difference in the significance of the comparisons using either method, and therefore the main results presented are following subtraction of the unstimulated cytokine response. Results using both methods can be found in Appendix 9. Geometric mean ratios of responses by BCG status were calculated using linear regression with the unstimulated response included in the regression analysis as a co-variate. Tests of interaction with sex were assessed as part of a linear regression model. The addition of boot-strapping to the linear regression model made little difference to the results, and therefore non-boot

strapped results are shown. Correlations between immunological parameters, clinical outcomes and BCG scar size were conducted using Spearman rank correlations.

Paired/longitudinal analysis of within-infant changes in outcome measures over time was conducted using the paired student t-test or Wilcoxon matched-pairs test, depending on the underlying distribution of the data. Results are presented as both unadjusted results, and adjusted for baseline levels, to allow for inter-individual variability in responses.

Hazard rates of invasive infectious disease in the first 10 weeks of life by BCG status were compared using Poisson regression with robust standard errors, to allow for within-child clustering.

Statistical significance was assessed at the two-sided 0.05 level, but interpretation of results was not be solely reliant on P-values. No correction for multiple-testing was applied to allow for better identification of overall trends; however, the risks of significant findings resulting from multiple comparisons were borne in mind when interpreting the results.

Statistical analysis was conducted using Stata version 14.1 (StataCorp, Texas, USA). Graphs were produced using GraphPad Prism version 6.0 (San Diego, California).

6.5 Paper 3: Investigating the non-specific effects of BCG vaccination on the innate immune system in neonates: study protocol for a randomized controlled trial.

London School of Hygiene & Tropical Medicine
Keppel Street, London WC1E 7HT
www.lshtm.ac.uk

Registry
T: +44(0)20 7299 4646
F: +44(0)20 7299 4656
E: registry@lshtm.ac.uk

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SECTION A – Student Details

Student	Sarah Prentice
Principal Supervisor	Stephen Cose
Thesis Title	Investigating the Non-Specific Effects of BCG in Neonates

If the Research Paper has previously been published please complete Section B, if not please move to Section C

SECTION B – Paper already published

Where was the work published?	Trials		
When was the work published?	April 2015		
If the work was published prior to registration for your research degree, give a brief rationale for its inclusion	Not applicable		
Have you retained the copyright for the work?*	Yes	Was the work subject to academic peer review?	Yes

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Where is the work intended to be published?	
Please list the paper's authors in the intended authorship order:	
Stage of publication	

SECTION D – Multi-authored work

For multi-authored work, give full details of your role in the research included in the paper and in the preparation of the paper. (Attach a further sheet if necessary)	I was responsible for the concept, design of the study, and manuscript preparation with advice from EW, HMD, PK, AME and SC

Student Signature: [redacted] **Date:** 25/03/2018

Supervisor Signature: [redacted] **Date:** 25/03/2018

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TRIALS

STUDY PROTOCOL Open Access

Investigating the non-specific effects of BCG vaccination on the innate immune system in Ugandan neonates: study protocol for a randomised controlled trial

Sarah Prentice[1,2,5*], Emily L Webb[3], Hazel M Dockrell[1,4,5], Pontiano Kaleebu[5], Alison M Elliott[1,2,5] and Stephen Cose[4,5]

Abstract

Background: The potential for Bacillus Calmette-Guérin (BCG) vaccination to protect infants against non-mycobacterial disease has been suggested by a randomised controlled trial conducted in low birth-weight infants in West Africa. Trials to confirm these findings in healthy term infants, and in a non-West African setting, have not yet been carried out. In addition, a biological mechanism to explain such heterologous effects of BCG in the neonatal period has not been confirmed. This trial aims to address these issues by evaluating whether BCG non-specifically enhances the innate immune system in term Ugandan neonates, leading to increased protection from a variety of infectious diseases.

Methods: This trial will be an investigator-blinded, randomised controlled trial of 560 Ugandan neonates, comparing those receiving BCG at birth with those receiving BCG at 6 weeks of age. This design allows comparison of outcomes between BCG-vaccinated and -naïve infants until 6 weeks of age, and between early and delayed BCG-vaccinated infants from 6 weeks of age onwards. The primary outcomes of the study will be a panel of innate immune parameters. Secondary outcomes will include clinical illness measures.

Discussion: Investigation of the possible broadly protective effects of neonatal BCG immunisation, and the optimal vaccination timing to produce these effects, could have profound implications for public healthcare policy. Evidence of protection against heterologous pathogens would underscore the importance of prioritising BCG administration in a timely manner for all infants, provide advocacy against the termination of BCG's use and support novel anti-tuberculous vaccine strategies that would safeguard such beneficial effects.

Trial registration: ISRCTN59683017: registration date: 15 January 2014

Keywords: Bacillus Calmette-Guérin, Heterologous effects, Innate immunity, Neonate, Invasive infectious disease

Background

Background and rationale

Bacillus Calmette-Guérin (BCG) immunisation, the only currently available tuberculosis (TB) vaccine, is one of the most frequently administered immunisations worldwide with more than 100 million children receiving it per year [1]. Although it provides protection against severe forms of TB in children, it has variable efficacy against adult pulmonary disease, with protection generally poor in high-risk areas such as sub-Saharan Africa and Asia [2]. There are currently concerted efforts in the scientific community to improve anti-TB protection either by enhancing existing BCG immunisation strategies or by developing an alternative vaccine [3].

However, it has been suggested that BCG may protect infants against a variety of non-mycobacterial pathogens and thus have beneficial effects beyond protection against TB [4]. The evidence for such a 'non-specific' effect of BCG is currently in equipoise. It is, therefore, important and pressing to interrogate this possibility

* Correspondence: Sarah.prentice@lshtm.ac.uk
[1]Wellcome Trust - Bloomsbury Centre for Global Health Research, London School of Hygiene and Tropical Medicine, Keppel Street, London WC1E 7HT, UK
[2]Clinical Research Department, London School of Hygiene and Tropical Medicine, Keppel Street, London WC1E 7HT, UK
Full list of author information is available at the end of the article

further so that any new vaccine or BCG schedule may be evaluated in terms of overall benefit to recipient, rather than in terms of TB-specific protection alone.

The possibility that BCG may have non-specific beneficial effects on diseases other than TB has been a controversial and highly-debated subject. Observations that BCG may have a greater impact on mortality than can be explained by protection against TB were first made following its introduction more than 80 years ago. Studies including more than 46,500 infants, carried out in the 1940s and 1950s in the USA and UK, showed on average a 25% (95% CI 6 to 41%) reduction in all cause mortality in children receiving BCG compared to those not receiving it [5-9]. This reduction was noted at the time to be larger than could be attributed to the expected reduction in rates of TB. However, as many of these studies were not strictly randomised or controlled, and this was a period of major public health improvements, the results were assumed to result from confounding effects. Similar arguments have been used to dismiss a number of observational studies carried out more recently, in Guinea-Bissau, which appear to show that infants who receive BCG at birth have lower all-cause morbidity and mortality than infants who do not [10-17].

Good quality, randomised controlled trials evaluating the possibility of non-specific effects of BCG are extremely limited. Only one trial has been conducted to specifically evaluate non-tuberculous mortality as a result of altered BCG vaccination schedule [18]. In this trial of low birth-weight infants in Guinea-Bissau, subjects randomised to receive BCG at birth had a 45% lower mortality rate (MRR 0.55 (0.34 to 0.89)) in the first 2 months of life than infants who had BCG immunisation delayed to, on average, 6 weeks of age. The reduction in deaths was due to protection from all-cause febrile illness, respiratory tract infections and diarrhoea, but not against TB (verbal autopsy data). However, although this study is the only trial designed primarily to investigate the impact of BCG on all-cause mortality, nine other randomised controlled trials have been conducted that delayed BCG vaccination past the neonatal period in high mortality areas [19-27]. None of these studies reported significant differences in mortality, either during the period when one intervention group had received BCG and the other group had not, or subsequently. Also, in contrast to the Guinea-Bissau trial, three large cohort studies appear to show that infants who receive BCG vaccination at the same time as Diphtheria Tetanus Pertussis (DTP) vaccination (at 6 weeks of age) have reduced longer-term all-cause mortality than those that have received BCG at birth [28]. Thus, it is currently unclear whether BCG has non-specific beneficial protective effects against diseases other than TB, and if so, what timing of administration would be optimal to induce these effects.

The possibility that BCG may have effects against non-tuberculous disease has also had limited acceptance in the scientific and public health communities due to the lack of a confirmed biological mechanism. Investigations into the hypothesis that BCG immunisation might skew the adaptive immune response from the T-helper type 2 (Th2) dominant environment of early neonatal life, toward a more protective T-helper type 1 (Th1) environment, have been inconclusive [29-32]. The evidence from the Guinea-Bissau randomised controlled trial, however, shows that any putative immunological mechanism would need to be: 1) effective at birth despite the immature neonatal immune system, 2) rapidly inducible (most protection at < 1 week post-immunisation) and 3) active against a range of pathogens. These features would suggest that BCG mediates its non-specific effects by stimulating the innate immune system. This is the hypothesis that we aim to interrogate during this study.

We plan to investigate three different aspects of the innate immune system. Firstly, we will investigate whether non-specific pro-inflammatory cytokine production is enhanced in infants who have received BCG by using *in vitro* stimulation with non-mycobacterial stimulants. Few studies exist investigating alterations in cytokine production to heterologous stimulants following neonatal BCG immunisation [33-35]. The few that have been reported have focused on adaptive cytokines, using a 6-day *in vitro* stimulation protocol, which is suboptimal for the quantification of innate cytokine production. No studies exist where samples have been collected prior to 5 months of age, thus early non-specific effects of BCG will have been missed. Lastly, *in vitro* stimulants used in previous studies have been antigens (for example, lipopolysaccharide or tetanus toxoid) and not whole organisms, potentially excluding the effect of other important pattern recognition receptor pathways. As part of this proposed study we aim to address these issues by focusing on the impact of BCG on innate cytokine production, conducting overnight stimulation using non-mycobacterial whole organism stimulants, and by using blood samples taken before 10 weeks of age.

Secondly, we will investigate whether BCG might mediate any non-specific beneficial effects by inducing a plasma hypoferraemia. Iron supply is critical for the virulence of most pathogens [36], with plasma hypoferraemia profoundly inhibiting the growth of bacteria [37,38], viruses [39], protozoa [40-43] and fungi [44,45]. As part of the innate acute-phase response, plasma hypoferraemia is induced by IL-6-driven release of hepcidin. Guinea pig models reveal that BCG also induces a rapid bacteriostatic hypoferraemia [46], although involvement of the IL-6/hepcidin pathway has never been studied. To our knowledge, no studies exist investigating the influence of BCG immunisation on the human iron-

inflammatory pathway. As part of this study we will investigate whether BCG immunisation in neonates induces alterations to the inflammatory iron axis, as a potential effector mechanism for heterologous protection.

Lastly we will investigate whether BCG induces epigenetic modification at the promoter region of pro-inflammatory cytokines in monocytes, thereby providing a mechanism for 'training' the innate immune system to respond in a persistently amplified manner to challenge by non-mycobacterial pathogens. BCG immunisation of naïve adults has been shown to produce trimethylation of histone-3 lysine 4 (H3K4) at the promoter region of TNF-α, IL-1β and IFN-γ in monocytes [47]. This led to enhanced cytokine production following *in vitro* stimulation with the heterologous pathogens *Staphylococcus aureus* (*S. aureus*), *Streptococcus pneumonia* (*S. pneumoniae*) and *Candida albicans* (*C. albicans*), which persisted to at least 3 months post-immunisation. We will investigate whether BCG immunisation produces similar epigenetic modification of monocytes in neonates.

Thus, we have designed a randomised controlled trial, comparing BCG administration at birth with administration at 6 weeks of age in healthy Ugandan neonates. We will use this to interrogate the impact of BCG vaccination on the innate immune response, as well on all-cause clinical illness outcomes. We believe this study will add significantly to the current debate regarding the non-specific effects of BCG vaccination as it aims to confirm a biological mechanism to explain such effects. Also, by being conducted in healthy neonates, in a geographical location distant from previous studies and by an independent research group, it will help to understand the global applicability of any non-specific effects.

Aims and objectives

The aims of our study are as follow:

1. To determine whether BCG immunisation at birth alters the innate immune response to heterologous pathogens in the short term (within 1 week)
2. To determine whether BCG immunisation at birth alters the innate immune response to heterologous pathogens in the longer term (at 6 weeks)
3. To determine whether BCG immunisation given at age 6 weeks has similar short- and longer-term effects on the innate immune response to heterologous pathogens compared to BCG immunisation at birth
4. To obtain data upon the effect of BCG on neonatal susceptibility to invasive infections in Ugandan infants

Aims 1, 2 and 3 will be addressed using sub-studies to interrogate 3 different elements of the innate immune system. The individual objectives for these studies are shown in Table 1. Clinical outcome measures from all 3 sub-studies will be combined to address Aim 4.

Study design

This study is an investigator-blinded randomised controlled trial of BCG vaccination given at birth versus BCG vaccination given at 6 weeks of age. Cord blood and two venous blood samples will be collected from participants to allow comparison of innate immune system parameters. All participants will be clinically followed-up until 10 completed weeks of age, to allow comparison of illness outcomes. This study design will allow comparison of outcomes between BCG-naïve and -vaccinated infants up to 6 weeks of age, and early with delayed BCG-vaccinated infants from 6 to 10 weeks of age, helping to identify whether there is a critical period for BCG-induced non-specific effects. The time-point of 6 weeks for the delayed BCG group has been chosen as it is the longest delay possible prior to the potential confounding influence of primary immunisations.

Methods

Setting and participants

Infants will be recruited on the day of birth from the maternity ward of Entebbe Grade B hospital, a government hospital located in Wakiso District in central Uganda. The region is populated mainly by semi-urban, rural and fishing communities. Neonatal mortality rates in Uganda remain high at 28/1,000 live births, with a large proportion attributable to invasive infectious diseases.

Eligibility criteria

The inclusion criteria for this study are:

1) Infant of a gestational age and birth weight sufficient to allow discharge directly home from hospital without requirement for supplemental oxygen or feeding
2) Delivery sufficiently uncomplicated to allow discharge directly from hospital without inpatient management
3) HIV-negative mother (based on antenatal records)
4) Residence within the study catchment area
5) Consenting mother

No specific weight or gestational age limit has been set for this study. Clinical responses to early BCG are suggested to have the greatest effect in infants of the lowest birth weight [18]; thus, it is important to include these infants in data collection. No increased rate of detrimental side-effects or reduction of immunological efficacy has been shown with BCG immunisation of premature infants [48]. Written informed consent will be obtained

Table 1 Objectives for immunological sub-studies

Sub-study	Primary objectives	Secondary objectives
Cytokine sub-study	Cross-sectional comparison of IL-1β, IL-6, TNF-α and IFN-γ cytokine levels following overnight *in-vitro* stimulation with *S. aureus, S. pneumoniae, E. coli, C. albicans* and Poly I:C/CpG between the two intervention groups:	Longitudinal analysis of within-infant changes in innate cytokine production following *in-vitro* stimulation with *S. aureus, S. pneumoniae, E. coli, C. albicans* and Poly I:C/CpG.
	1. Shortly after birth intervention (BCG vaccination/ no vaccination): Aim 1	
	2. Six weeks post-birth intervention (immediately prior to first dose of primary vaccination): Aim 2	
	3. Shortly after 6-week intervention (BCG vaccination/ no vaccination): Aim 3	
	4. Three weeks post-6-week intervention (immediately prior to second dose of primary vaccinations): Aim 3	
Iron sub-study	Cross-sectional comparison of transferrin saturation and hepcidin levels between the two intervention groups:	Cross-sectional comparison of serum iron, total iron binding capacity, ferritin, transferrin, haemoglobin and red cell parameters at the above time-points.
	1. Shortly after birth intervention (BCG vaccination/no vaccination): Aim 1	Longitudinal analysis of within-infant changes to iron status following *in-vivo* non- specific stimulation (provided by primary vaccinations)
	2. Six weeks post-birth intervention (shortly after first dose of primary vaccination): Aim 2	
	3. Shortly after 6-week intervention (BCG vaccination/ no vaccination): Aim 3	
	4. Three weeks post-6-weeks intervention (shortly after second dose of primary vaccination): Aim 3	
Epigenetic sub-study	Cross-sectional comparison of monocyte histone-3 lysine 4 trimethylation (H3K4me3) at the promoter region of pro-inflammatory cytokines between the 2 intervention groups:	Longitudinal analysis of within-infant changes in monocyte epigenetic modification.
	1. Shortly after birth intervention (BCG vaccination/ no vaccination): Aim 1	
	2. Six weeks post-birth intervention (immediately prior to first dose of primary vaccination): Aim 2	

from the mothers of all infants prior to their enrolment in the study.

Neonates will be excluded from the study if:

1) Cord blood is not obtained
2) They have major congenital malformations
3) The infant is clinically unwell, as judged by a midwife
4) Known maternal TB or active TB within the family (based on direct questioning of mother during recruitment)
5) Maternal or family member positive for any of the following TB screening symptoms:
   - Cough > 2 weeks
   - Recent haemoptysis
   - >3 kg weight loss in past month
   - Recurrent fevers/chills or night sweats for the past 3 days or more

Intervention and randomisation

All infants will receive 0.05 ml of BCG-Statens Serum Institute (SSI, Copenhagen, Denmark) (Danish Strain 1331) intra-dermally into the right deltoid. This will be given either at birth (Early intervention arm) or at 6 weeks of age (Delayed intervention arm).

Intervention and blood sampling time-point allocation will be determined by block randomisation, stratified by sex. This will be carried out by an independent statistician, prior to the trial commencement, using Stata (StataCorp, College Station, TX, USA) to generate the allocation sequence. Allocations will be concealed within sequentially numbered, sealed opaque envelopes, prepared by two research assistants who are independent of the trial. Upon delivery of an eligible infant, assignment of allocation will be carried out by midwives who will select the next sequential envelope according to the infant's gender.

Blinding

This study will be single blind. Mothers will not be blinded to intervention allocation due to lack of feasibility (BCG produces a visible reaction) and to reduce confusion if a child who is lost to follow-up presents to a community immunisation clinic.

Staff involved in administering BCG immunisation to the participants, either at birth or at 6 weeks of age, will

not be involved in clinical follow-up or assessment of outcomes.

Investigators performing clinical assessment of children will be blinded to intervention allocation by means of a plaster placed over the area corresponding to BCG vaccination site. This will be placed by a nurse not involved in clinical assessment, prior to physician assessment. If a child is presenting due to concerns about the immunisation site it will be left uncovered and the unblinding documented. Illness events arising from concerns or complications directly related to the BCG immunisation will not be included in the analysis of illness events, but will be presented separately.

Immunological investigations will be conducted on blood samples identified only by study number. The intervention allocation code will only be broken once laboratory analysis is complete and the data have been cleaned and locked.

Study procedures

Overview

Figure 1 shows the SPIRIT (Standard Protocol Items: Recommendations for Interventional Trials) diagram for the trial procedures. On presentation to labour ward, mothers in active labour will be screened for their eligibility and informed consent will be taken. Following delivery the infant will be assessed for eligibility and placental cord blood collected. Infants who are eligible for the study will be randomised as described above, to receive BCG vaccination either immediately or at 6 weeks of age. All infants will be followed-up until 10 completed weeks of age. During this time 2 × 2 ml venous blood samples and 2 stool samples will be collected and all routine immunisations will be given (Oral Polio Vaccination (OPV) at birth and primary immunisations at 6 and 10 weeks of age). Clinical follow-up of the infants will be carried out by weekly telephone interviews to check the well-being of participants, and physician review and anthropometry at each routine clinic visit for blood samples/routine immunisations (on average four visits per participant). Unwell participants presenting to the study clinic or Entebbe Grade B hospital will also be reviewed and managed by the study team, free of charge. Study follow-up is complete once the child has completed 10 weeks of age.

Consent

Sensitisation of parents to the study will occur during antenatal classes via posters, group discussions and during individual midwife-led consultations. Mothers will then be approached for consent by trained midwives when presenting in active labour to Entebbe Grade B hospital. The study will be explained in detail verbally and the information sheet provided (or read to illiterate mothers). Information sheets will be available in English and Luganda. Consent will also be taken to allow for storage of excess samples and use of data in future research studies. Although consent during labour is not optimal, it is necessary to enable cord blood collection. However, consent will be verbally reconfirmed with mothers following delivery prior to any intervention. This method of consent and recruitment has been piloted in the same hospital and shown to be an appropriate and successful method.

Data collected

Demographic details, anthropometric measurements and socio-economic indices will be collected at enrolment including gender, gestational age, birth weight, occipito-frontal circumference and length, maternal age and parity, parental ethnicity, parental educational level attained, accommodation type and assets. Global Positioning System (GPS) co-ordinates of the participant's home address will also be collected to aid follow-up.

During routine clinic visits anthropometric and vital sign measurements will be collected. All mothers will be interviewed about illness episodes in the participant since they were last seen in clinic and any current concerns. Physical examination findings will be documented.

A standardised illness episode case report form will be completed whenever a child presents unwell to the research clinic or paediatric ward at Entebbe Grade B hospital. This will include anthropometric and vital sign measurement, symptoms and signs, investigation results, final diagnosis and outcome.

All participants will be interviewed by telephone on a weekly basis by a fieldworker using a standardised case report form to ensure the health of the infant. Any infants for whom there are concerns will be reviewed in clinic. This intensive follow-up will enhance identification of clinical illness episodes, which are secondary outcomes for the study. More importantly, however, it will allow early identification and management of any cases of perinatal TB, particularly in the delayed intervention group. Any suspected or confirmed cases of TB occurring during the study will be reported to the ethics committees and Data Safety Monitoring Board (DSMB), who will decide whether the study needs to be stopped early for safety.

Direct electronic data entry will occur for all case report forms. This will be verified and optimized by co-documentation with paper case report forms at the beginning of the study. Data will be maintained in encrypted, password protected forms, to maintain confidentiality.

Blood samples collected

All participants in the study will have 10 ml placental cord blood collected at birth; divided into 5 ml of

	STUDY PERIOD											
	Enrolment	Allocation	Follow-up (weeks of age)									
TIMEPOINT	Labour	Birth	1	2	3	4	5	6*	7	8	9	10
ENROLMENT:												
Eligibility screen Mother	X											
Eligibility screen Infant	X											
Informed Consent	X											
Allocation		X										
INTERVENTION:												
BCG at birth (n=280)		X										
BCG at 6 weeks (n=280)								X				
DATA COLLECTION:												
Demographic and birth details	X	X										
Weekly telephone follow-up			X	X	X	X	X	X	X	X	X	X
Routine physician review **			X					XX				X
Parental Recall of Illness Episodes **			X					XX				X
Illness episode documentation			◆	—	—	—	—	—	—	—	—	◆
PROCEDURES:												
Cord blood collection		X										
2ml Blood Sample ***			X					XX				X
Stool Sample								X				X
Primary Immunisations ****		X						X				X

* Exact time-points of blood sampling at 6 weeks of age will vary according to sub-study (please see Table 2).
** Exact timing of physician review and parental interview regarding illness episodes will vary between participants, coinciding with blood sampling time-point and primary immunisations.
***Each infant will only be bled at two of the possible four sampling time-points
**** Primary immunisations are Oral Polio Vaccination at birth, Diptheria Tetanus Pertussis/Haemophilus Influenzae B/Hepatitis B and Oral Polio Vaccine at 6 weeks and 10 weeks

Figure 1 Standard Protocol Items: Recommendations for Interventional Trials (SPIRIT) diagram of study procedures.

heparinised and 5 ml of ethylenediaminetetraacetic acid (EDTA)-anticoagulated blood. They will then have 2-ml venous blood samples collected at 2 time-points between birth and their exit from the study at 10 completed weeks of age. Each sub-study has up to four possible time-points where blood samples are collected, but each infant will only be bled at two of these time-points (randomly allocated) to avoid undue stress for the baby

and the mother. The time-points have been selected to enable interrogation of the changes to the innate immune system induced by BCG both acutely following vaccination and in the longer term. The timing of the blood samples in the iron sub-study differs slightly from those in the cytokine and epigenetic sub-studies (see Table 2). These differences are necessitated by the systemic nature of iron metabolism. As hepcidin is produced mainly in the liver this precludes analysis of iron metabolism following *in-vitro* non-specific stimulation. Thus, the iron sub-study will use routine primary immunisations as *in-vivo* non-specific stimuli and measure the resulting changes to iron parameters.

Stool samples

Stool samples will be collected at the 6-week and 10-week time-points and stored to allow for future analysis, funding permitting.

Other samples collected

Whenever an unwell participant presents to the study team investigations and treatments will be conducted as directed by the attending clinician. Investigations will include cultures for accurate diagnosis of febrile illness. An extra 2-ml blood sample will be taken from any participant under-going phlebotomy provided that this will not compromise the child's health or well-being. This will allow a sub-study to be conducted to compare primary immunological outcomes in unwell children according to BCG status.

Laboratory procedures

Cytokine sub-study

Overnight whole blood stimulation with the non-specific stimulants *S. aureus, S. pneumoniae, E. coli, C. albicans* and polyinosinic:polycytidylic acid/C-phosphate-G (Poly I:C/CpG) will be carried out using fresh sodium-heparinised blood. Measurement of the pro-inflammatory cytokines IL-1β, IL-6, TNF-α and IFN-γ by ELISA (BD-OptEIA, Becton, Dickinson and Company, Oxford, UK) will then be conducted on the harvested supernatant following storage at −80°C. These stimulants have been chosen because they are the most common pathogens isolated from septic neonates in Uganda [49] and because they represent a range of pathogen types and toll-like receptor pathways.

Epigenetic sub-study

The levels of trimethylation of H3K4 at the promoter region of pro-inflammatory cytokines will be assessed using chromatin immunoprecipitation followed by qPCR. Peripheral blood mononuclear cell (PBMC) isolation for this work will occur by density-centrifugation over histopaque (Sigma-Aldrich, Dorset, UK).

Iron sub-study

Measures of iron status will be conducted on the plasma fraction of lithium-heparinised blood following storage at −80°C. Serum iron, Unbound Iron Binding Capacity (UIBC), Total Iron Binding Capacity (TIBC), Transferrin Saturation (TSAT) and ferritin will be measured using the automated Cobas Integra (Roche Diagnostics, Switzerland). The hormone hepcidin will be quantified using ELISA (Bachem-25, Bachem, Switzerland).

Red cell parameters will be measured from fresh EDTA whole blood using a Coulter $A^{C\cdot}T$ 5 Diff CP haematology analyser (Beckman Coulter, Inc, CA, USA).

Primary outcomes

Cytokine sub-study

- IL-1β, IL-6, IL-10, TNF-α and IFN-γ cytokine levels following *in-vitro* stimulation with *S. aureus, S. pneumoniae, E. coli, C. albicans* and Poly I:C/CPG.

Epigenetic sub-study

- H3K4 trimethylation at the region of pro-inflammatory cytokines in peripheral blood monocytes

Iron sub-study

- Hepcidin levels
- TSAT

Primary outcomes in each sub-study will be compared between the 2 intervention groups both acutely following

Table 2 Blood sample time-points (T) according to immunological sub-study

	Blood sample T1 (first week of life)	Blood T2 (6 weeks of age)	Blood sample T3 (6 weeks of age)	Blood sample T4 (10 weeks of age)
Cytokine sub-study	5 days after birth	Immediately before primary immunisations	5 days after primary immunisations	Immediately before primary immunisations
Iron sub-study	5 days after birth	1 day after primary immunisations	5 days after primary immunisations	1 day after primary immunisations
Epigenetic sub-study	5 days after birth	Immediately before primary immunisations		

BCG (up to 1 week after birth/6 weeks of age) and at time-points distant from vaccination (6 and 10 weeks of age).

Secondary outcomes

- Physician-diagnosed infectious disease
- Parental-reported infectious disease
- Culture-positive infectious disease
- Mortality

The above clinical outcomes for the three sub-studies will be analysed together to increase power.

The iron sub-study will also have the following secondary outcomes:

- Serum iron
- TIBC
- Ferritin
- Transferrin
- Haemoglobin
- Red cell parameters

In a secondary analysis, longitudinal within-infant changes in primary outcomes will also be analysed for each sub-study.

Sample size considerations

Each sub-study is powered for its own primary outcomes. The overall sample size is the summation of the participants required for each sub-study.

Cytokine sub-study: n = 240

Due to paucity of published data in this area, an approach based on standard deviation (SD) difference in average population cytokine levels has been used. Forty-eight subjects per intervention group (BCG immunisation at birth or at 6 weeks of age) will be needed at each time point to show a 0.66 SD difference in average population cytokine levels with 90% power and 5% significance. Sixty infants per intervention group per time point will be recruited to allow for attrition. As each recruited infant will be bled at 2 time-points, 240 infants will be recruited in total to allow for the 4 time-points.

Epigenetic sub-study: n = 80

The only previous study in this area (which was performed in adults) required 20 subjects per intervention arm [47]. We will recruit 40 subjects to each intervention arm to allow for attrition and also due to the requirement for a full 2-ml blood sample for epigenetic analysis, which is unlikely to be obtained for all subjects. Due to funding constraints, epigenetic analysis will be restricted to the first two sampling time-points, and each infant will be bled at both time-points, eighty subjects will be recruited in total.

Iron sub-study: n = 240

Sample size determination was performed using TSAT as it is the only primary outcome parameter currently of clinical relevance. Average neonatal TSAT in low-income settings is 55% [50]. Fifty infants in each group at each time point will be needed to show a 30% reduction in transferrin saturation (reduction to average TSAT levels in low income settings) with 90% power and 5% significance. Sixty subjects will be recruited to each intervention group at each time point to allow for attrition. As each recruited infant will be bled at 2 time-points, 240 infants will be recruited in total.

Overall sample size: n = 560

Combined analysis of clinical end-points from all three sub-studies will be conducted as secondary analysis. Based on data from a previous study in Entebbe [51] we expect 80% power to detect a $\geq$ 40% reduction in physician-diagnosed invasive infections with 5% significance. The effect of BCG is unlikely to be this pronounced, but this preliminary data combined with the primary immunological outcomes, should provide sufficient evidence to determine whether expanding the cohort would be valuable.

Data management

Description of the data

This is a randomised controlled trial with datasets generated from clinical questionnaires and laboratory assays. A combination of direct electronic capture and paper forms will be used, linked by a unique participant identifier. Microsoft Access (Redmond, WA, USA) will be utilised to produce the study database. Data will be exported from Microsoft Access to Stata (StataCorp, College Station, TX, USA) for statistical analysis.

Quality assurance

A detailed data dictionary with range checks will be used to reduce data entry errors. Quality control checks will be run by the data clerk, on a weekly basis, who will highlight any queries to the principal investigator. Data will only be uploaded onto the master database once any queries highlighted by quality control checks have been resolved.

Statistical analysis

Group characteristics will be compared using Pearson's Chi-squared test for categorical variables and the *t*-test for continuous variables. Cross-sectional comparisons between intervention groups at each time-point will be carried-out using the *t*-test for differences between

means. Non-normally distributed outcome data will be log-transformed before analysis; Mann–Whitney two-tailed test will be used for persistently skewed data. If potential confounders remain unbalanced between the groups despite randomisation: for instance season of birth, these will be adjusted for using multiple linear regression analysis. Paired/longitudinal analysis of within infant changes in parameters over time will be conducted using the paired student *t*-test or Wilcoxon matched-pairs test. Incidence rate of invasive infectious disease in the first 10 weeks of life will be compared by Poisson regression with a random effects model to allow for within-child clustering. Statistical significance will be assessed at the 2-sided 0.05 level but interpretation of results will not be solely reliant on *P*-values.

Trial monitoring

This clinical trial will be conducted according to Good Clinical Practice standards. An internal study monitor will oversee the day-to-day running of the trial locally, with external oversight and monitoring co-ordinated by the London School of Hygiene and Tropical Medicine. This may include internal audit by the Clinical Trials Quality Assurance Manager and external audits by a third party. A Trial Steering Committee (TSC) and an independent DSMB have been set up for this study. The DSMB will look at a number of clinical outcome measures, documented in 'real time' during the study, to assess whether the study needs to be stopped early for safety.

Safety reporting for this trial will follow standard Uganda Virus Research Institute and London School of Hygiene and Tropical Medicine procedures. This includes notification of Serious Adverse Events (SAEs) to the local ethics committee within 24 hours, notification of Suspected Unexpected Serious Adverse Reactions (SUSARs) to the sponsor within 7 days if life-threatening or 15 days if non-life-threatening. The manufacturer of the BCG vaccine, Staten Serum Institute, will also be notified of any SAE/SUSAR.

Ethics

As this trial will alter the timing of BCG from the current Ugandan guidelines (BCG at birth) in half of the study infants, a thorough risk-benefit analysis of a 6-week delay in vaccination has been conducted. In summary, we feel that the risks of delay are minimal for the following reasons:

- Neonatal TB is rare and the chances of infants in the delayed BCG arm becoming infected during a 6-week delay period are extremely small. At least 7 previous studies have been conducted in areas of high TB prevalence that randomised infants to delayed BCG vaccination past 6 weeks of age [19-24]. None of these studies showed an increase in TB incidence in the delayed vaccination group either in the period prior to vaccination or during follow-up (cumulative n for delayed BCG vaccination = 849, median follow-up time 1 year).
- A recent study using an Entebbe based birth-cohort showed a prevalence of latent TB infection of 9.7% in children under 5 years old [52]. This suggests that in our population, a 6-week delay in BCG administration risks 0.63 infants becoming infected with latent TB. However, the strongest risk factor for latent TB acquisition in Entebbe is a known contact with a TB case (odds ratio (OR) 2.62 (1.29 to 5.30), unpublished data). Thus, the exclusion of infants at risk of TB from mother or a household contact will reduce this risk to negligible. Active weekly follow-up of infants will occur to ensure they remain healthy and the trial will be stopped early if cases of TB are found to be higher in the delayed BCG arm.

There is also evidence that delay in BCG vaccination from birth to 6 weeks may be beneficial for participants because:

- The optimal timing of BCG vaccination for immunity against TB is not known. There is some evidence that delaying BCG past the neonatal period may improve the magnitude and duration of anti-TB immunity, thus providing direct benefit to participants in the delayed vaccination arm [19-24].
- The incidence of vaccination-induced complications, including BCG-induced abscesses, suppurative lymphadenitis and osteomyelitis are reduced by approximately one third in infants who receive BCG vaccination after the neonatal period [21].

All infants in the study, whether in the early or delayed BCG group will benefit from regular physician reviews and free access to medical review and treatment if participants become unwell. They will also benefit from receiving all other primary vaccinations at the correct time as part of the study. The most recent survey of vaccination rates in Uganda showed that 56% of infants have not received their first set of primary immunisations (diphtheria/tetanus/pertussis/hepatitis B/*Haemophilus influenzae* (HiB) and oral polio vaccine) by 12 weeks of age, with 26% still not having received it by 1 year of age. This produces a substantial risk for those children of contracting serious, preventable illnesses, which participation in the study will negate.

Thus, we believe the general benefits of taking part in the study will outweigh the extremely small risks from a 6-week delay in BCG vaccination. The full risk-benefit analysis for this study can be found in Additional file 1.

This trial has been approved by ethics boards at the Uganda Virus Research Institute on AIDS (Ref: GC/127/13/11/432), the Uganda National Council for Science and Technology (Ref: HS 1524), The Office of the President of Uganda and the London School of Hygiene and Tropical Medicine (Ref: 6545). This study will be conducted according to the principles of the Declaration of Helsinki.

Study limitations

The primary immunisation schedule imposes a number of constraints on the design of this study, as blood samples need to be timed to limit the potentially confounding influence of non-BCG vaccinations on innate immune responses. This is particularly relevant for comparison of the longer-term non-specific effects of BCG between the Early and Delayed intervention arms at 10 weeks, where BCG will have been given more recently in the Delayed intervention arm. As we are investigating the acute response to non-tuberculous stimulants, we believe that this should not be a problem, as any bystander effect of BCG vaccination itself is likely to be lost by 4 weeks of age. However, we are actively seeking funding for a longer-term follow-up time-point that should help to clarify this issue as well as to provide information about the duration of any non-specific effects of BCG vaccination on the innate immune system.

Although it is important to understand the biological mechanism underlying any non-specific effects of BCG vaccination, ultimately the impact on all-cause clinical illness episodes and mortality will be the outcome measures that are likely to have impacts on public healthcare policy. This study has limited power to detect differences in such outcomes, due to its small sample size. However, if suggested by the immunological and preliminary clinical data in this study, additional funding will be sought to expand the cohort to allow full interrogation of clinical outcomes.

Discussion

Global acceptance of the hypothesis that BCG immunisation affords non-specific protective effect when given during infancy has been limited due to paucity of randomised controlled trial data and because of a lack of a confirmed biological mechanism to explain such effects in the neonatal period. We aim to address these issues by carrying out this randomised controlled trial in Uganda, providing variety of location and research group from much of the previous work, and investigating the impact of BCG immunisation on the innate immune system in neonates. Interrogation of the possible heterologous protection afforded by BCG immunisation, and the optimal timing of immunisation to achieve beneficial effects, is important to ensure that any new anti-TB vaccine or alteration in timing of BCG administration is evaluated in terms of overall benefit to recipient, rather than solely in terms of TB-specific protection alone.

Trial status

The study commenced recruitment in September 2014. Two hundred and forty participants had been recruited as of March 2015. The trial is projected to complete recruitment by August 2015.

Additional file

Additional file 1: Risk-benefit analysis of altering BCG vaccination from birth to 6 weeks of age.

Abbreviations

BCG: Bacillus Calmette-Guérin; *C. albicans*: *Candida albicans*; CpG: C-phosphate-G; DSMB: Data Safety Monitoring Board; DTP: Diphtheria Tetanus Pertussis; EDTA: Ethylenediaminetetraacetic acid; ELISA: Enzyme Linked Immunosorbent Assay; *E. coli*: *Escherichia coli*; GPS: Global Positioning System; HepB: Hepatitis B; HiB: *Haemophilus Influenzae B*; H3K4: Histone-3 lysine 4; ISRCTN: International Standard Randomised Controlled Trial Number; IFN-γ: Interferon gamma; IL: Interleukin; OPV: Oral Polio Vaccine; OR: Odds ratio; PBMC: Peripheral blood mononuclear cells; Poly I:C: Polyinosinic:polycytidylic acid; qPCR: Quantitative polymerase chain reaction; SAE: Serious Adverse Event; *S. aureus*: *Staphylococcus aureus*; *S. pneumonia*: *Streptococcus pneumonia*; SSI: Statens Serum Institute; SUSAR: Suspected Unexpected Serious Adverse Reactions; TB: Tuberculosis; Th1: T-helper type 1; Th2: T-helper type 2; TIBC: Total Iron Binding Capacity; TNF-α: Tumour necrosis factor-alpha; TSAT: Transferrin Saturation; TSC: Trial Steering Committee; UIBC: Unbound Iron Binding Capacity; UVRI: Uganda Virus Research Institute.

Competing interests

The authors do not have any competing interests, financial or otherwise, to report.

Authors' contributions

SP conceived this study, with the assistance of SC and HD as PhD supervisors. SP will lead the enrolment of participants, data collection, laboratory analysis, data collection and drafting manuscripts. EW will supervise the statistical analysis. HD, PK, AE and SC will provide supervision and expertise for all aspects of the study and participate in manuscript drafts. All authors have read and approved the final manuscript.

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The independent data monitoring committee for this study comprises Professor Andrew Nunn (chair), Dr Philippa Musoke, and Professor Elly Katibira. The TSC comprises Professor Jonathan Levin (chair), Mr Frank Mugabe, Professor Helen McShane, Dr Moses Muwanga and Mr Dan Nsubika. We would like to thank the members of both committees for their input to the study.

Author details

[1]Wellcome Trust - Bloomsbury Centre for Global Health Research, London School of Hygiene and Tropical Medicine, Keppel Street, London WC1E 7HT, UK. [2]Clinical Research Department, London School of Hygiene and Tropical Medicine, Keppel Street, London WC1E 7HT, UK. [3]Department of Infectious Disease Epidemiology, London School of Hygiene and Tropical Medicine, Keppel Street, London WC1E 7HT, UK. [4]Department of Infection and Immunology, London School of Hygiene and Tropical Medicine, Keppel Street, London WC1E 7HT, UK. [5]MRC/Uganda Virus Research Institute on AIDS, Plot 51-59, Nakiwogo Road, PO Box 49, Entebbe, Uganda.

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7. Results: Main trial

7.1 Study population

7.1.1 Participant recruitment and follow-up

Details regarding the participant recruitment and follow-up are shown in in Figure 7.1.

A total of 1148 women were approached in early labour for potential recruitment into the study. Of those approached, 123 (11%) lived outside of the study area and 57 (5%) were not interested in the participating in the study. A further 114 (10%) were not recruited to the study because the mothers were known to have HIV infection. This is consistent with known rates of HIV positivity in Ugandan antenatal clinic attendances.[288] Additionally, 294 (25%) delivered by C-section, excluding their infants from participation. This is slightly higher than contemporaneous C-section rates for the whole of Uganda, likely reflective of the fact that this study was performed in a referral hospital.[289]

Of those randomised, 83% completed follow-up. These are better follow-up rates than in other studies conducted in the area,[245, 290] likely as a result of the active, weekly patient follow-up. The distribution of randomised infants who did not complete follow-up was essentially equal at 17% of infants randomised to receive BCG at birth and 18% of infants randomised to receive BCG at 6 weeks.

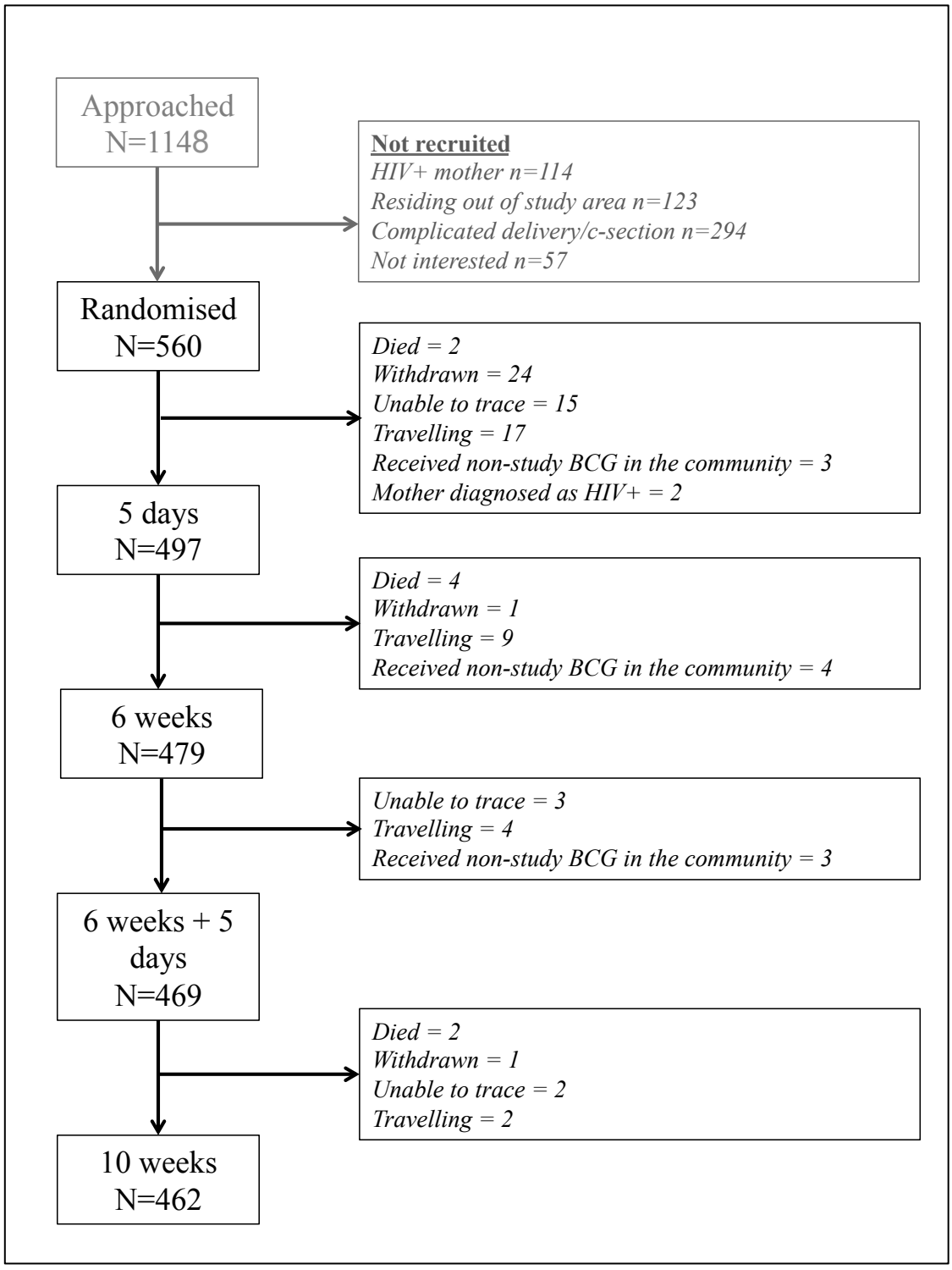
The main loss to follow-up occurred early in the trial with 63 randomised infants never attending any routine clinic appointment. This was largely due to paternal withdrawal of consent when the father had not been present at the delivery. A number of infants could never be traced (e.g. false contact details had been provided) or were travelling to rural villages and could not attend routine visits (despite affirming that they planned to stay in the study area upon consenting to the study). These participants were assumed to have not fully consented to the study and therefore their cord samples, though processed, were not analysed. Two mothers were newly diagnosed as HIV positive on routine post-natal screening. These were both discovered within 24 hours of birth, but subsequent to randomisation allocation. The infants were excluded from the study and vaccinated with BCG at birth, as per the current Ugandan national guidelines. Three infants randomised to the delayed BCG group were subsequently found to have had non-study BCG in the community prior to their first routine visit, and were discontinued from the study.

Follow-up for infants that had attended one study visit tended to be complete, with few additional losses to follow-up for withdrawal of consent, travel or difficulties in tracing.

Seven further instances of non-study BCG receipt in the delayed group occurred. Samples collected from these infants prior to BCG receipt were retained in the study, but no further samples were collected. Clinical follow-up was censored from the date of BCG vaccination.

Eight infants died over the course of the study. These will be discussed further in the Clinical Outcomes section (7.5).

Figure 7.1. Study flow diagram



HIV+, Human Immunodeficiency Virus positive; n, number; BCG, Bacille Calmette Guerin.

7.1.2 Participant demographics

Demographic variables for participants completing the study are shown in Table 7.1. There were no significant differences in any anthropometric or demographic variable between the two groups, showing that randomisation procedures occurred effectively.

Table 7.1. Participant anthropometric and demographic variables

Variable	BCG at birth (n=232)	BCG at 6 weeks (n=230)	p-value
Female sex, n (%)	113 (49%)	118 (51%)	0.58
Birth weight (grams)	3251	3222	0.47
OFC (cm)	34.5	34.5	0.62
Maturity, n (%)			0.10
Mature	228 (98%)	220 (96%)	
Premature	4 (2%)	10 (4%)	
Postmature	0	0	
Maternal Age (years)	24.1	23.9	0.61
Marital Status, n (%)			0.21
Married/living as married	201 (87%)	188 (83%)	
Single	30 (13%)	39 (17%)	
Parity (median)	2	2	0.73
Number of rooms in house (median)	1	1	0.10
Number of people in house (median)	4	4	0.20
Roof material, n (%)			0.80
Dry banana leaves	11 (4.7%)	6 (2.6%)	
Grass	2 (0.9%)	3 (1.3%)	
Iron sheets	216 (93.1%)	217 (94.8%)	
Tiles	1 (0.4%)	1 (0.4%)	
Tin	2 (0.9%)	2 (0.9%)	
Wall material, n (%)			0.30
Bricks	220 (94.8%)	214 (93.0%)	
Iron sheet	10 (4.3%)	10 (4.4%)	
Metal	1 (0.4%)	0	
Wattle	0	2 (0.9%)	
Wood	1 (0.4%)	4 (1.7%	
Cooking fuel used, n (%)			0.11
Charcoal	199 (85.8%)	209 (90.9%)	
Firewood	19 (8.2%)	10 (4.4%)	
Electricity/gas	14 (6.0%)	9 (3.9%)	
Paraffin	0	2 (0.9%)	
Maternal education, n (%)			0.80
None	4 (2%)	7 (3%)	
Primary	76 (33%)	75 (32%)	
Secondary	125 (54%)	125 (54%)	
Tertiary	27 (11%)	24 (11%)	
Iron supplements during pregnancy, n=yes, (%)	213 (92%)	209 (91%)	0.72
Maternal smoking in pregnancy, n=no, (%)	232 (100%)	229 (99.6%)	0.32
Maternal alcohol in pregnancy, n=no, (%)	199 (86%)	202 (88%)	0.52
Mean age at blood sampling (days)			
S1	7.76	8.01	0.76
S2e/c	42.90	43.03	0.80
S2i	44.00	44.38	0.29
S3	49.12	49.73	0.25
S4c	70.86	73.45	0.09
S4i	72.31	73.13	0.17

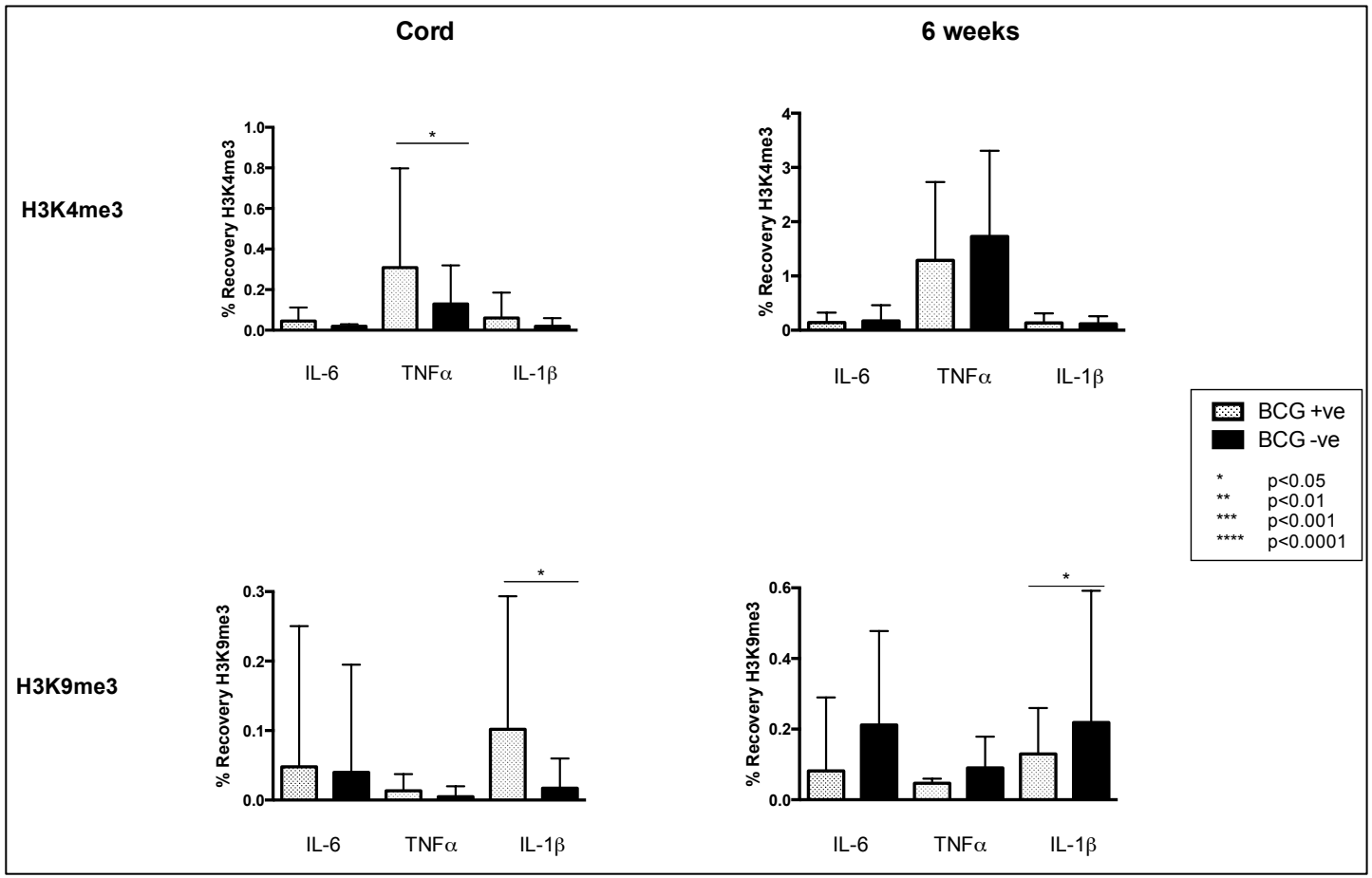
7.2 Epigenetic sub-study results

The epigenetic sub-study was conducted to investigate whether H3K4me3 (stimulatory) and H3K9me3 (inhibitory) epigenetic modifications at the promoter region of pro-inflammatory cytokines differed in the first 6 weeks of life, in infants randomised to receive BCG at birth and BCG unvaccinated infants. Epigenetic modification was assessed in PBMCs using chromatin immunoprecipitation followed by qPCR, as described in the Methods section 6.3.1.3

7.2.1 H3K4me3 and H3K9me3 epigenetic modification at the promoter region of pro-inflammatory cytokines at 6 weeks was lower in BCG vaccinated infants compared to unvaccinated infants

Cross-sectional comparison of the amount of stimulatory H3K4me3 and inhibitory H3K9me3 at the promoter regions of pro-inflammatory cytokines at 6 weeks of age showed a consistent trend to being lower in infants that received BCG vaccination compared to infants who did not (Figure 7.2, and Table 1.2.1 in Appendix 9). This was statistically significant for H3K9me3 at the promoter region of TNFα. However, significant between-group differences were also seen in the baseline blood sample (cord blood), with a tendency toward higher median H3K4me3 and H3K9me3 levels in the group receiving BCG at birth. As randomisation appears to have occurred appropriately in the study, this is likely a chance finding. As a result of this baseline variability, analysis of within-infant changes to the amount of epigenetic modification from baseline to 6 weeks of age is likely to provide more information regarding any effects of BC

Figure 7.2. Cross-sectional comparisons of epigenetic modification at the promoter region of pro-inflammatory cytokines by BCG status



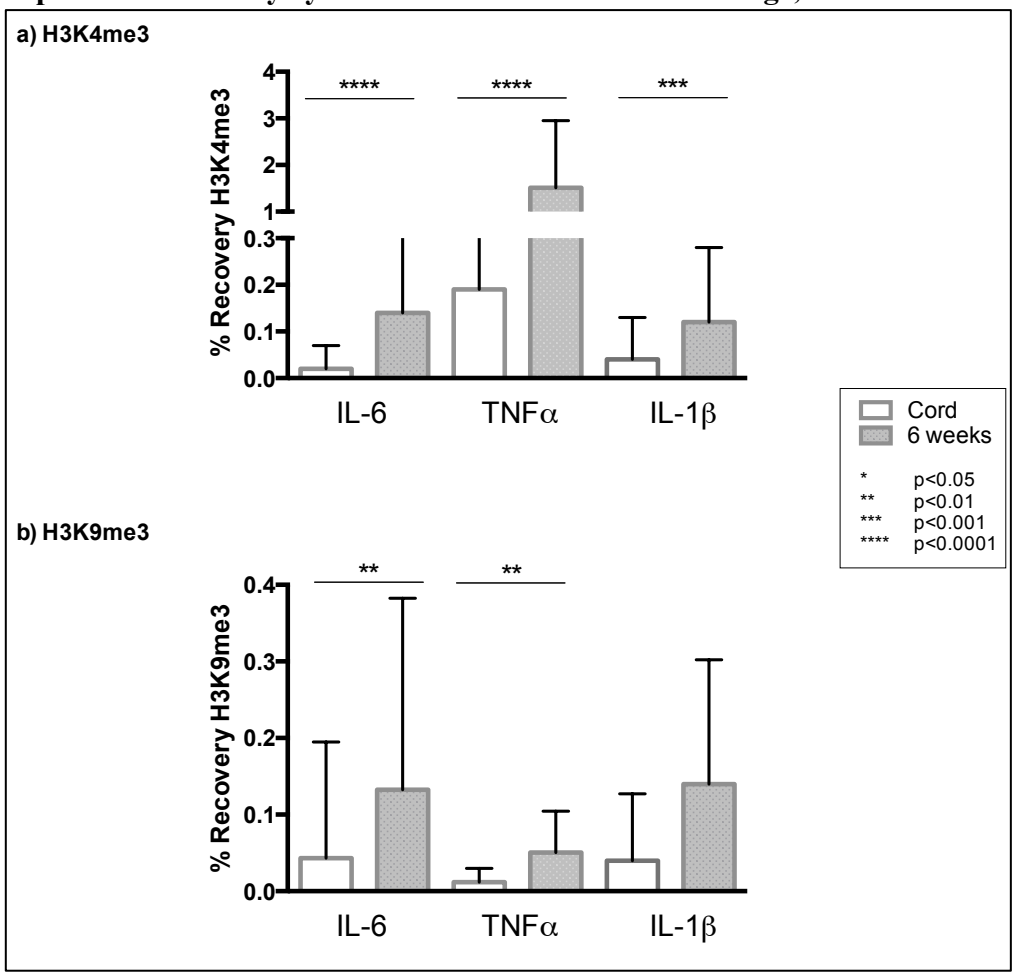
Between-group comparisons of medians in cord blood and at 6 weeks of age, conducted using the Mann-Whitney U test. BCG vaccinated (+ve) n=16, BCG unvaccinated (-ve) n=15. H3K4me3, histone-3 lysine-4 trimethylation; H3K9me3, histone-3 lysine-9 trimethylation; IL, interleukin; TNF, tumor necrosis factor

7.2.2 H3K4me3 and H3K9me3 epigenetic modification increased between birth and 6 weeks of age, regardless of BCG status

Epigenetic modification at the promoter regions of all pro-inflammatory cytokines was higher at 6 weeks of age compared to baseline for all infants (Figure 7.3, and Table 1.3.1 in Appendix 9). This increase was highly significant for the stimulatory mark H3K4me3 at all pro-inflammatory cytokines, and for the inhibitory mark H3K9me3 for IL-6 and TNFα. H3K9me3 at the promoter region of IL-1β showed the same trend but this did not reach statistical significance.

This global increase in epigenetic modification likely reflects ontological changes occurring in the first 6 weeks of life.

Figure 7.3. Comparison of levels of epigenetic modification at the promoter region of pro-inflammatory cytokines at birth and 6 weeks of age, in all infants



Comparisons of median levels of epigenetic modification between cord and 6 weeks of age, conducted using the Wilcoxon matched-pairs test. n=31. H3K4me3, histone-3 lysine-4 trimethylation; H3K9me3, histone-3 lysine-9 trimethylation; IL, interleukin; TNF, tumor necrosis factor.

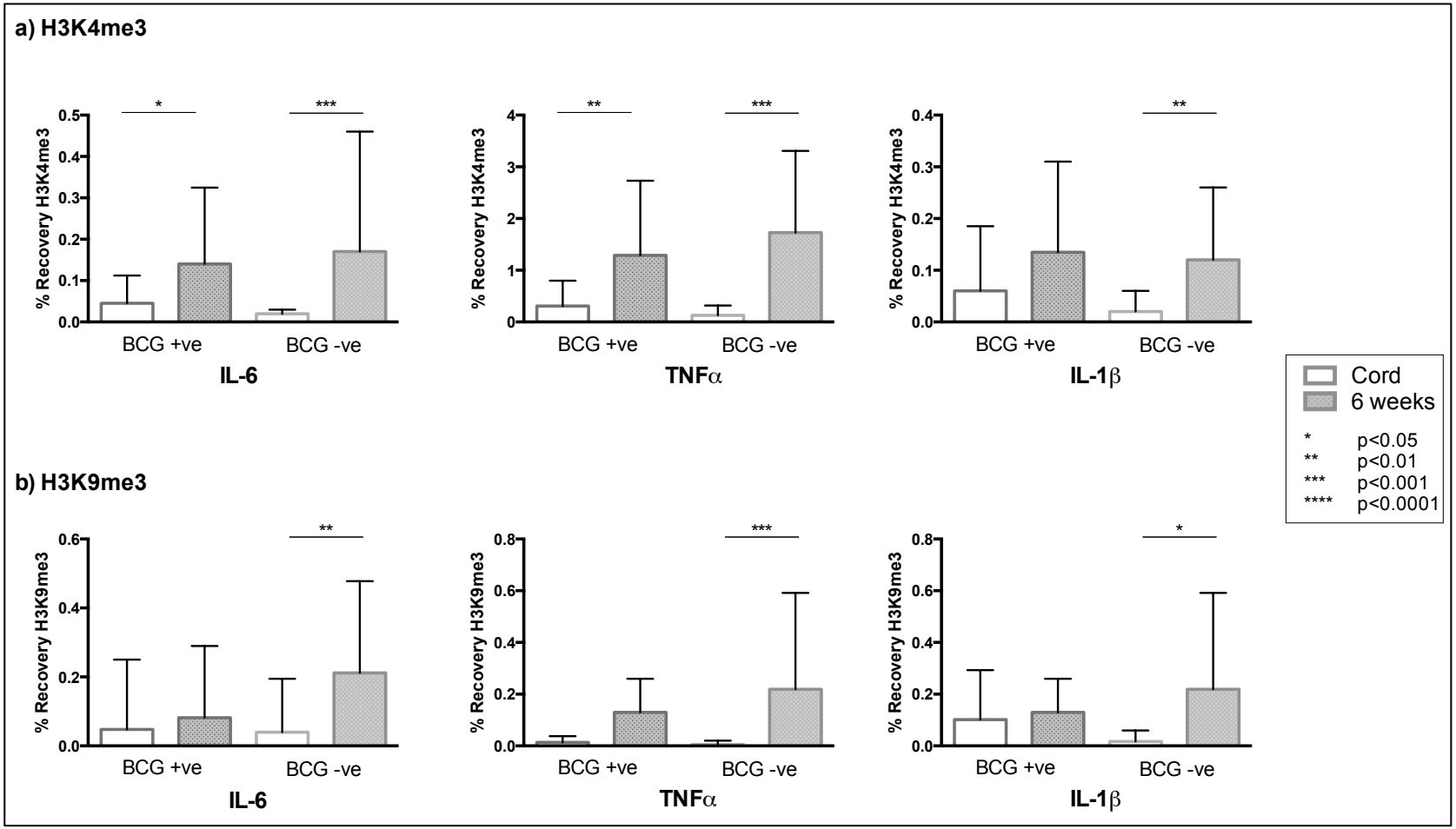
7.2.3 Increases in epigenetic modification between birth and 6 weeks were lower in BCG vaccinated infants than unvaccinated infants.

Median H3K4me3 was significantly increased at 6 weeks compared to baseline in both BCG vaccinated and unvaccinated infants, but the increase was consistently greater and more significant in unvaccinated infants (Figure 7.4a, and Table 1.3.2 in Appendix 9). Median H3K9me3 was significantly increased in 6 week samples compared to baseline only in BCG unvaccinated infants. Increase in median H3K9me3 from baseline to 6 weeks at the promoter region of pro-inflammatory cytokines occurred, but was small and not statistically significant (Figure 7.4b, and Table 1.3.2 in Appendix 9).

Comparison of median within-infant changes to epigenetic modification at the promoter region of pro-inflammatory cytokines, from birth to 6 weeks showed a consistently larger increase over time in BCG unvaccinated than BCG vaccinated infants (Figure 7.5, and Table 1.4.1 in Appendix 9). This was statistically significant only for increases in H3K9me3 at the TNFα promoter.

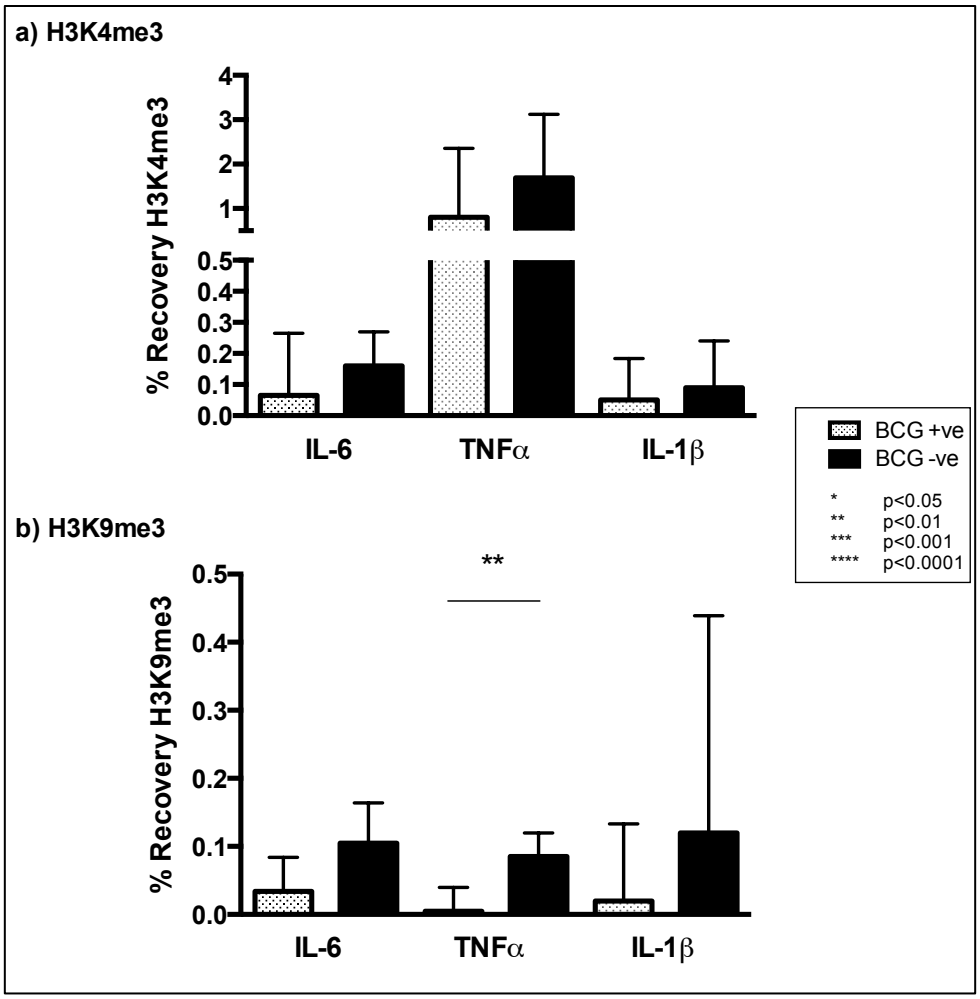
Thus, these experiments suggest that BCG reduces the constitutive increase of both stimulatory H3K4me3 and inhibitory H3K9me3 histone modifications at the promoter region of pro-inflammatory cytokines. The reduction in H3K9me3 inhibitory marks appears to be more pronounced (especially for TNFα).

Figure 7.4. Comparison of levels of epigenetic modification at the promoter region of pro-inflammatory cytokine at baseline and 6 weeks of age, by BCG status



Comparisons of median levels of epigenetic modification between cord and 6 weeks of age, conducted using the Wilcoxon matched-pairs test. BCG vaccinated (+ve) n=16, BCG unvaccinated (-ve) n=15. H3K4me3, histone-3 lysine-4 trimethylation; H3K9me3, histone-3 lysine-9 trimethylation; IL, interleukin; TNF, tumor necrosis factor.

Figure 7.5. The impact of BCG vaccination on within-infant changes in epigenetic modification at the promoter region of pro-inflammatory cytokines between birth and 6 weeks



Comparisons of median within-infant change to the levels of epigenetic modification between cord and 6 weeks of age, conducted using the Mann-Whitney U test. BCG vaccinated (+ve) n=16, BCG unvaccinated (-ve) n=15. H3K4me3, histone-3 lysine-4 trimethylation; H3K9me3, histone-3 lysine-9 trimethylation; IL, interleukin; TNF, tumor necrosis factor.

7.2.4 Individual variability in the changes to epigenetic modification over time was high, particularly in BCG vaccinated infants

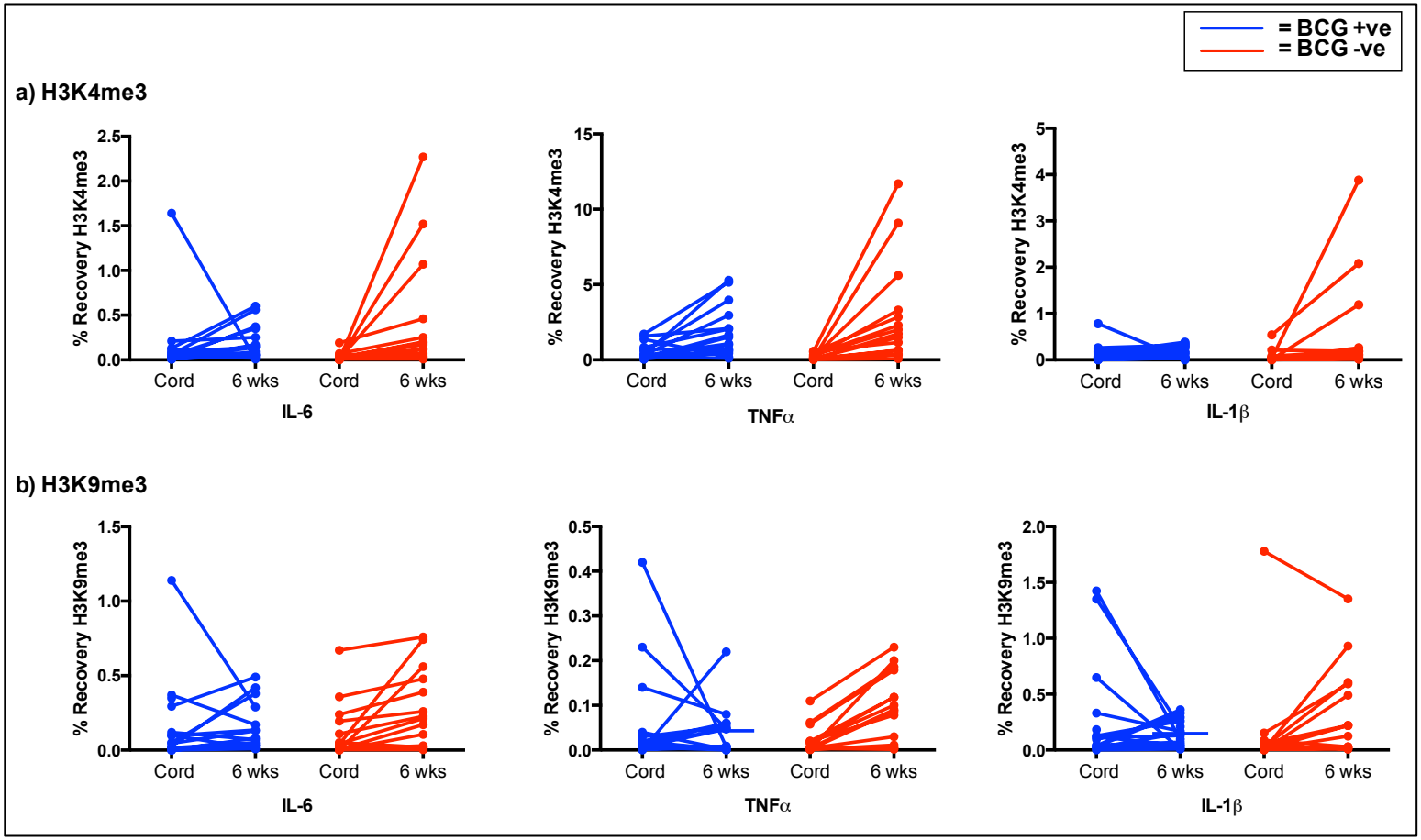
When changes to H3K4me3 and H3K9me3 were viewed at an individual level, variability in response was seen (Figure 7.6).

Overall, as suggested by the observations of grouped medians, increases in H3K4me3 and H3K9me3 occurred in most infants between cord and 6 weeks, and these appeared to be larger in BCG unvaccinated infants. However, there were notable exceptions where histone modification decreased over time. These occurred particularly in the BCG vaccinated infants. In many infants the BCG vaccinated and unvaccinated infants had equivalent changes in the amount of histone modification. Large increases in epigenetic modification in the BCG unvaccinated group, and decreases in the BCG vaccinated group, only occurred in certain infants. Exploratory analyses was therefore conducted to investigate whether these represent a distinct subset of infants based on:

1) sex,
2) response to BCG as measured by scar size at 10 weeks (10 weeks post-BCG at birth or 4 weeks post-BCG at 6 weeks). Median BCG scar size by group was used to define large and small scars,
3) incidence of infections prior to the 6 week blood sample.

The small sub-group numbers, and post-hoc nature of the analysis, means that these results should be viewed as hypothesis-generating for future larger studies, not as conclusive.

Figure 7.6. Individual changes in epigenetic modification at the promoter region of pro-inflammatory cytokines from birth to 6 weeks, by BCG status



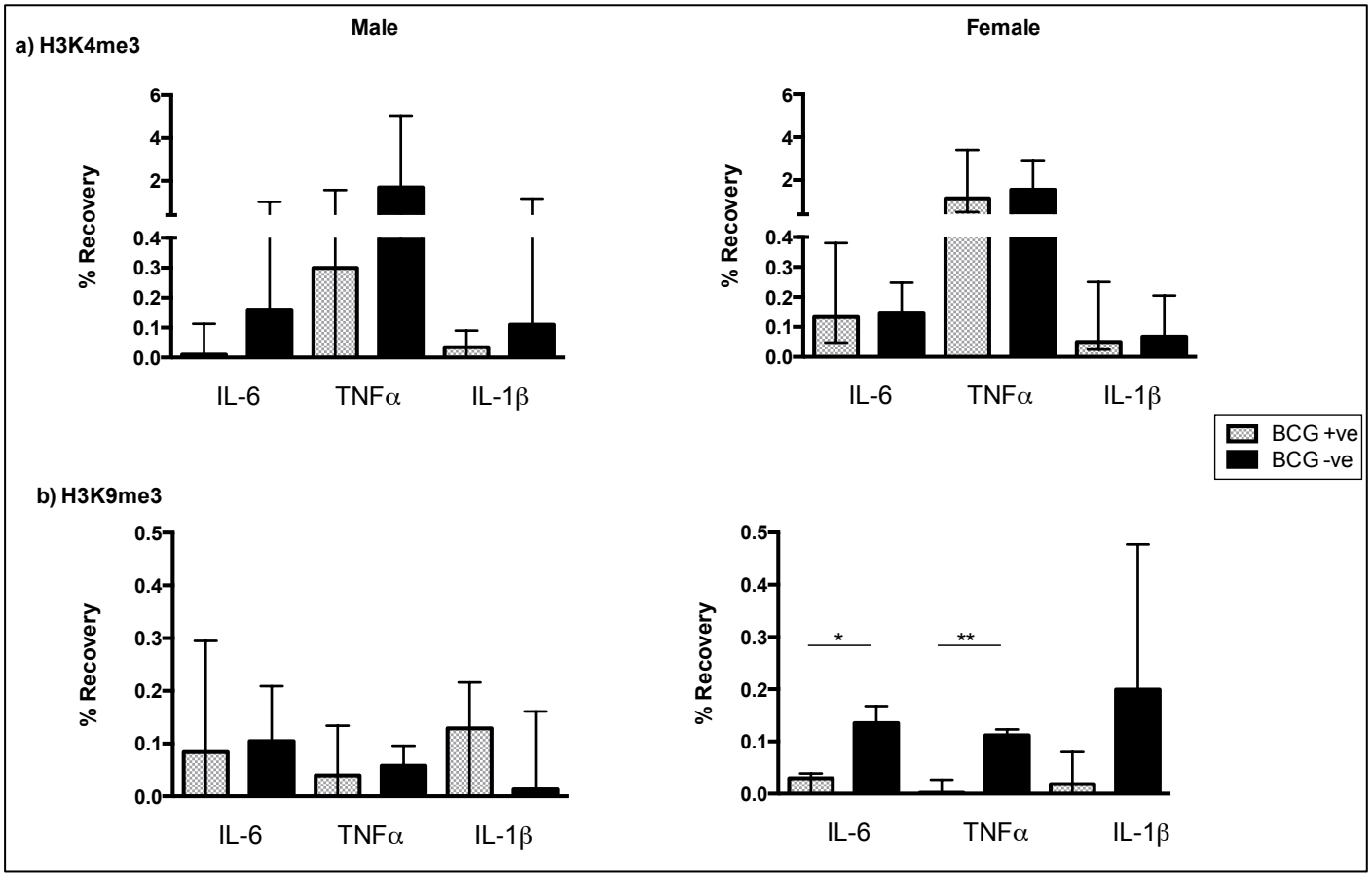
Individual changes to amount of epigenetic modification at the promoter regions of pro-inflammatory cytokines. BCG vaccinated (+ve, shown in blue) n=16, BCG unvaccinated (-ve, shown in red) n=15. H3K4me3, histone-3 lysine-4 trimethylation; H3K9me3, histone-3 lysine-9 trimethylation; IL, interleukin; TNF, tumor necrosis factor.

7.2.4.1 Patterns of median within-infant changes to epigenetic modification at promoter regions of pro-inflammatory cytokines between birth and 6 weeks, differed between males and females

When analysed by sex, the impact of BCG on epigenetic modifications did not appear to be consistent between males and females (Figures 7.7a) and 7.7b), Table 1.5.2 in Appendix 9). BCG vaccination in male infants tended to reduce the constitutive increase in H3K4me3 at the promoter regions of all cytokines, whereas BCG vaccination in female infants appeared to have little impact. Conversely, BCG vaccination in female infants tended to reduce the constitutive increase in H3K9me3, reaching statistical significance for IL-6 and TNFα, whereas BCG vaccination in male infants had little effect (and even a tendency toward greater increase at the promoter region of IL-1β).

These findings may suggest that the overall effect of BCG vaccination at birth in boys is to reduce pro-inflammatory cytokine production from heterologous stimuli (decreased stimulatory H3K4me3, equivalent or higher inhibitory H3K9me3). Conversely the effect in girls may result in increased pro-inflammatory cytokine production (equivalent stimulatory marks H3K4me3, lower inhibitory H3K9me3). However, the limited significance of the findings, and small study numbers means these patterns of results should be interpreted with caution.

Figure 7.7. The impact of BCG vaccination on within-infant changes to epigenetic modification at the promoter region of pro-inflammatory cytokines between birth and 6 weeks, analysed by sex

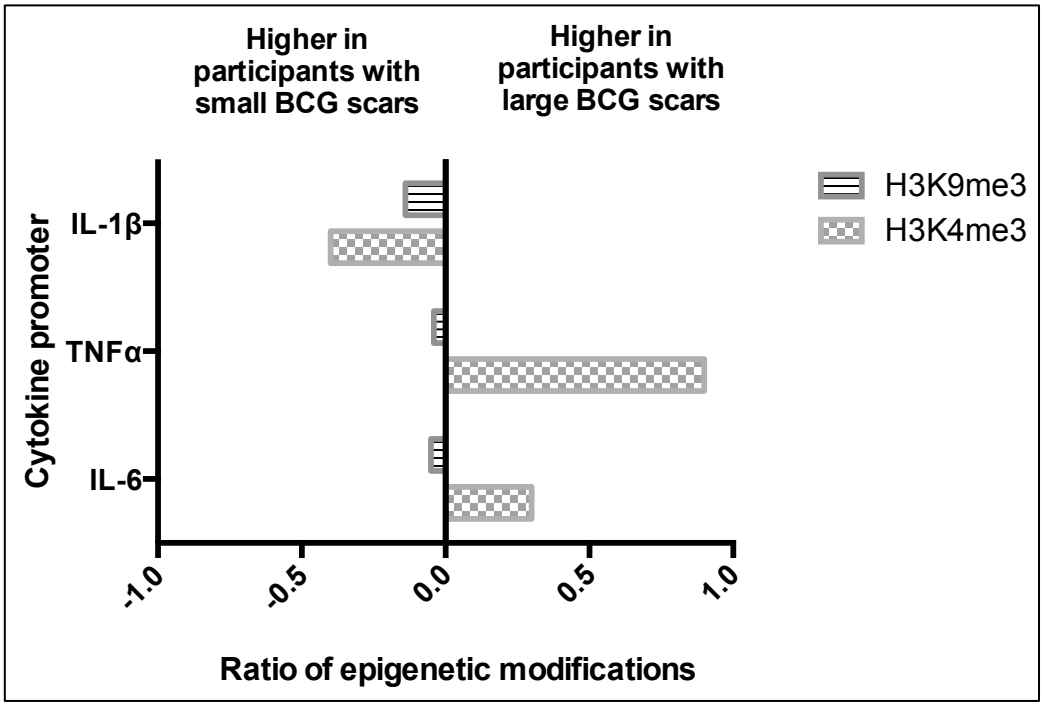


Comparisons of median within-infant change to the levels of epigenetic modification between cord and 6 weeks of age, by BCG status and sex, conducted using the Mann-Whitney U test. Male BCG vaccinated n=6, Male BCG unvaccinated n=7. Female BCG vaccinated n=10, Female BCG unvaccinated n=8. H3K4me3, histone-3 lysine-4 trimethylation; H3K9me3, histone-3 lysine-9 trimethylation; IL, interleukin; TNF, tumor necrosis factor. *=p<0.05; **=p<0.01; ***=p<0.001; ****=p<0.0001.

7.2.4.2 Infants who had a larger BCG scar at 10 weeks showed a non-significant trend toward increased H3K4me3 and decreased H3K4me9

Infants with a larger BCG scar at 10 weeks of age, regardless of when they received BCG vaccination, had a consistent non-significant trend toward lower H3K9me3 at all pro-inflammatory cytokines, and higher H3K4me3 at IL-6 and TNFα (Figure 7.8, and Table 1.6.1 Appendix 9). This could suggest that increased pro-inflammatory cytokine production (due to an increased H3K4me3:H3K9me3 ratio) is associated with larger scar size from BCG vaccination.

Figure 7.8. The impact of BCG scar size at 10 weeks on differences of median within-infant changes of epigenetic modification at the promoter region of pro-inflammatory cytokines between birth and 6 weeks



Comparisons of median within-infant change to the levels of epigenetic modification between cord and 6 weeks of age by scar size, conducted using the Mann-Whitney U test and expressed as ratios of infants with large BCG scars:small BCG scars. Large scar size in BCG vaccinated infants (≥5mm) n=11, small scar size in BCG vaccinated infants (≤4mm) n=5. Large scar size in BCG unvaccinated infants (≥4mm) n=8, small scar size in BCG unvaccinated infants (≤3mm) n=6. H3K4me3, histone-3 lysine-4 trimethylation; H3K9me3, histone-3 lysine-9 trimethylation; IL, interleukin; TNF, tumor necrosis factor.

If this is a true finding, it could be interpreted either:

a) that a larger BCG scar reaction occurs in children with a pre-existing propensity to increased pro-inflammatory cytokine production (i.e. that a large scar is simply a marker of a more reactive immune system) or

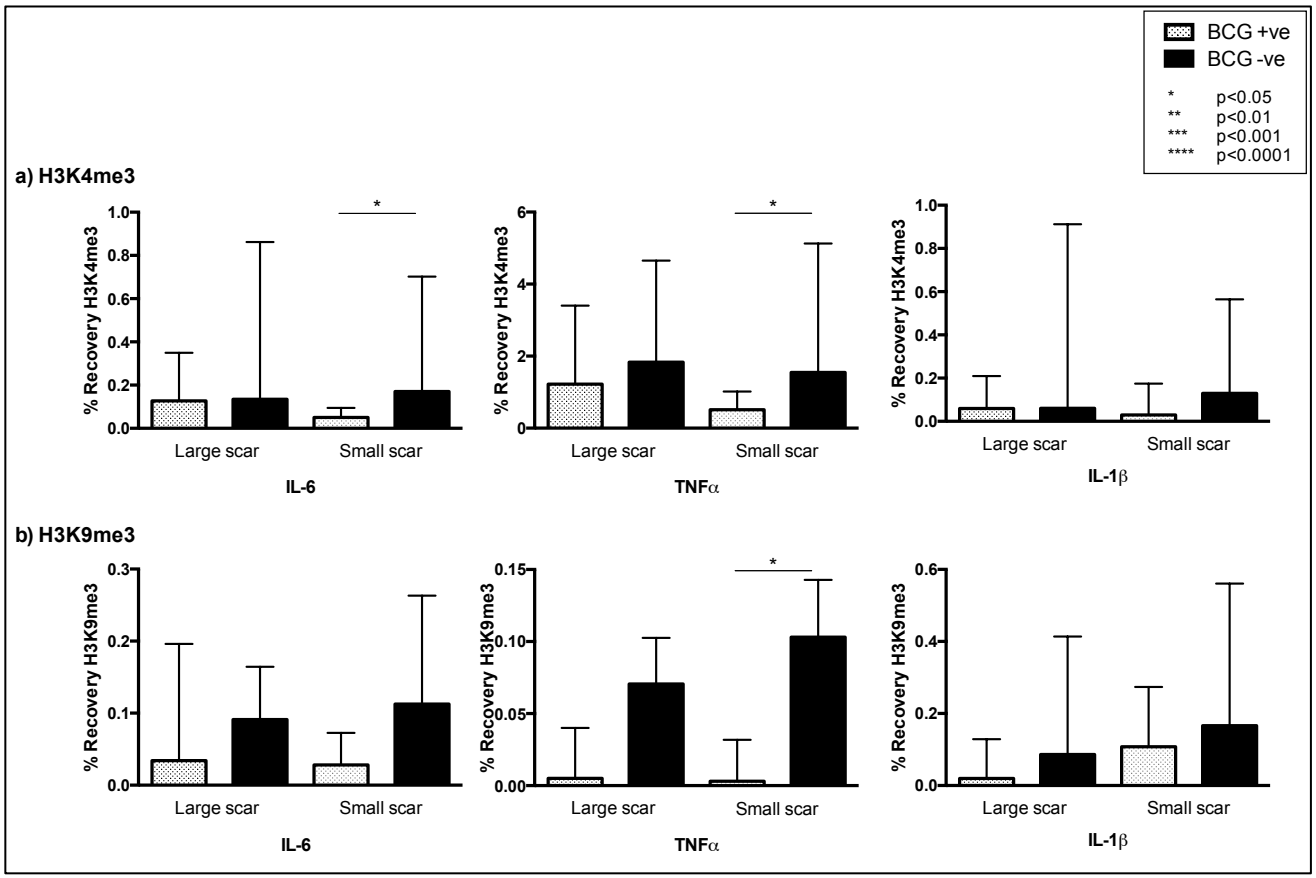
b) that enhanced responsiveness to BCG (shown by a larger scar) increases the pro-inflammatory response because it has a larger effect on epigenetic modification (i.e. greater inhibition H3K9me3 epigenetic modification and therefore increased pro-inflammatory cytokine responses). This does not exactly fit with the previously described evidence, which suggests that H3K4me3 is lower in BCG vaccinated infants not higher, as may be associated with infants with larger scars.

7.2.4.3 BCG-associated reduction in the constitutive increase in epigenetic modification was more pronounced in infants who ultimately had a small BCG scar

The effect of BCG vaccination in reducing the degree of epigenetic modification at the promoter region of pro-inflammatory cytokines between birth and 6-weeks was most pronounced in infants who ultimately had a small BCG scar at 10 weeks of age (Figure 7.9, and Table 1.6.2 Appendix 9). H3K4me3 at the promoter region of IL-6, and H3K4me3 and H3k9me3 at the promoter region of TNFα, were significantly reduced in BCG vaccinated infants with small scars, compared to BCG unvaccinated infants who ultimately had a low scar upon receipt of immunisations. This trend was consistent for H3K4me3 and H3k9me3 at other cytokine promoters, but did not reach statistical significance (Table 1.6.2 Appendix 9). For infants who had large scars at 10 weeks of age, changes to H3K4me3 over time were very similar in BCG vaccinated and BCG unvaccinated infants. Increases in H3K9me3 showed a trend to being lower in BCG vaccinated infants compared to BCG naïve infants who ultimately had a larger BCG scar, but the differences were smaller than seen in infants who ultimately had a small BCG scar.

These findings argue that increased potential for NSE following BCG vaccination might not be measurable by BCG-induced scar size, but rather the reverse. This suggests that scar size post-BCG is a proxy for the immune-activation state of the infant, and that the effects of BCG boosting pro-inflammatory responses are more marked in infants with less reactive immune systems. This could underlie the enhanced clinical beneficial effects of BCG that appear to occur in low birth-weight and pre-term infants. Again, the small numbers in these sub-analyses mean that the results should be viewed as exploratory rather than conclusive.

Figure 7.9. The impact of BCG status and BCG response on within-infant changes to epigenetic modification at the promoter region of pro-inflammatory cytokines between birth and 6 weeks of age



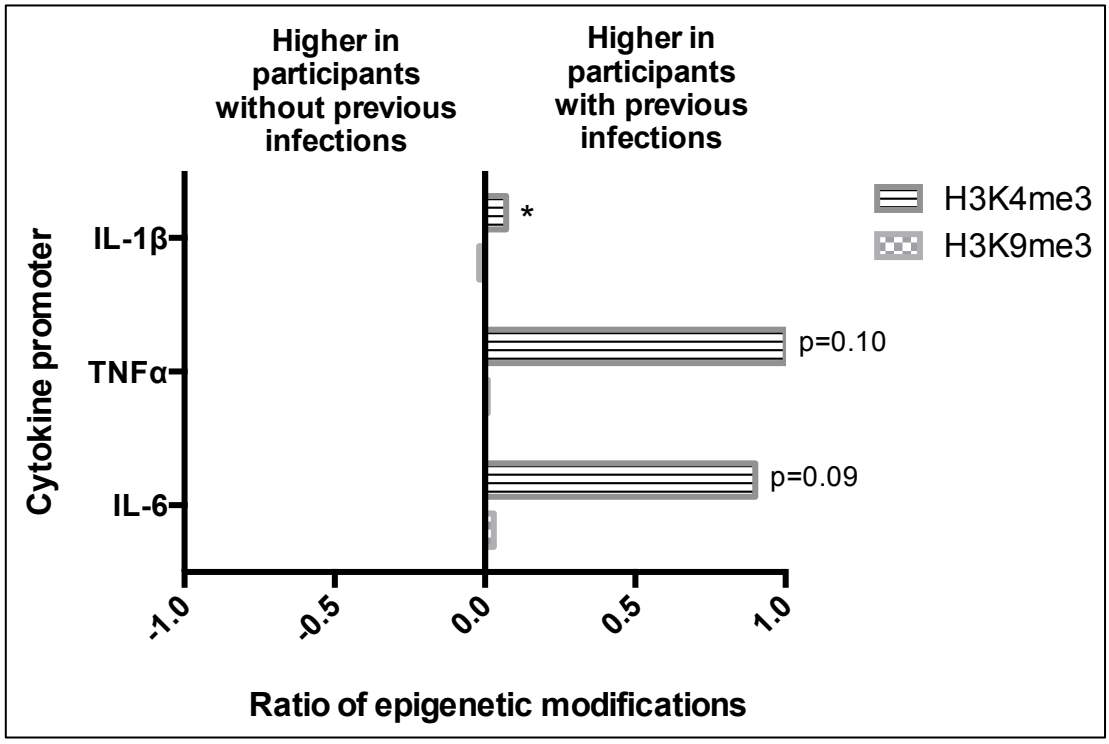
Comparisons of median within-infant change to the levels of epigenetic modification between cord and 6 weeks of age by BCG vaccination status and response as measured by scar size at 10 weeks of age, conducted using the Mann-Whitney U test. Large scar size in BCG vaccinated infants (≥5mm) n=11, small scar size in BCG vaccinated infants (≤4mm) n=5. Large scar size in BCG unvaccinated infants (≥4mm) n=8, small scar size in BCG unvaccinated infants (≤3mm) n=6. H3K4me3, histone-3 lysine-4 trimethylation; H3K9me3, histone-3 lysine-9 trimethylation; IL, interleukin; TNF, tumor necrosis factor.

7.2.4.4 Within-infant changes in H3K4me3 from birth to 6 weeks tended to be higher in infants presenting with an infectious disease during the same period

If BCG vaccination induces changes to long-term epigenetic modification at the promoter region of pro-inflammatory cytokines, it could be argued that exposure to other pathogens may also produce changes.

Nine out of the 31 participants (four BCG vaccinated, 5 BCG unvaccinated) in this sub-study either presented to clinic with an infectious illness, or reported symptoms consistent with an infection, between birth and 6 weeks of age. In these infants, there was a clear trend to greater increases in H3K4me3 at the promoter region of all pro-inflammatory cytokines between baseline and 6 weeks, compared to infants that had not had an infection between baseline and 6 weeks (Figure 7.10, and Table 1.7.1 in Appendix 9). This was statistically significant for changes in H3K4me3 at the promoter region of IL-1β, and close to statistical significance for IL-6 and TNFα. Minimal differences in H3K9me3 epigenetic modification were seen.

Figure 7.10. Differences in the within-infant changes to levels of epigenetic modification at the promoter region of pro-inflammatory cytokines over time by infection status

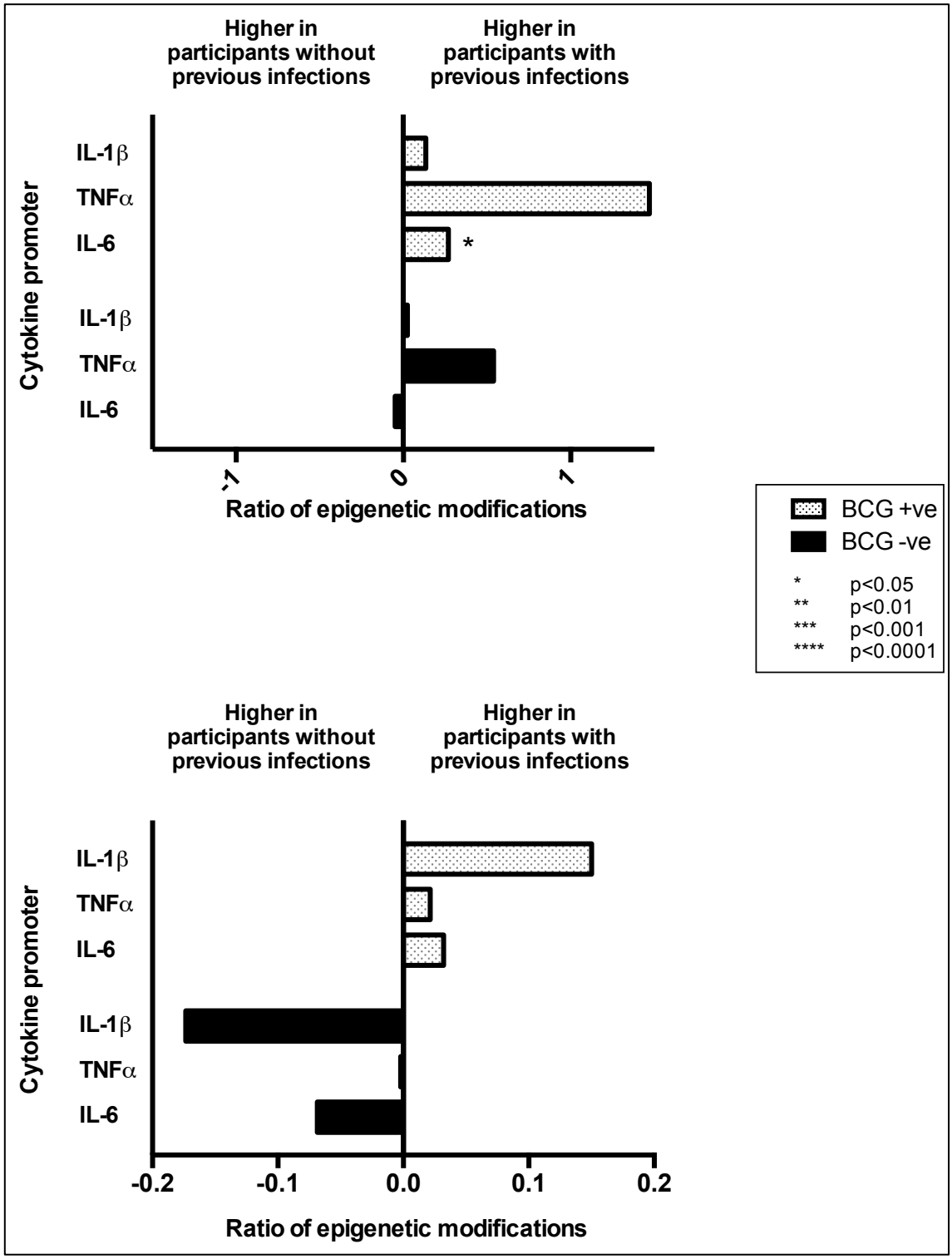


Comparisons of median within-infant change to the levels of epigenetic modification between cord and 6 weeks of age, by presence or absence of infection in the 6 week follow-up period, conducted using the Mann-Whitney U test and expressed as ratios of infants with infections:infants without infections. Infants with infections n=9 Infants with no infections n=22. H3K4me3, histone-3 lysine-4 trimethylation; H3K9me3, histone-3 lysine-9 trimethylation; IL, interleukin; TNF, tumor necrosis factor. *=p<0.05; **=p<0.01; ***=p<0.001; ****=p<0.0001.

This suggests that exposure to infectious pathogens increases stimulatory epigenetic modification at the promoter region of pro-inflammatory cytokines, compared to infants that have no infections. The lack of similar effects on H3K9me3 (inhibitory) epigenetic modification suggests that an increase in pro-inflammatory cytokine production from immune cells upon further pathogen challenge would occur. Thus, these findings suggest that the developing neonatal innate immune system may 'learn' to respond in an up-regulated manner to subsequent challenge from a variety of stimuli. However, as the exact timing of changes to epigenetic modification in comparison to infectious episodes are not known, it could be that increases in H3K4me3 pre-date infections, and the clinical features of the infection are a result of an enhanced pro-inflammatory milieu.

When analysed by BCG status, infants who had been BCG vaccinated at birth that subsequently went on to get infections had greater increases in both H3K4me3 and H3K9me3 over time than infants who had not had infections (Figure 7.11). In BCG unvaccinated infants, the increase of H3K4me3 over time was higher in infants who had an interim infection, but the increase in H3K9me3 over time was lower. Thus in the context of interim infection, BCG is associated with increased inhibitory and stimulatory marks, whereas without BCG, the increase is seen only in stimulatory marks. This exploratory analysis could suggest that BCG unvaccinated infants may, on balance, have a tendency toward more pro-inflammatory responses, whereas the increase in H3K9me3 may temper these responses in BCG vaccinated infants.

Figure 7.11. The differential effects of BCG on changes to epigenetic modification between birth and 6 weeks of age induced by interim infections



Comparisons of median within-infant change to the levels of epigenetic modification between cord and 6 weeks of age, by BCG status and presence or absence of infection in the 6 week follow-up period, conducted using the Mann-Whitney U test and expressed as ratios of participants with infections to participants without infections. BCG vaccinated (+ve) infants with infections n=4, with no infections n=12. BCG naïve (-ve) infants with infections n=5, with no infections n=10. H3K4me3, histone-3 lysine-4 trimethylation; H3K9me3, histone-3 lysine-9 trimethylation; IL, interleukin; TNF, tumor necrosis factor.

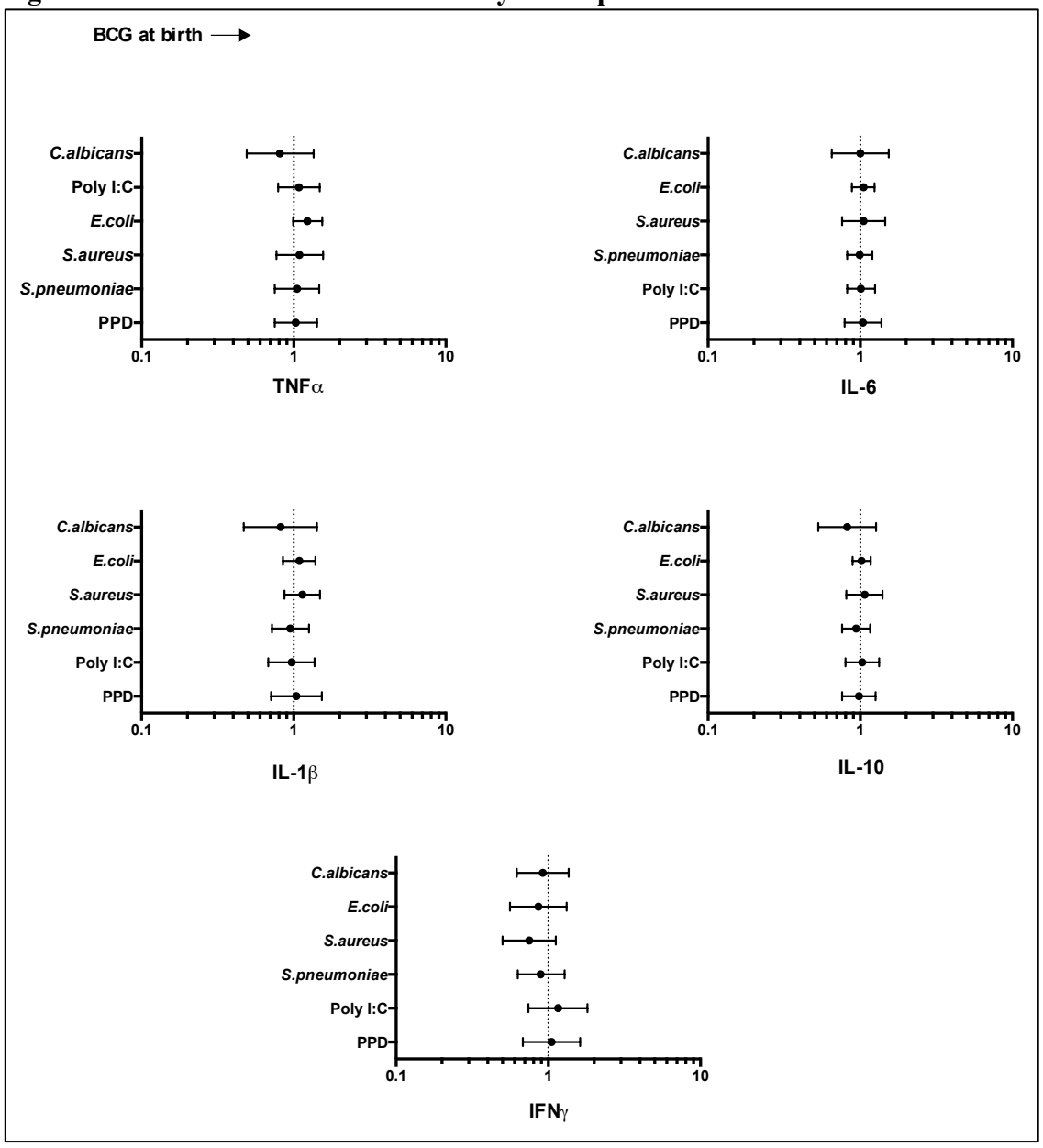
7.3 Cytokine sub-study results

The cytokine sub-study was conducted to compare innate cytokine concentrations following *in vitro* whole blood stimulation with heterologous pathogens, between infants BCG vaccinated at birth and infants BCG vaccinated at 6 weeks of age. Cytokine concentrations in stimulated supernatants were assessed using ELISA as described in Methods section 6.3.1.2.

7.3.1 Baseline samples

Cord blood samples served as a proxy for pre-intervention, baseline infant samples. There were no significant differences in cytokine concentrations following heterologous stimulation with any stimuli between the two intervention groups (Figure 7.12, and Tables 2.2.1 and 2.2.2, Appendix 9). This suggests that the randomisation produced balanced groups.

Figure 7.12. Geometric mean ratios of cytokine production in cord blood



Ratios of geometric mean cytokine concentrations in cord blood, comparing infants BCG vaccinated at birth with those BCG vaccinated at 6 weeks of age. Cytokine production was measured by competitive ELISA following 24-hour whole blood stimulation with the heterologous stimuli. N≥102 per group. Exact numbers for each stimulus and cytokine can be found in Table 2.1 Appendix 9. The unstimulated cytokine response for each infant was included as a covariate in the regression model. A ratio of 1 indicates equivalence. Ratio >1 shows concentration is higher in infants BCG vaccinated at birth, ratio <1 shows higher concentrations in infants BCG vaccinated at 6 weeks. TNF, tumor necrosis factor; IL, interleukin; IFN, interferon; PPD, purified protein derivative; Poly I:C, polyinosinic;polycytidylic acid, *S.pneumoniae, Streptococcus pneumonia; S.aureus, Staphylococcus aureus; E.coli, Escherichia coli; C.albicans, Candida albicans.*

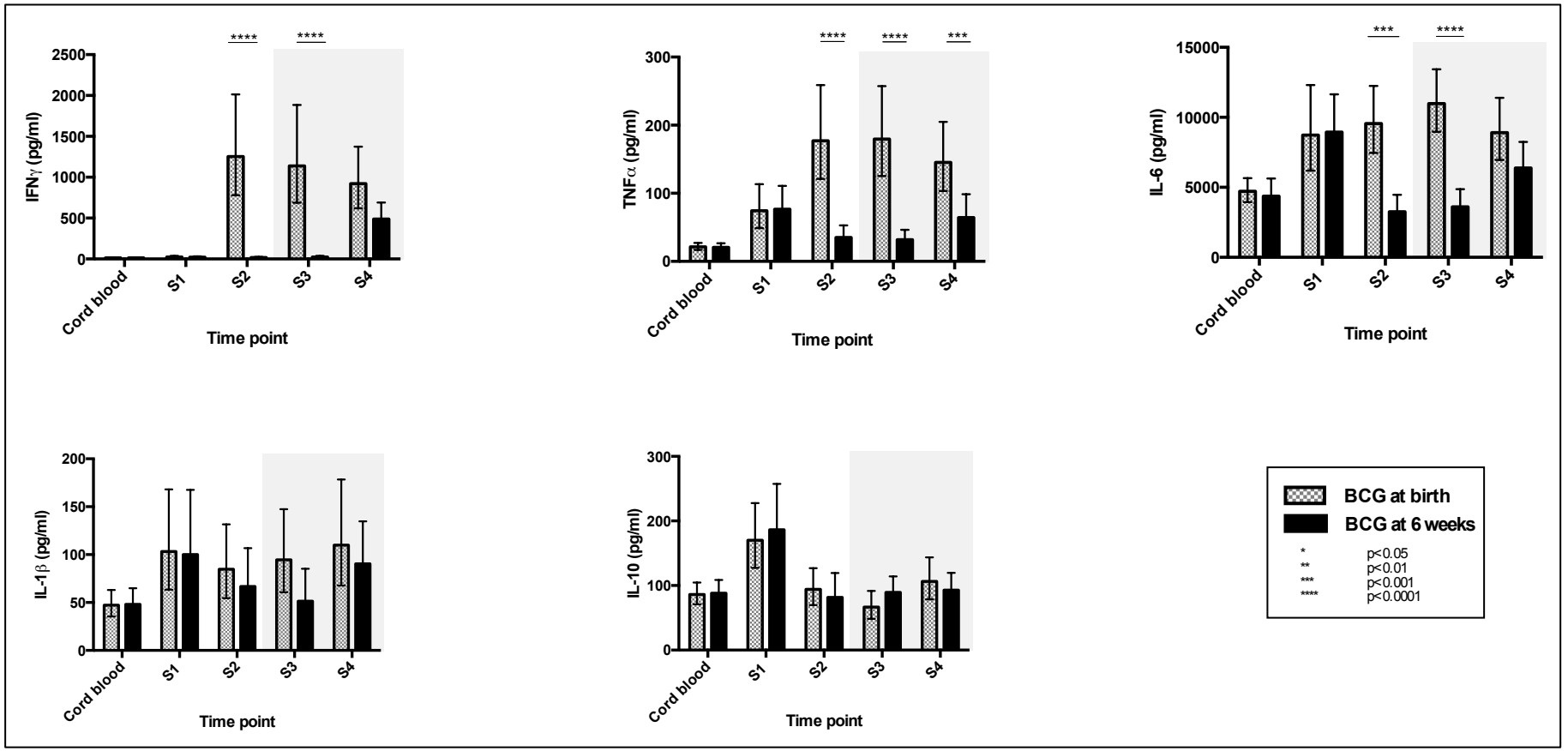
7.3.2 BCG-specific responses show that intervention allocation occurred correctly

In vitro cytokine production following stimulation with PPD is illustrated in Figure 7.13 (Tables 2.2.1 and 2.2.2 in Appendix 9).

IFNγ production following PPD stimulation in infants receiving BCG at birth was low in cord blood and at 5 days post-vaccination (S1), but strongly induced by 6 weeks of age. A similar pattern of response was seen following BCG vaccination at 6 weeks of age in the delayed group, with increased PPD-stimulated IFNγ production seen at 4 weeks post-BCG vaccination (S4), but not at 5 days post-BCG vaccination (S3). PPD-induced IFNγ production was therefore significantly higher in infants receiving BCG vaccination at birth at all time points after 5 days of age, although the difference was less strong by 10 weeks of age as the IFNγ levels in infants receiving delayed BCG were beginning to increase. These findings are as expected, and provide immunological confirmation that BCG vaccination was given according to infant randomisation allocation. TNFα and IL-6 showed a similar pattern of responses to IFNγ, but no significant differences in PPD-induced IL-1β and IL-10 production were seen at any time-point.

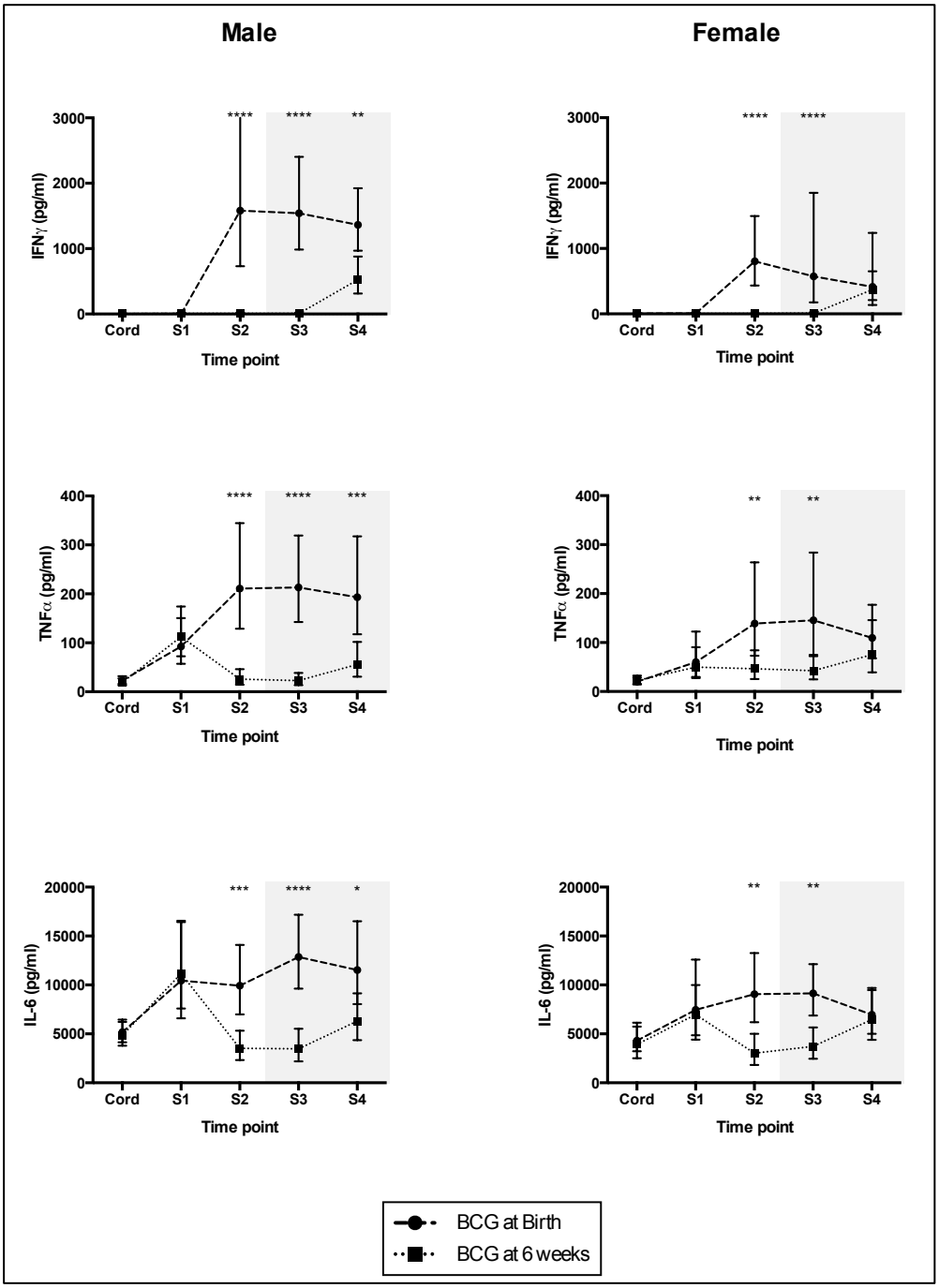
As shown in Figure 7.14, the PPD-induced innate cytokine responses are more pronounced in male infants. The interaction of sex and BCG vaccination timing on PPD-induced cytokine responses was only significant for PPD-induced TNFα production at S3 (test for interaction p=0.03).

Figure 7.13. Cytokine concentrations following PPD stimulation by BCG status



Geometric mean and standard error of cytokine concentrations following 24-hour whole blood stimulation with PPD, comparing infants BCG vaccinated at birth with those BCG vaccinated at 6 weeks of age. Cytokine production was measured by competitive ELISA. All responses were adjusted for the cytokine production following RPMI (negative control) stimulation. Comparison of uncorrected values gave similar results. S1, 5 days of age; S2, 6 weeks of age, pre-EPI vaccinations; S3, 6 weeks of age 5 days after EPI-1 +/- BCG in delayed group; S4, 10 weeks of age, pre-EPI-2; BCG, Bacille Calmette Guerin; EPI, Expanded Programme of Immunisations; RPMI, Roswell Park Memorial Institute; TNF, tumor necrosis factor; IL, interleukin; IFN, interferon; PPD, purified protein derivative; Poly I:C, polyinosinic;polycytidylic acid, *S.pneumoniae, Streptococcus pneumonia; S.aureus, Staphylococcus aureus; E.coli, Escherichia coli; C.albicans, Candida albicans.*

Figure 7.14. PPD stimulated cytokine concentrations, by BCG vaccination timing and sex



Geometric mean and standard error of cytokine concentrations following 24-hour whole blood stimulation with PPD, comparing infants BCG vaccinated at birth with those BCG vaccinated at 6 weeks of age, separated by sex. Cytokine production was measured by competitive ELISA. All responses were adjusted for the cytokine production following RPMI (negative control) stimulation. Comparison of uncorrected values gave similar results. S1, 5 days of age; S2, 6 weeks of age, pre-EPI vaccinations; S3, 6 weeks of age 5 days after EPI-1 +/- BCG in delayed group; S4, 10 weeks of age, pre-EPI-2; BCG, Bacille Calmette Guerin; EPI, Expanded Programme of Immunisations; RPMI, Roswell Park Memorial Institute; TNF, tumor necrosis factor; IL, interleukin; IFN, interferon; PPD, purified protein derivative; Poly I:C, polyinosinic;polycytidylic acid, *S.pneumoniae, Streptococcus pneumonia; S.aureus, Staphylococcus aureus; E.coli, Escherichia coli; C.albicans, Candida albicans.* Note: The data displayed are representative of the cross-sectional geometric mean cytokine levels at each time-point. The time-points are shown as joined up only to more clearly convey the changes to geometric mean cytokine production over time. It does not represent within-infant changes.

7.3.3 Cross-sectional comparison of geometric mean cytokine production in response to *in vitro* heterologous stimulation

7.3.3.1 Pro-inflammatory cytokine production in response to heterologous bacterial pathogens was significantly higher at 6 weeks of age, 5 days following EPI-1 in all infants and BCG vaccination in the delayed group (S3), in infants BCG vaccinated at birth

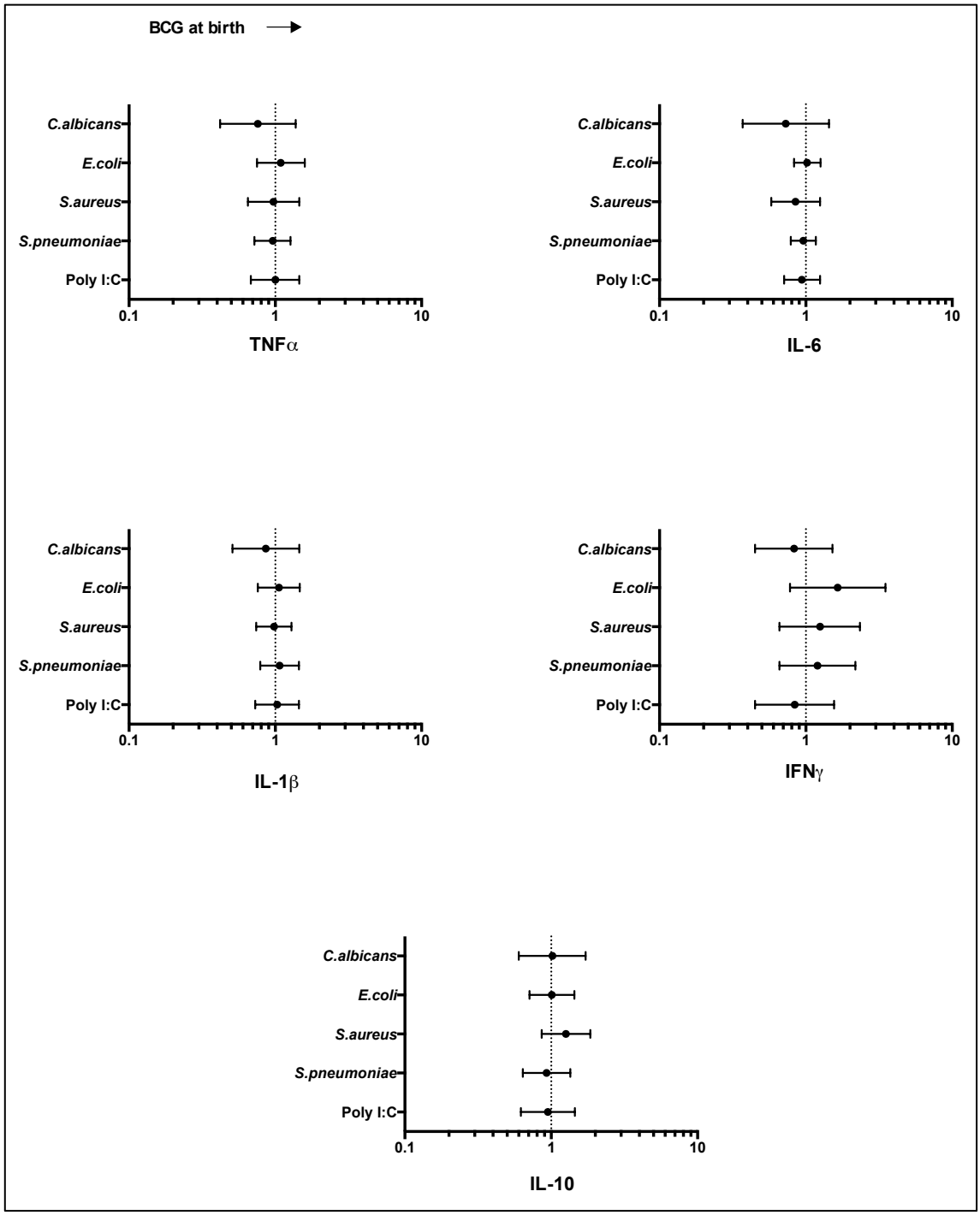
Figures 7.15 – 7.18 show the geometric mean ratios (GMR) of *in vitro* cytokine responses to heterologous stimuli, comparing infants that were BCG vaccinated at birth with infants that were BCG vaccinated at 6 weeks, at each of the four post-natal blood sampling time-points. The geometric mean level data can be found in Tables 2.2.1 and 2.2.2, Appendix 9.

No significant differences were seen at 5 days of age (S1) or 6 weeks of age – pre-EPI-1/BCG in delayed group (S2) in geometric mean cytokine production following heterologous stimulation.

At 6 weeks, 5 days following EPI-1 in all infants and BCG vaccination in the delayed group (S3), TNFα production in response to stimulation with Gram-positive bacteria was significantly higher in infants BCG vaccinated at birth (*S.pneumoniae* GMR 1.33 (1.01-1.76), p=0.046, *S.aureus* GMR 1.54 (1.00-2.41), p=0.05). A similar trend was seen with TNFα and IL-1β production following *E.coli* stimulation, and with IL-6 production following Gram positive and negative bacterial stimulation, although these did not reach conventional statistical significance. No significant differences or clear trends in TNFα, IL-6 and IL-1β production following viral or fungal stimulation were seen, nor with IFNγ or IL-10 production following any pathogen stimulation, by BCG group.

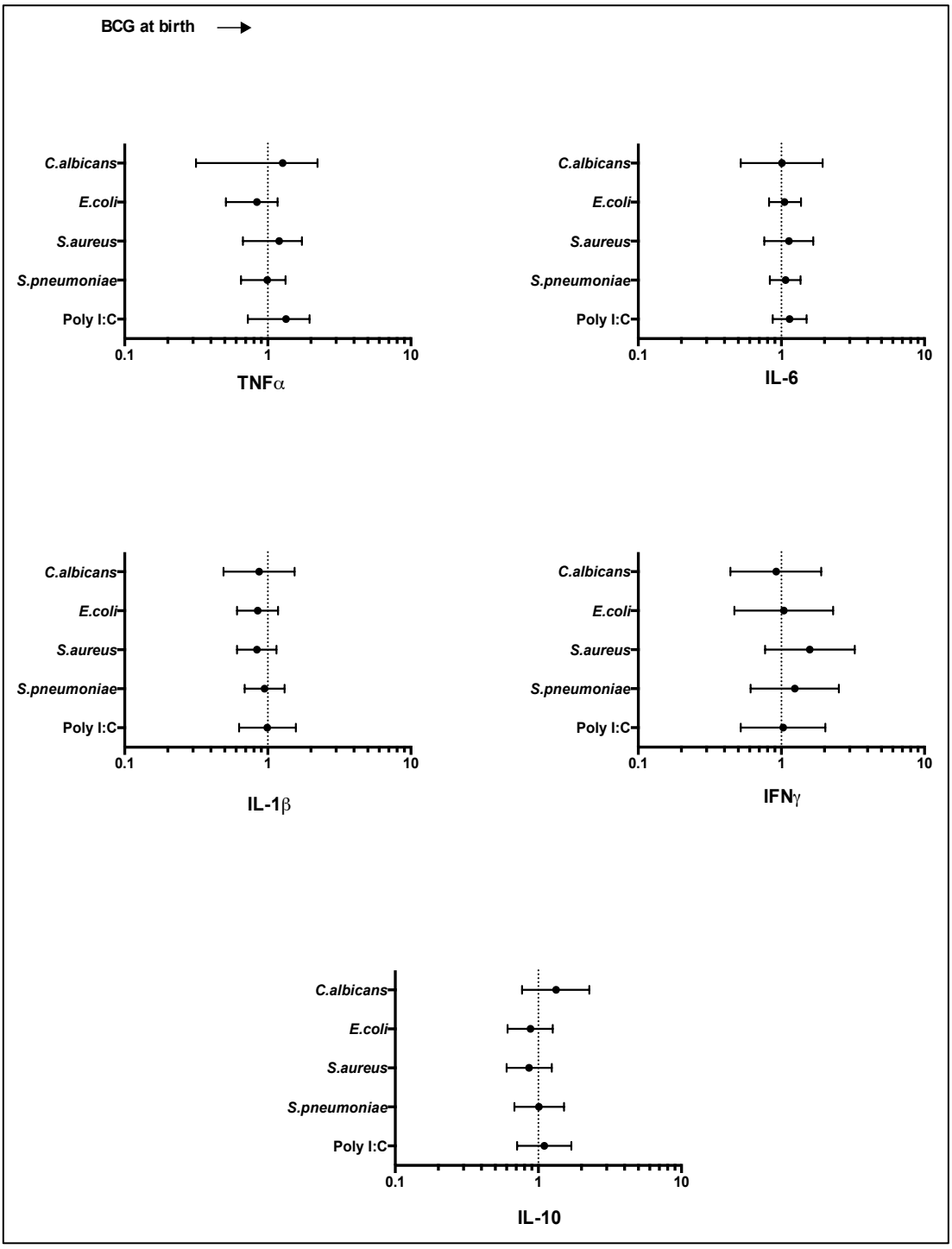
At 10 weeks of age, pre-EPI-2 vaccinations (S4), no statistically significant differences in cytokine production following *in vitro* stimulation with any heterologous pathogen by BCG group were seen. For bacterial pathogens the trend in TNFα and IL-6 was reversed from S3, however, with cytokine production appearing higher in infants who received BCG at 6 weeks of age, but this was not statistically significant.

Figure 7.15. Geometric mean cytokine ratios comparing BCG vaccinated with unvaccinated infants at 5 days of age



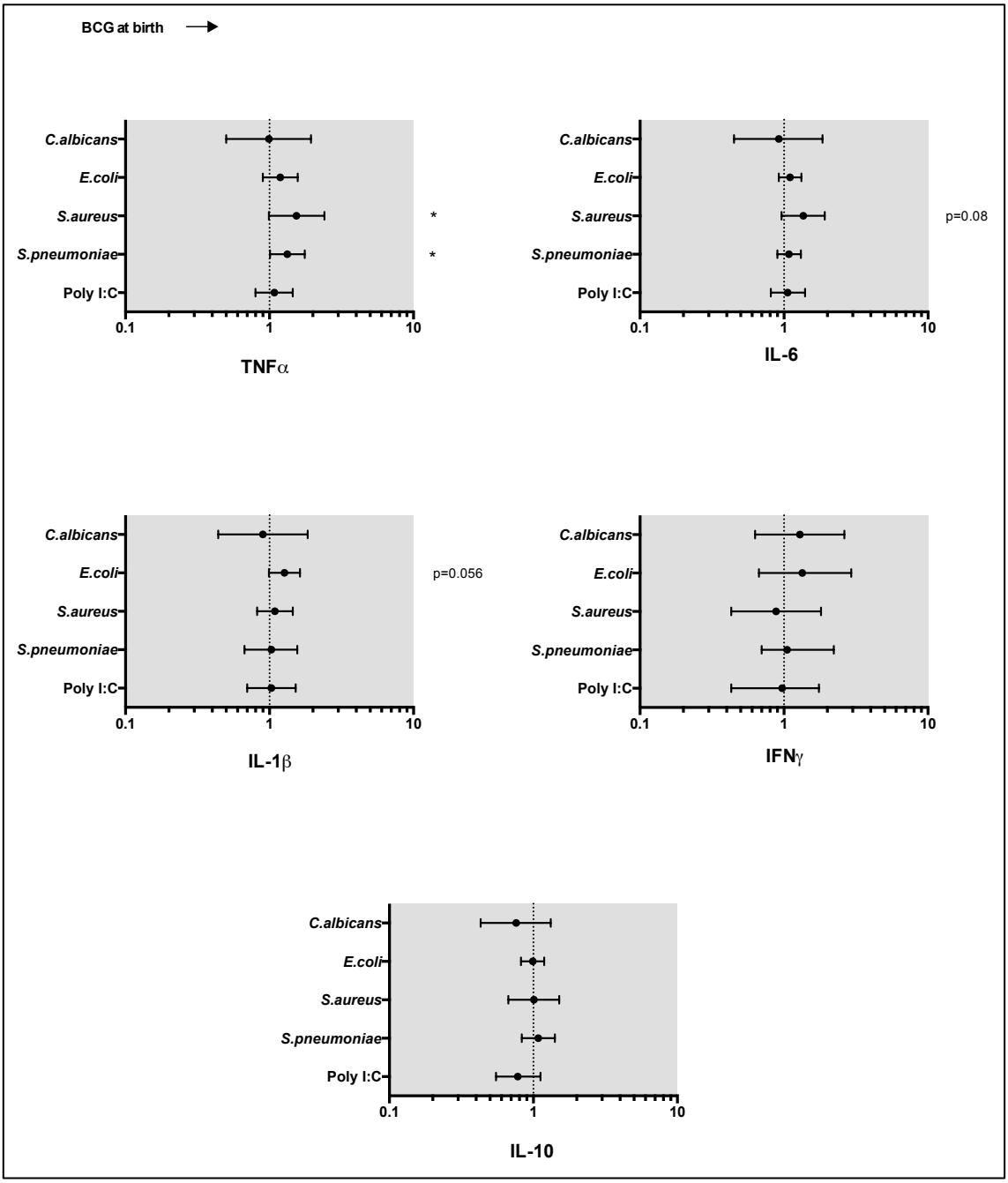
Ratios of geometric mean cytokine concentrations in blood taken at 5 days of age (S1), comparing infants BCG vaccinated at birth with those BCG vaccinated at 6 weeks of age. Cytokine production was measured by competitive ELISA following 24-hour whole blood stimulation with the heterologous stimuli. N≥49 per group. Exact numbers for each stimulus and cytokine can be found in Table 2.1 Appendix 9. The unstimulated cytokine response for each infant was included as a covariate in the regression model. A ratio of 1 indicates equivalence. Ratios >1 show concentration is higher in infants BCG vaccinated at birth, ratios <1 show higher concentrations in infants BCG vaccinated at 6 weeks. TNF, tumor necrosis factor; IL, interleukin; IFN, interferon; PPD, purified protein derivative; Poly I:C, polyinosinic;polycytidylic acid, *S.pneumoniae, Streptococcus pneumonia; S.aureus, Staphylococcus aureus; E.coli, Escherichia coli; C.albicans, Candida albicans.* Note; the clear background denotes that comparisons at this time-point are between BCG vaccinated and BCG unvaccinated infants.

Figure 7.16. Geometric mean cytokine ratios comparing BCG vaccinated with unvaccinated infants at 6 weeks of age (prior to EPI-1 vaccinations)



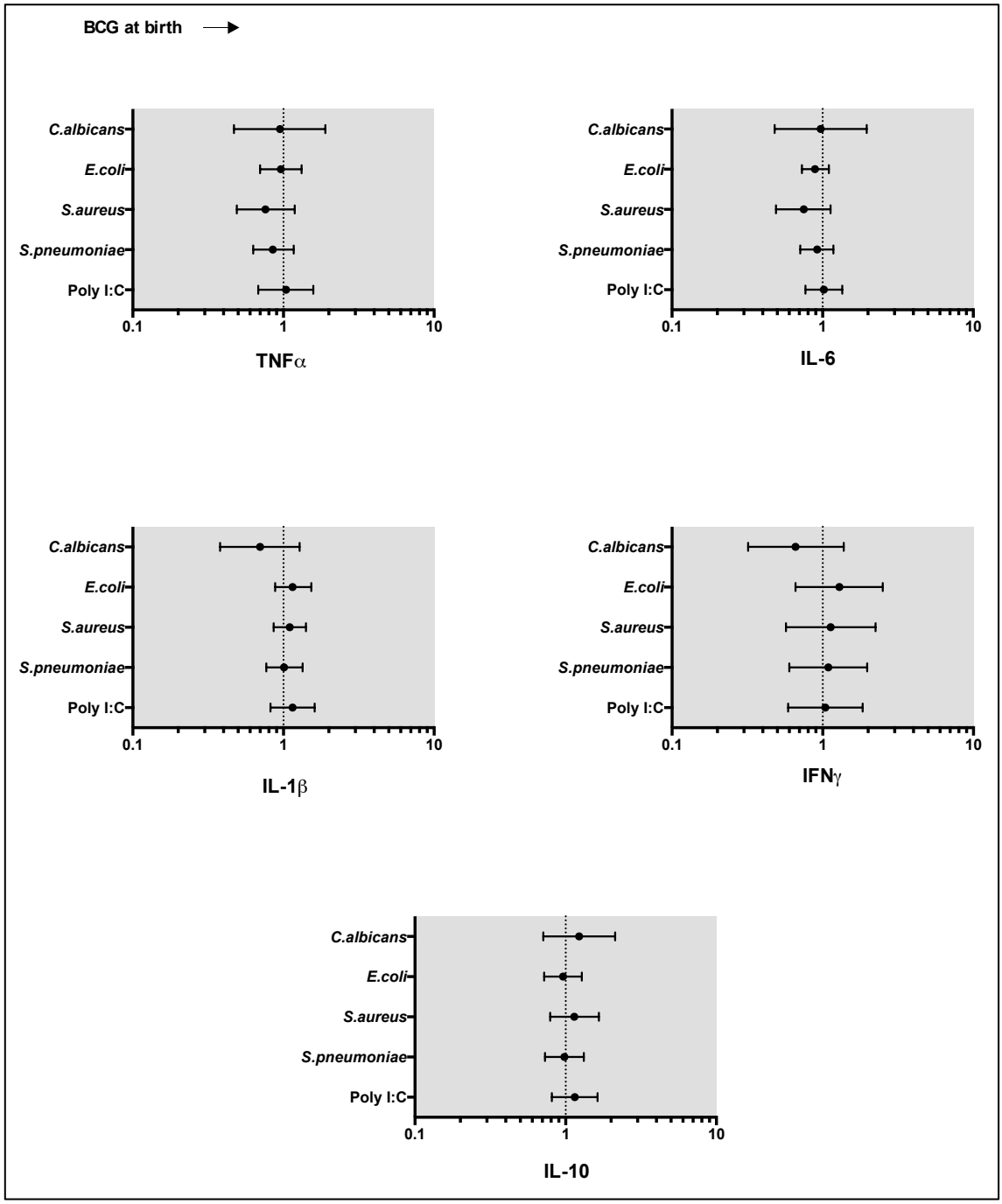
Ratios of geometric mean cytokine concentrations in blood taken at 6 weeks of age, prior to EPI-vaccinations (S2), comparing infants BCG vaccinated at birth with those BCG vaccinated at 6 weeks of age (i.e. comparing BCG vaccinated with BCG unvaccinated infants). Cytokine production was measured by competitive ELISA following 24-hour whole blood stimulation with the heterologous stimuli. N≥41 per group. Exact numbers for each stimulus and cytokine can be found in Table 2.1 Appendix 9. The unstimulated cytokine response for each infant was included as a covariate in the regression model. A ratio of 1 indicates equivalence. Ratios >1 show higher concentrations in infants BCG vaccinated at birth, ratios <1 show higher concentrations in infants BCG vaccinated at 6 weeks. BCG, Bacille Calmette Guerin; EPI, Expanded programme of immunisations; TNF, tumor necrosis factor; IL, interleukin; IFN, interferon; PPD, purified protein derivative; Poly I:C, polyinosinic;polycytidylic acid, *S.pneumoniae, Streptococcus pneumonia; S.aureus, Staphylococcus aureus; E.coli, Escherichia coli; C.albicans, Candida albicans.* Note; the clear background denotes that comparisons at this time-point are between BCG vaccinated and BCG unvaccinated infants.

Figure 7.17. Geometric mean cytokine ratios in blood taken at 6 weeks of age, 5 days after EPI-1 vaccinations (and BCG vaccination in the delayed group)



Ratios of geometric mean cytokine concentrations in blood taken at 6 weeks of age, 5 days post EPI-1 vaccinations +/- BCG in the delayed group (S3), comparing infants BCG vaccinated at birth with those BCG vaccinated at 6 weeks of age. Cytokine production was measured by competitive ELISA following 24-hour whole blood stimulation with the heterologous stimuli. N≥36 per group. Exact numbers for each stimulus and cytokine can be found in Table 2.1 Appendix 9. The unstimulated cytokine response for each infant was included as a covariate in the regression model. A ratio of 1 indicates equivalence. Ratios >1 show higher concentrations in infants BCG vaccinated at birth, ratios <1 show higher concentrations in infants BCG vaccinated at 6 weeks. BCG, Bacille Calmette Guerin; EPI, Expanded programme of immunisations; TNF, tumor necrosis factor; IL, interleukin; IFN, interferon; PPD, purified protein derivative; Poly I:C, polyinosinic;polycytidylic acid, *S.pneumoniae, Streptococcus pneumonia; S.aureus, Staphylococcus aureus; E.coli, Escherichia coli; C.albicans, Candida albicans.* Note: the grey background denotes that comparisons at this time-point are between infants BCG vaccinated at birth and infants BCG vaccinated at 6 weeks.

Figure 7.18. Geometric mean cytokine ratios at 10 weeks of age (pre-EPI-2), comparing infants BCG vaccinated at birth with those vaccinated at 6 weeks



Ratios of geometric mean cytokine concentrations in blood taken at 10 weeks of age, pre-EPI-2 vaccinations (S4), comparing infants BCG vaccinated at birth with those BCG vaccinated at 6 weeks of age. Cytokine production was measured by competitive ELISA following 24-hour whole blood stimulation with the heterologous stimuli. N≥35 per group. Exact numbers for each stimulus and cytokine can be found in Table 2.1 Appendix 9. The unstimulated cytokine response for each infant was included as a covariate in the regression model. A ratio of 1 indicates equivalence. Ratios >1 show higher concentrations in infants BCG vaccinated at birth, ratios <1 show higher concentrations in infants BCG vaccinated at 6 weeks. BCG, Bacille Calmette Guerin; EPI, Expanded programme of immunisations; TNF, tumor necrosis factor; IL, interleukin; IFN, interferon; PPD, purified protein derivative; Poly I:C, polyinosinic;polycytidylic acid, *S.pneumoniae, Streptococcus pneumonia; S.aureus, Staphylococcus aureus; E.coli, Escherichia coli; C.albicans, Candida albicans.* Note: the grey background denotes that comparisons at this time-point are between infants BCG vaccinated at birth and infants BCG vaccinated at 6 weeks.

As non-significant trends toward higher cytokine concentration at S3 and lower cytokine concentration at S4 in infants BCG vaccinated at birth were found, combined analysis was performed to see if this increased the power to detect significant differences (Table 2.4.1 Appendix 9). Combining the responses to all heterologous stimuli for each individual cytokine did not strengthen any association with BCG vaccination timing. Combining the pro-inflammatory cytokine concentrations (TNFα, IL-6, IL-1β and IFNγ) from all heterologous stimuli also did not show any significant global effect of BCG vaccination, although the same trend of higher pro-inflammatory cytokine concentration at S3 in BCG at birth vs. BCG at 6 weeks infants, and lower cytokine concentration at S4 in BCG at birth vs. BCG at 6 weeks infants, was retained. Similarly, comparing the TNFα:IL-10 ratio (as a proxy for the ratio of pro-inflammatory:anti-inflammatory responses) for the combined responses to all pathogens was not significantly different at any time-point, although the trend toward higher ratios was seen in those receiving BCG at birth compared with 6 weeks at S3 and lower ratios at S4.

When cytokine responses to bacteria were investigated, there was a trend toward higher pro-inflammatory cytokine production to bacteria at S3 in infants who received BCG at birth. This was strengthened when limited to production of TNFα and IL-6 (GMR 3.65 (1.20-11.11), p=0.02).

7.3.3.2 Increased pro-inflammatory cytokine production to bacterial stimuli at 6 weeks of age, 5 days following EPI-1 in all infants and BCG vaccination in the delayed group (S3), was more pronounced in male infants, BCG vaccinated at birth.

When analysed by sex, the impact of BCG timing on cytokine production to heterologous stimuli was more pronounced in males (Tables 2.3.1 and 2.3.2 Appendix 9). At S3 the higher TNFα production to *S.pneumoniae* and *S.aureus*, and the higher IL-6 production to *S.aureus*, in infants BCG vaccinated at birth, was significant only in males (GMRs 1.54 (1.03-2.31), p=0.04, 1.72 (1.00-3.24), p=0.05, and 1.77 (1.13-2.76), p=0.01, respectively). Male infants receiving BCG at birth also showed significantly higher IL-1β production following *E.coli* stimulation (GMR 1.58 (1.07-2.33), p=0.02). For these cytokine and stimuli, the trend was similar in females, and no significant interaction of sex with the impact of BCG vaccination timing on heterologous cytokine production was seen (Tables 2.3.1 and 2.3.2, Appendix 9). At S3 male infants receiving BCG at birth also had significantly higher IL-1β production when combining responses from all pathogens (p=0.03), and all bacterial stimuli (p=0.02) and a sex-differential effect was seen with a non-significant trend to lower geometric mean responses in females who received BCG at birth (test for interaction p=0.04).

When analysed by sex, higher IFNγ production following a) *E.coli* stimulation (GMR 3.57 (1.35-9.52), p=0.01), b) combined analysis of all pathogens (GMR 2.87 (1.87-6.03), p=0.006) c) combined analysis of bacterial pathogens (GMR 2.47 (1.14-5.31), p=0.003), was seen in male infants BCG vaccinated at birth compared to unvaccinated infants at 5 days of age (S1). A non-significant trend toward opposite effects was seen in female infants and the test for interaction of sex was significant, p=0.03. Similarly the impact of BCG timing on TNFα production to Poly I:C at S1 differed by sex. TNFα production following Poly I:C was non-significantly higher in male infants vaccinated at birth compared to unvaccinated infants, but was non-significantly lower in female infants; the test for interaction was significant, p=0.009.

At 10 weeks of age (S4), male infants who receive BCG vaccination at birth had significantly lower geometric mean IL-6 concentrations following *E.coli* stimulation (GMR 0.72 (0.53-0.97), p=0.03), and upon combined bacterial stimuli analysis (GMR 0.83 (0.58-0.99), p=0.05), compared to male infants receiving BCG at 6 weeks of age. Female infants did not show this effect of BCG vaccination timing, with a trend toward the opposite responses, tests for interaction p=0.04 and 0.15 respectively. IL-10 production following Poly I:C stimulation at S4 was significantly higher in male infants

receiving BCG vaccination at birth compared to 6 weeks of age (GMR 1.60 (1.03-2.46) p=0.04). A trend towards lower production was seen in female infants BCG vaccinated at birth and the interaction of sex on the impact of BCG timing approaches conventional statistical significance (p=0.08).

A summary of the statistically significant differences in heterologous cytokine responses is shown in Table 7.2.

Table 7.2. Summary of statistically significant differences in cytokine production following heterologous stimulation, between infants BCG vaccinated at birth and at 6 weeks of age Blue = Concentrations higher with BCG vaccination at birth Red = Concentrations higher with BCG at 6 weeks

	S1 5 days of age	S2 6 weeks, pre-EPI-1	S3 6 weeks, 5 days post-EPI-1 (and BCG in delayed group)	S4 10 weeks, pre-EPI-2
TNFα			***S.pneumoniae*** Overall, but more in males ***S.aureus*** Overall, but more in males	
IL-6			***S.aureus*** Overall, but more in males	**E.coli** Males only **Combined: bacterial pathogens** Males only
IL-1β			***E.coli*** Males only **Combined: all pathogens** Males only **Combined: bacterial pathogens** Males only	
IFNγ	***E.coli*** Males only **Combined: all pathogens** Males only **Combined: bacterial pathogens** Males only			
IL-10				**Poly I:C** Males only
All pro-inflammatory cytokines				
TNFα and IL-6			**Combined: bacterial pathogens** Overall, but more in males	
TNFα:IL-10 ratio				

EPI, Expanded Programme of Immunisations; BCG, Bacille Calmette Guerin; *S.pneumoniae, Streptococcus pneumoniae; S.aureus, Staphylococcus aureus; E.coli, Escherichia coli;* TNF, tumor necrosis factor; IL, interleukin; IFN, interferon; Poly I:C, polyinosinic:polycytidylic acid.

7.3.4. Correlations between stimulated cytokine production and PPD-specific responses

7.3.4.1 PPD stimulated cytokine production was strongly correlated to BCG scar size at 10 weeks

Table 7.3 shows significant correlations between PPD-stimulated cytokine production and BCG scar size measured at 10 weeks of age. IFNγ, TNFα and IL-6 production following PPD stimulation was associated with a significantly increased scar size at 10 weeks, when all infants were analysed together. These correlations were particularly strong at 6 weeks of age, with strength of association weakening by 10 weeks of age. These findings suggest that stronger acute PPD responses, particularly to IFNγ are associated with larger scar size. However, they may also be reflective of the design of the study, with infants BCG vaccinated at birth having higher PPD responses at S2 and S3 and larger scars at 10 weeks due to increased time for scar development compared to infants BCG vaccinated at 6 weeks of age. The lack of similar significant correlations when looking only at infants BCG vaccinated at birth corroborates the later explanation – suggesting PPD responses are surrogate markers for BCG vaccination, but that the level of response itself is not strongly associated with scar size at 10 weeks. Sex did not affect correlations markedly.

At S3 correlation between TNFα, IL-6 and IFNγ production in response to heterologous stimuli are also seen, with higher cytokine production to heterologous stimuli associated with larger BCG scar size at 10 weeks. Again, as infants with BCG at birth are associated with higher cytokine production at S3 compared to infants BCG vaccinated at 6 weeks (see section 7.3.3), this association may be reflecting merely the fact that infants BCG vaccinated at birth have larger scars at 10 weeks of age due to the increased time to scar development compared to BCG vaccination at 6 weeks.

Table 7.3. Correlations between stimulated cytokine production and scar size at 10 weeks of age

	All infants				BCG at birth				BCG at 6 weeks			
Correlations with scar size	**S1**	**S2**	**S3**	**S4**	**S1**	**S2**	**S3**	**S4**	**S1**	**S2**	**S3**	**S4**
TNFα												
PPD		0.39***	0.22*	0.19 (p=0.07)								
E.coli			0.23*			0.38*						
Poly I:C									-0.32*			
IL-6												
PPD		0.30**	0.33**									
S.pneumoniae								-0.44*				
Poly I:C			0.26*				0.45 **					
IFNγ												
PPD		0.53****	0.36***	0.21 (p=0.06)								
S.pneumoniae		0.27*										
S.aureus		0.33**				0.35*						
E.coli			0.24*									
Poly I:C			0.27*									

Spearman rank correlations between stimulated cytokine concentrations and BCG scar size measured at 10 weeks of age. Statistically significant data only shown with Rho value and significance level. *=p<0.05; **=p<0.01; ***=p<0.001; ****=p<0.0001. S1, 5 days of age; S2, 6 weeks of age, pre EPI-1; S3, 6 weeks of age, 5 days post-EPI-1 +/- BCG in delayed group; S4, 10 weeks of age, pre-EPI-2. BCG, Bacille Calmette Guerin; *S.pneumoniae, Streptococcus pneumoniae; S.aureus, Staphylococcus aureus; E.coli, Escherichia coli;* TNF, tumor necrosis factor; IL, interleukin; IFN, interferon; Poly I:C, polyinosinic:polycytidylic acid.

7.3.4.2 PPD-induced IFNγ production was correlated to IFNγ production in response to other pathogens, and to other cytokine production in response to heterologous stimulation in infants BCG vaccinated at 6 weeks, after BCG

When all study infants were analysed together, increased IFNγ production to PPD stimulation was strongly associated with increased IFNγ production following heterologous stimulation. This occurred consistently at all time-points except for S3. This suggests that the strength of IFNγ production in response to different pathogens is relatively consistent in individual infants. At S3, although correlations with heterologous stimulated IFNγ and PPD-IFNγ were not seen, correlations with TNFα, IL-6 and IL-1β production from a variety of heterologous stimuli did occur.

When divided by BCG status, significant correlations with PPD-IFNγ and IFNγ production from heterologous stimuli were only seen in infants BCG vaccinated at birth at S2 and S4. In infants who were BCG vaccinated at 6 weeks, correlations with PPD-IFNγ and IFNγ production from heterologous stimuli were only seen at S1 and S2 (i.e. prior to BCG vaccination). Conversely, these infants had consistent and strong correlations with stimulated TNFα, IL-6, IL-1β and IL-10 production in response to various heterologous stimuli only at S3 and S4 (after BCG vaccination).

It is interesting that the only time-point where significant correlations between BCG-specific IFNγ production and non-specific IFNγ production were not seen (S3) was the time-point when there were significant differences between BCG vaccinated and un-vaccinated infants.

Table 7.4 Correlations between BCG-specific stimulated responses and non-specific cytokine responses to heterologous stimuli

	All infants				BCG at birth				BCG at 6 weeks			
Correlations with PPD-IFNγ response	**S1**	**S2**	**S3**	**S4**	**S1**	**S2**	**S3**	**S4**	**S1**	**S2**	**S3**	**S4**
TNFα												
S.pneumoniae			0.29**						0.27*		0.28*	
S.aureus			0.20 (p=0.06)									
E.coli	0.24*		0.23*			0.55****		0.37*	0.31*			
Poly I:C	0.20*		0.22*								0.36*	
C. albicans											0.56****	
IL-6												
S.pneumoniae			0.25*								0.30*	0.34*
S.aureus			0.22 (p=0.05)	0.24*								0.35*
E.coli				0.25*								0.34*
Poly I:C		0.17*										
C. albicans											0.28*	
IL-1β												
S.pneumoniae											0.29*	0.29*
E.coli			0.25*	0.33**		0.30*		0.38**		-0.37**		0.32*
Poly I:C											0.32*	
C.albicans											0.46***	
IL-10												
S.pneumoniae											0.32*	0.41**
S.aureus				0.27**								0.41**
Poly I:C								-0.41**			0.31*	
C.albicans								-0.35*			0.41**	0.37**
IFNγ												
S.pneumoniae	0.30**	0.28**		0.23*		0.52***		0.53***	0.39**	0.30*		
S.aureus	0.32***	0.31**		0.28**		0.46**		0.54***	0.43**	0.34*		
E.coli	0.24**			0.27**		0.34*		0.50***	0.32*			
Poly I:C	0.34***	0.23*		0.32**		0.33*		0.46**	0.42*	0.37**		
C.albicans	0.21*			0.25*				0.45**	0.31*		0.34*	

Spearman rank correlations between stimulated cytokine concentrations and BCG-specific stimulated responses (PPD-induced IFNγ production). Statistically significant data only shown, with Rho value and significance level. *=p<0.05; **=p<0.01; ***=p<0.001; ****=p<0.0001. S1, 5 days of age; S2, 6 weeks of age, pre EPI-1; S3, 6 weeks of age, 5 days post-EPI-1 +/- BCG in delayed group; S4, 10 weeks of age, pre-EPI-2. BCG, Bacille Calmette Guerin; *S.pneumoniae, Streptococcus pneumoniae; S.aureus, Staphylococcus aureus; E.coli, Escherichia coli;* TNF, tumor necrosis factor; IL, interleukin; IFN, interferon; Poly I:C, polyinosinic:polycytidylic acid.

7.3.4.3 No clear correlations between specific or non-specific stimulated cytokine production and infection, by BCG status, were seen

When all infants were analysed together, higher cytokine production following heterologous stimulation with non-specific pathogens at S1 and S2 was associated with greater incidence of infections (see Table 7.5). As these were the only significant correlations it is impossible to determine whether the increased stimulated cytokine responses were a result of having had more infections, or whether a propensity to greater cytokine responses increased the likelihood of clinically apparent infections. Of note, lower production of IL-6 and IFNγ in response to *E.coli* at S3 was also shown. This pattern, of a change in direction of responses at S3, is similar to that seen in the cytokine data and may suggest that the associations of heterologous stimulated cytokine responses and infections may simply be displaying the effects of BCG, rather than suggesting a causal link. Clear patterns of correlations were harder to assess when looking by BCG status, but infants vaccinated with BCG at 6 weeks had a tendency to higher incidence of infection with higher heterologous stimulated cytokines at S1 and S2 and reduced stimulated cytokine responses at S3. These findings did not occur in infants who were BCG vaccinated at birth.

Table 7.5 Correlations between infection incidence and *in vitro* stimulated cytokine responses

	All infants				BCG at birth				BCG at 6 weeks			
Correlations with infections	**S1**	**S2**	**S3**	**S4**	**S1**	**S2**	**S3**	**S4**	**S1**	**S2**	**S3**	**S4**
TNFα												
S.pneumoniae		0.26**		0.21**						0.32*		
S.aureus	0.20*										-0.30*	
C. albicans	0.22*	0.24*			0.38**	0.36**						
IL-6												
S.pneumoniae								0.43**	0.32*			
E.coli			-0.21*				0.46**					
C. albicans								0.38*				
IL-1β												
S.aureus		0.22*										
Poly I:C	0.22*	0.21*										
IL-10												
S.aureus		0.27**										
E.coli	0.24*											
Poly I:C	0.19*	0.22*										
IFNγ												
PPD									0.39**			
S.aureus									0.28*		-0.28*	
E.coli			-0.23*								-0.29*	

Spearman rank correlations between stimulated cytokine concentrations and BCG scar size measured at 10 weeks of age. Statistically significant data only shown with Rho value and significance level. *=p<0.05; **=p<0.01; ***=p<0.001; ****=p<0.0001. S1, 5 days of age; S2, 6 weeks of age, pre EPI-1; S3, 6 weeks of age, 5 days post-EPI-1 +/- BCG in delayed group; S4, 10 weeks of age, pre-EPI-2. BCG, Bacille Calmette Guerin; *S.pneumoniae, Streptococcus pneumoniae; S.aureus, Staphylococcus aureus; E.coli, Escherichia coli;* TNF, tumor necrosis factor; IL, interleukin; IFN, interferon; Poly I:C, polyinosinic:polycytidylic acid.

7.3.5 Within-infant changes in cytokine production over time in response to *in vitro* heterologous pathogen stimulation

Infants were randomised to two of the four post-natal blood sampling time-points to allow within-infant changes to stimulated cytokine production over time to be investigated as secondary outcomes (Tables 2.5.1-2.6.5, Appendix 9). It was hoped that this would allow for some of the high variability in cytokine responses known to occur in infant samples, and therefore increase the power to detect significant differences between vaccination groups. However, sample sizes for this analysis were small, particularly when analysed by sex (see Table 2.1.2 for exact numbers), often with n<10 per group. Although some within-infant differences in heterologous stimulated cytokine production by BCG group reached statistical significance, the large number of comparisons due to multiple stimuli, multiple cytokines and overlapping time-points, made patterns of change difficult to detect. Therefore, I limited the use of within-infant change data to answering specific questions presented by the epigenetic and cross-sectional cytokine data, namely:

1. Does the reduction in the constitutive increase of H3K4me3 and H3K9me3 at TNFα, and possibly IL-6 and IL-1β promoters, between cord blood and 6 weeks (shown in the epigenetic sub-study), result overall in increased or decreased production of these cytokines in response to heterologous stimuli?
2. Are the significant differences in pro-inflammatory cytokine production seen at S3 due to an extension of what is happening at S2 (e.g. the prolonged effect of BCG given at birth, as BCG at 6 weeks had not started to have an effect) or a reversal (e.g. an early significant effect of BCG at 6 weeks or an interaction with EPI-1)?

In exploring these questions, comparisons of differences in the geometric mean cytokine concentrations per time-point were made. The two ways of measuring changes over time have different strengths and limitations (within-infant changes reduce the effect of inter-individual variability in parameters, but the low participant numbers limit the power to detect differences, while changes to mean cytokine concentrations between different time-points are affected by individual variability in responses, but the larger number of participants contributing data increases the power to detect differences). It was therefore reasoned that good agreement between the two methods would provide some reassurance that the pattern of changes seen were reflective of true changes. Again, due to small numbers, multiple testing, and the limited statistical significance,

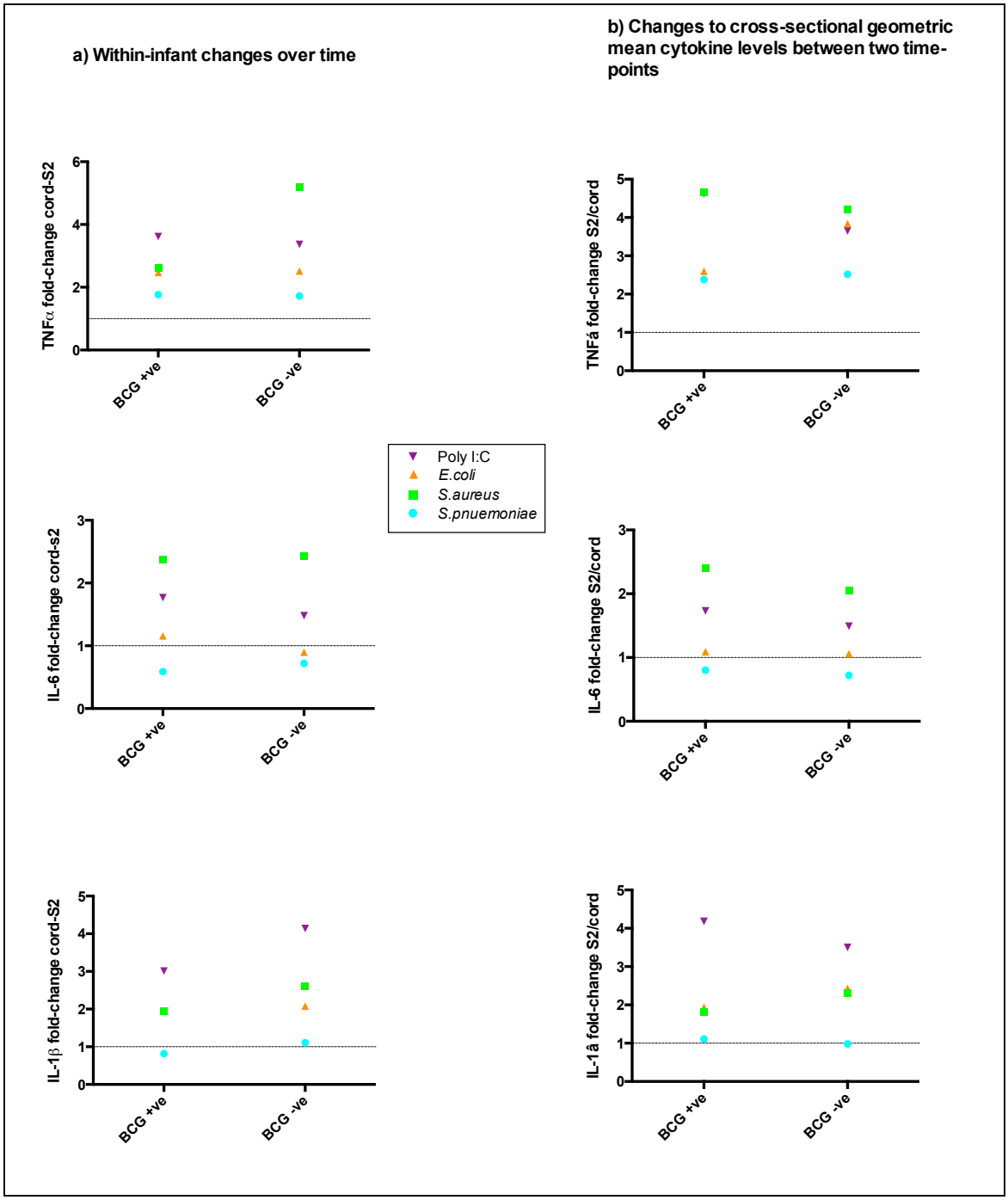
these results should be viewed as exploratory/hypothesis generating only, rather than conclusive findings.

7.3.5.1 TNFα production in response to heterologous pathogens over the first 6 weeks of life tended to be reduced in BCG vaccinated infants but increased in unvaccinated infants, particularly in boys

Exploratory analysis of within-infant changes in TNFα, IL-6 and IL-1β production following heterologous stimulation between cord blood and 6 weeks of age was conducted to investigate whether the suppression of constitutive H3K4me3 and H3K9me3 increases over time induced by BCG vaccination results in an increased or decreased pro-inflammatory bias (Figure 7.19). When analysed together, or by sex (data not shown), no clear differences in the change in cytokine concentrations between infants BCG vaccinated at birth and BCG vaccinated at 6 weeks were seen.

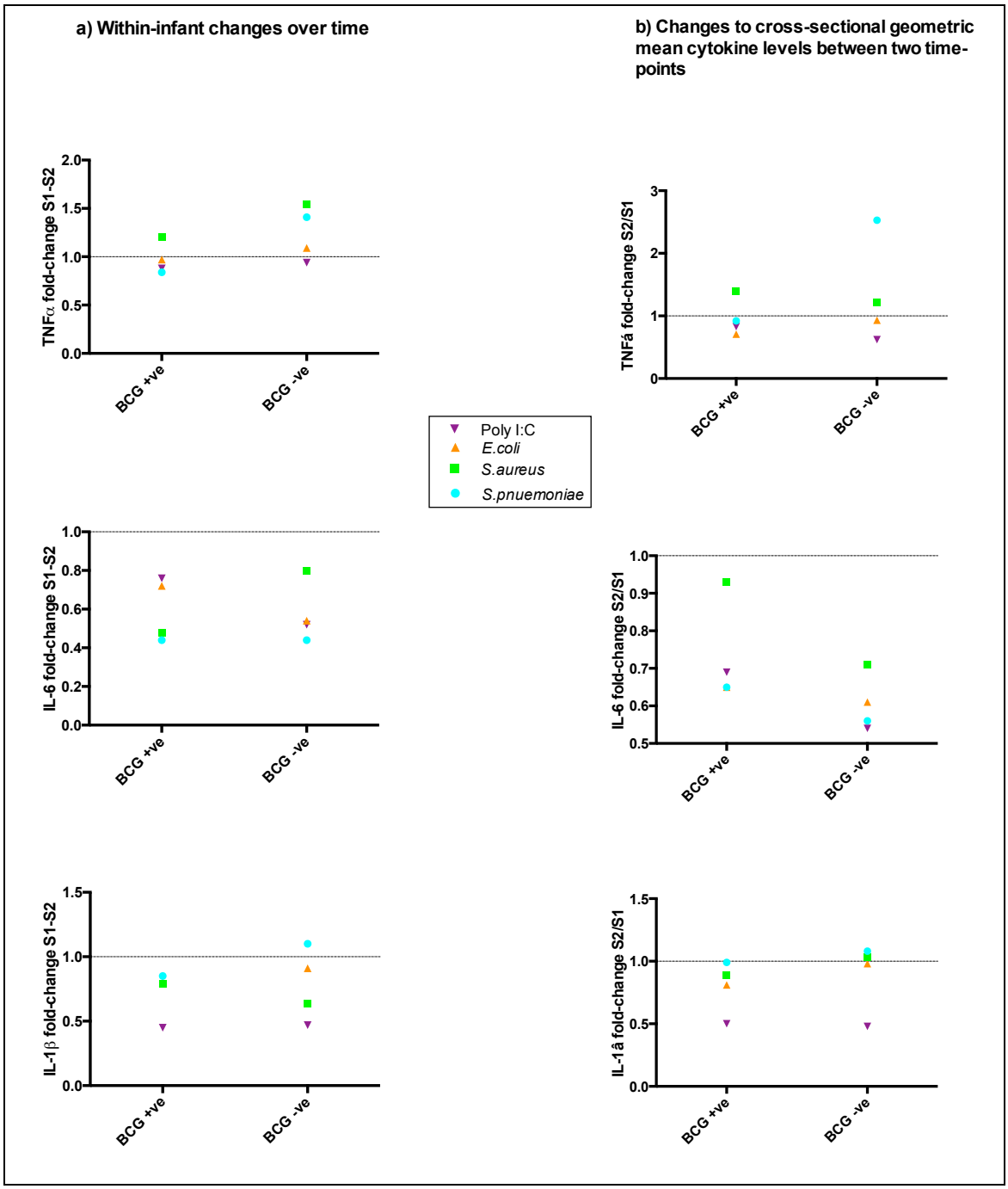
This may indicate that the BCG-induced changes to epigenetic modification occurring between birth and 6 weeks do not translate to measurable changes in cytokine production following *in vitro* heterologous pathogen challenge. However, the use of cord blood as a proxy for pre-vaccination infant blood samples, may not be valid as cytokine levels might be affected by events during labour.[291] As PPD responses to BCG were not increased by 5 days of age (see section 7.3.2), it could be argued that the epigenetic modifications induced by BCG might also not have occurred by this time (and epigenetic changes were not seen after 2 weeks in adult studies[168]). This might suggest that S1 may be valid as a proxy baseline infant sample. Changes in cytokine production between 5 days post-vaccination (S1) and 6 weeks of age (S2) were, therefore, explored (Figure 7.20 and 7.21). Between these two time points, male infants who had received BCG vaccination at birth tended to reduce their production of TNFα in response to heterologous pathogens, whereas male infants who had not received BCG tended to increase TNFα production. These changes were particularly marked for bacterial pathogens. A similar, though less marked, trend was seen in female infants. This may suggest that the decreased H3K4me3 and H3K9me3 at the promoter region of TNFα seen between cord blood and S2, led to a reduction in TNFα production upon heterologous pathogen challenge. However, none of the changes were statistically significantly different between the two intervention groups, so results should be viewed as hypothesis-generating only.

Figure 7.19. Fold-change in stimulated cytokine production from cord blood to 6 weeks of age, pre-EPI-1, by BCG status



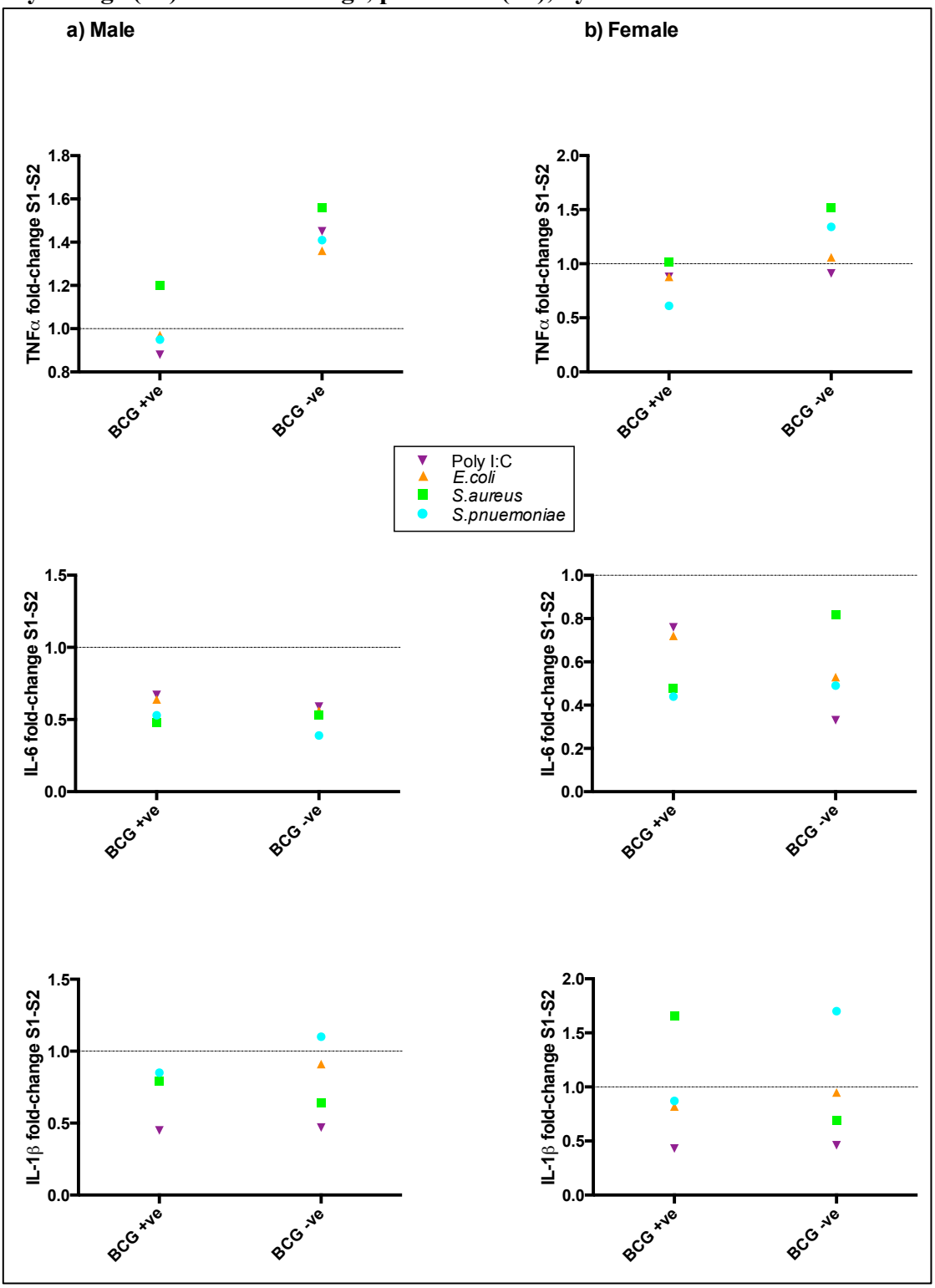
Results are displayed as geometric mean fold-change in within-infant changes over time (Figure 7.19a) and fold-change in the cross-sectional geometric means of the two time-points (Figure 7.19b). Fold-change was calculated as the later time-point/earlier time-point, therefore 1 indicates equivalence. Change <1 shows a reduction in stimulated cytokine production over time, >1 shows an increase in production over time. Within-infant changes n≥44, cross-sectional changes n≥45 S2, 6 weeks of age, pre-EPI vaccinations; EPI, Expanded Programme of Immunisations; BCG, Bacille Calmette Guerin; IL, interleukin; TNF, tumor necrosis factor; Poly I:C, polyinosinic:polycytidylic acid; *E.coli, Escherichia coli; S. aureus, Staphylococcus aureus; S.pneumoniae, Streptococcus pneumonia.*

Figure 7.20. Fold-change in stimulated cytokine production from 5 days of age (S1) to 6 week of age, pre-EPI-1 (S2), by BCG status



Results are displayed as geometric mean fold-change in within-infant changes over time (Figure 7.20a) and fold-change in the cross-sectional geometric means of the two time-points (Figure 7.20b). Fold-change was calculated as the later time-point/earlier time-point, therefore 1 indicates equivalence. Change <1 shows a reduction in stimulated cytokine production over time, >1 shows an increase in production over time. Within-infant changes n≥15, cross-sectional changes n≥43. S1, 5 days of age; S2, 6 weeks of age, pre-EPI vaccinations; EPI, Expanded Programme of Immunisations; BCG, Bacille Calmette Guerin; IL, interleukin; TNF, tumor necrosis factor; Poly I:C, polyinosinic:polycytidylic acid; *E.coli, Escherichia coli; S. aureus, Staphylococcus aureus; S.pneumoniae, Streptococcus pneumonia.*

Figure 7.21. Within-infant fold-change in stimulated cytokine production from 5 days of age (S1) to 6 week of age, pre-EPI-1 (S2), by BCG status and sex



Geometric mean fold-change in within-infant changes over time. Fold-change was calculated as the later time-point/earlier time-point, therefore 1 indicates equivalence. Change <1 shows a reduction in stimulated cytokine production over time, >1 shows an increase in production over time. Male within-infant changes n≥8, female within-infant changes n≥7. S1, 5 days of age; S2, 6 weeks of age, pre-EPI vaccinations; EPI, Expanded Programme of Immunisations; BCG, Bacille Calmette Guerin; IL, interleukin; TNF, tumor necrosis factor; Poly I:C, polyinosinic:polycytidylic acid; *E.coli, Escherichia coli; S. aureus, Staphylococcus aureus; S.pneumoniae, Streptococcus pneumonia.*

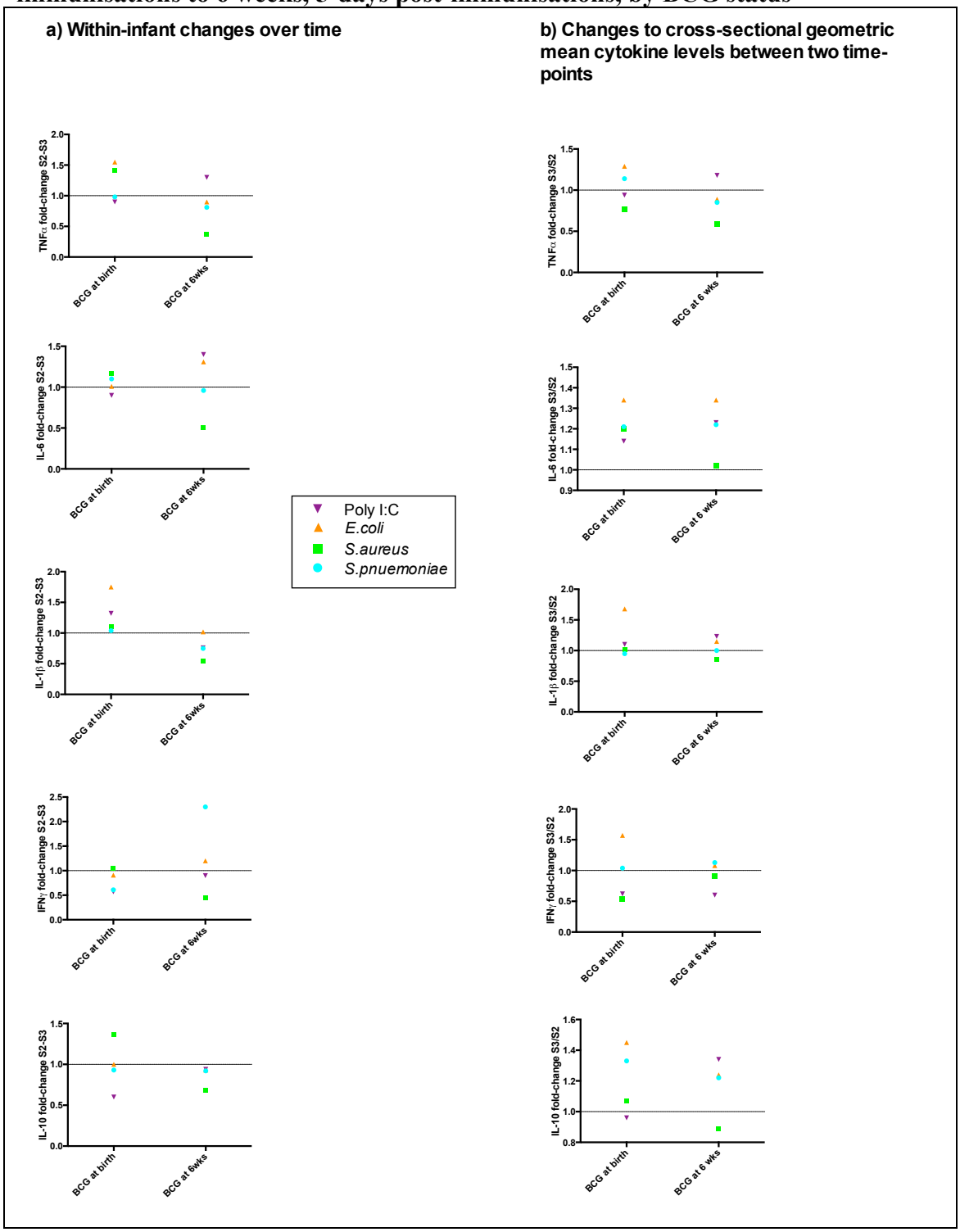
7.3.5.2 The effect of BCG and routine-EPI vaccinations at 6 weeks on changes to cytokine production in response to heterologous stimuli

Changes to cytokine production induced by the receipt of BCG at 6 weeks of age in the delayed group, and routine immunisations in all of the infants, were assessed by comparing S2 (6 week pre-immunisations) and S3 (6 weeks, 5 days post-immunisations) blood sampling time points. These are shown in Figure 7.22. When analysed together, a very slight tendency toward lower TNFα and IL-1β production following BCG vaccination at 6 weeks was suggested. When analysed by sex, this reduction was shown to be pronounced in male infants (Figure 7.23) and particularly so for the production of TNFα, IL-6 and IL-1β following bacterial stimulants (Figure 7.24).

The within-infant changes suggest that following receipt of routine immunisations, infants who were BCG vaccinated at birth had an increase in pro-inflammatory cytokine production to bacterial stimuli (fold-change >1). Infants who received BCG with routine immunisations appeared to have had reduced pro-inflammatory cytokine production in response to bacterial stimulation (fold-change <1). Interestingly, this is the same trend as following BCG at birth, i.e. BCG as last vaccination appeared to suppress the production of TNFα, in particular, but possibly also of IL-6 and IL-1β, in response to heterologous stimuli in male infants. It should be noted that the timing of these changes was different though (with no effects of BCG at birth seen at 5 days of age), and an interaction with routine-EPI vaccinations cannot be ruled out.

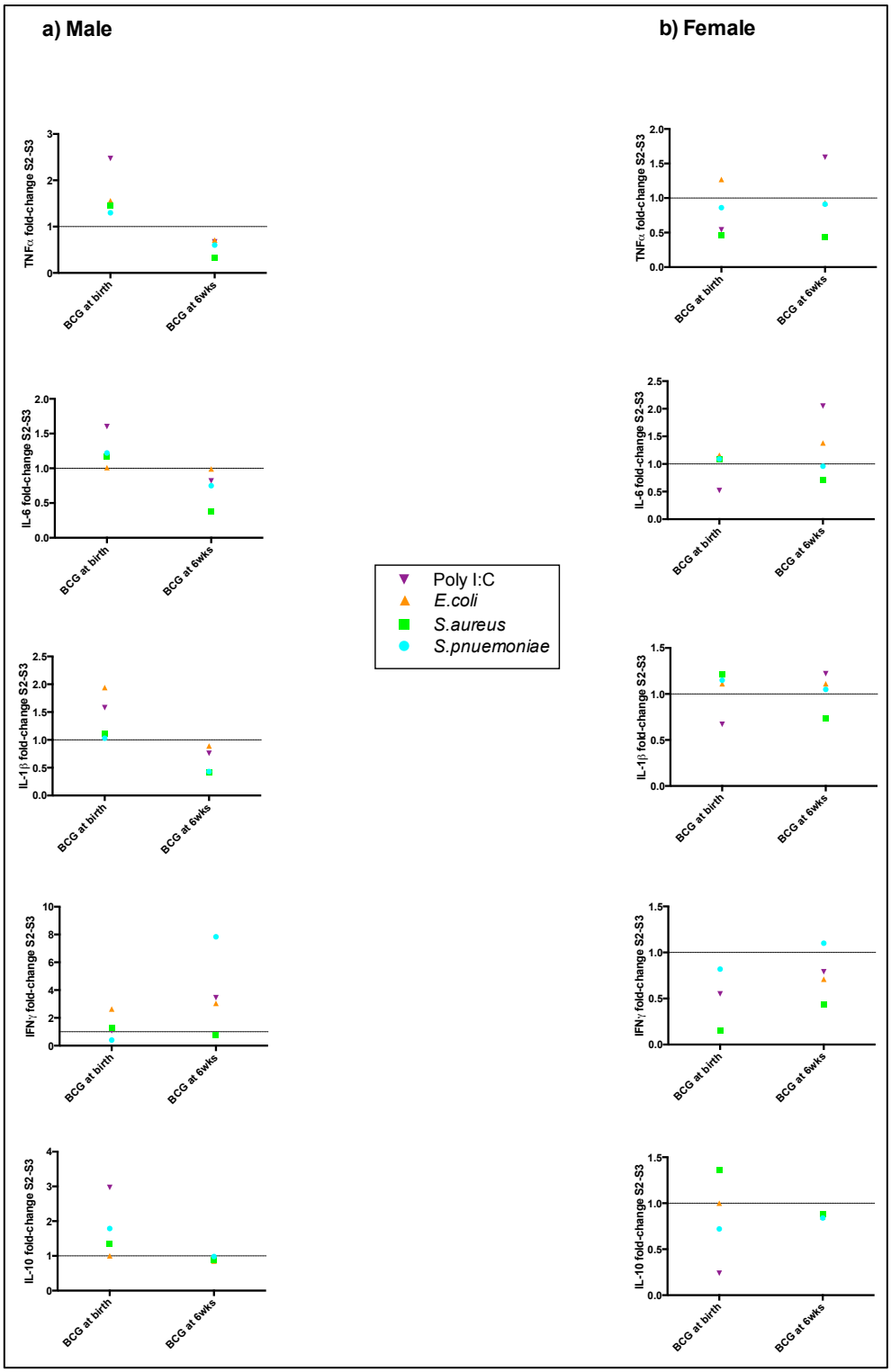
To explore the duration of the effects of delayed BCG vaccination, heterologous cytokine production before immunisations were received at 6 weeks (S3) was compared with production at 10 weeks of age (S4). No clear trends consistent between within-infant changes and changes in geometric mean cytokine data, either overall or by sex, were obvious (data not shown).

Figure 7.22. Fold-change in stimulated cytokine production from 6 weeks pre-immunisations to 6 weeks, 5-days post-immunisations, by BCG status



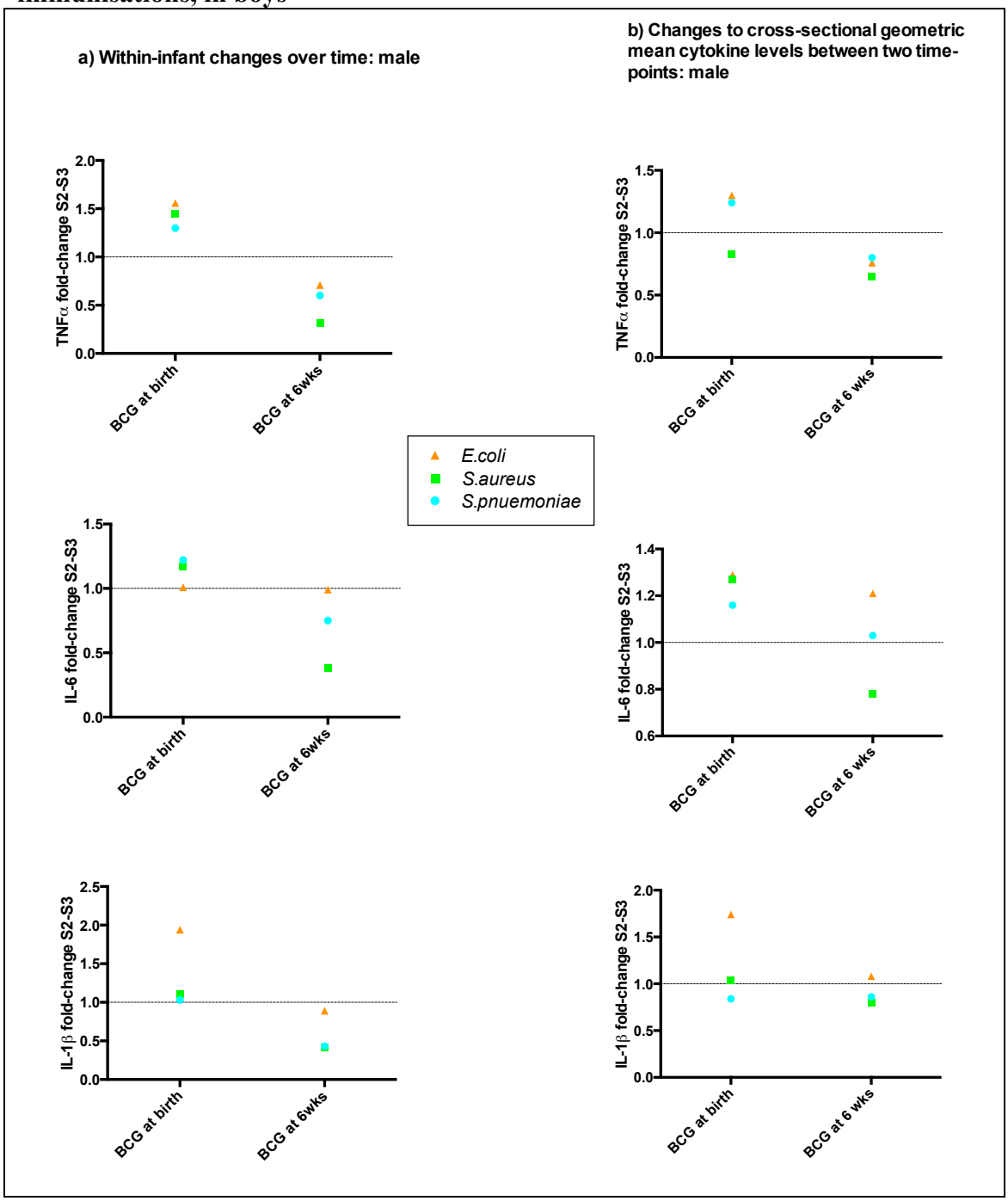
Results are displayed as geometric mean fold-change in within-infant changes over time (Figure 7.22a) and fold-change in the cross-sectional geometric means of the two time-points (Figure 7.22b). Fold-change was calculated as the later time-point/earlier time-point, therefore 1 indicates equivalence. Change <1 shows a reduction in stimulated cytokine production over time, >1 shows an increase in production over time. Within-infant changes n≥10, cross-sectional changes n≥39. S2, 6 weeks of age, pre-EPI vaccinations; S3, 6 weeks of age 5 days post-EPI-1/BCG in the delayed group; EPI, Expanded Programme of Immunisations; BCG, Bacille Calmette Guerin; IL, interleukin; TNF, tumor necrosis factor; Poly I:C, polyinosinic:polycytidylic acid; *E.coli, Escherichia coli; S. aureus, Staphylococcus aureus; S.pneumoniae, Streptococcus pneumonia.*

Figure 7.23. Within-infant fold-change in stimulated cytokine production from 6 weeks pre-immunisations to 6 weeks, 5-days post-immunisations, by BCG status and sex



Geometric mean fold-change in within-infant changes over time. Fold-change was calculated as the later time-point/earlier time-point, therefore 1 indicates equivalence. Change <1 shows a reduction in stimulated cytokine production over time, >1 shows an increase in production over time. Male within-infant changes n≥6, female within-infant changes n≥4. S1, 5 days of age; S2, 6 weeks of age, pre-EPI vaccinations; EPI, Expanded Programme of Immunisations; BCG, Bacille Calmette Guerin; IL, interleukin; TNF, tumor necrosis factor; IFN, interferon; Poly I:C, polyinosinic:polycytidylic acid; *E.coli, Escherichia coli; S. aureus, Staphylococcus aureus; S.pneumoniae, Streptococcus pneumonia.*

Figure 7.24. Fold-change in pro-inflammatory cytokine production in response to bacterial stimulation from 6 weeks pre-immunisations to 6 weeks, 5-days post-immunisations, in boys



Results are displayed as geometric mean fold-change in within-infant changes over time (Figure 7.24a) and fold-change in the cross-sectional geometric means of the two time-points (Figure 7.24b). Fold-change was calculated as the later time-point/earlier time-point, therefore 1 indicates equivalence. Change <1 shows a reduction in stimulated cytokine production over time, >1 shows an increase in production over time. Within-infant changes n≥6, cross-sectional changes n≥18. S2, 6 weeks of age, pre-EPI vaccinations; S3, 6 weeks of age 5 days post-EPI-1/BCG in the delayed group; EPI, Expanded Programme of Immunisations; BCG, Bacille Calmette Guerin; IL, interleukin; TNF, tumor necrosis factor; Poly I:C, polyinosinic:polycytidylic acid; *E.coli, Escherichia coli; S. aureus, Staphylococcus aureus; S.pneumoniae, Streptococcus pneumonia.*

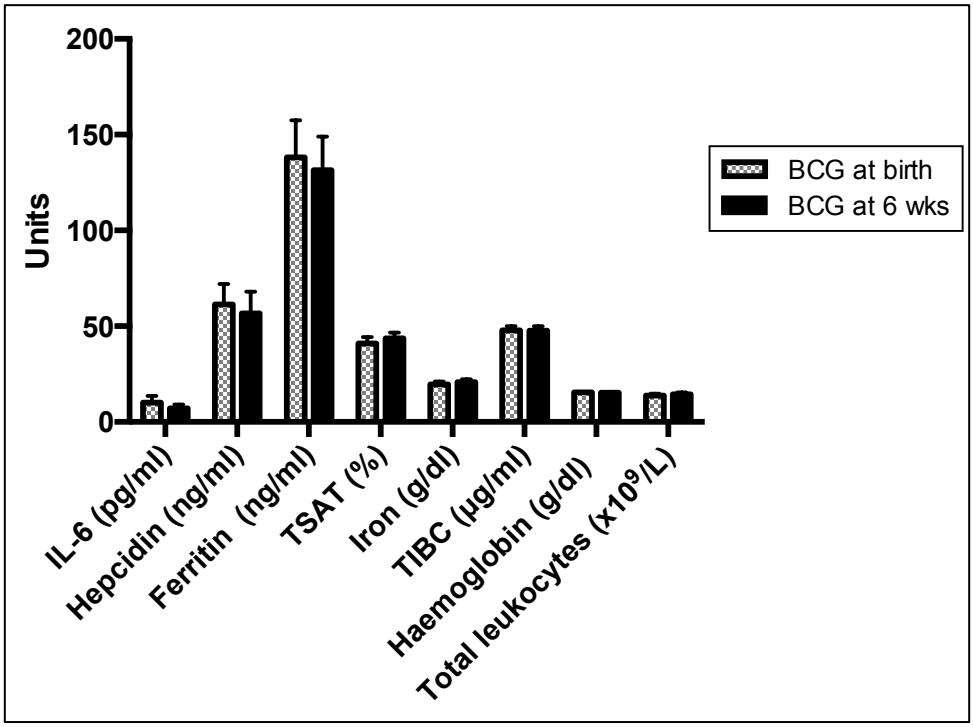
7.4 Iron sub-study

The iron sub-study was conducted to compare components of the inflammatory-iron axis following *in vivo* heterologous stimulation with EPI vaccinations, between infants BCG vaccinated at birth and infants BCG vaccinated at 6 weeks of age. Iron parameters, cell counts and red cell indices were measured by automated analyser, with IL-6 and hepcidin levels measured by ELISA, as described in Methods section 6.3.1.1.

7.4.1 Baseline samples

There were no significant differences in inflammatory-iron parameters in cord blood samples between the two intervention groups (Figure 7.25, and Tables 3.2.1 and 3.2.2, Appendix 9). This suggests that randomisation occurred appropriately.

Figure 7.25 Inflammatory-iron parameters in cord blood samples by BCG randomisation group



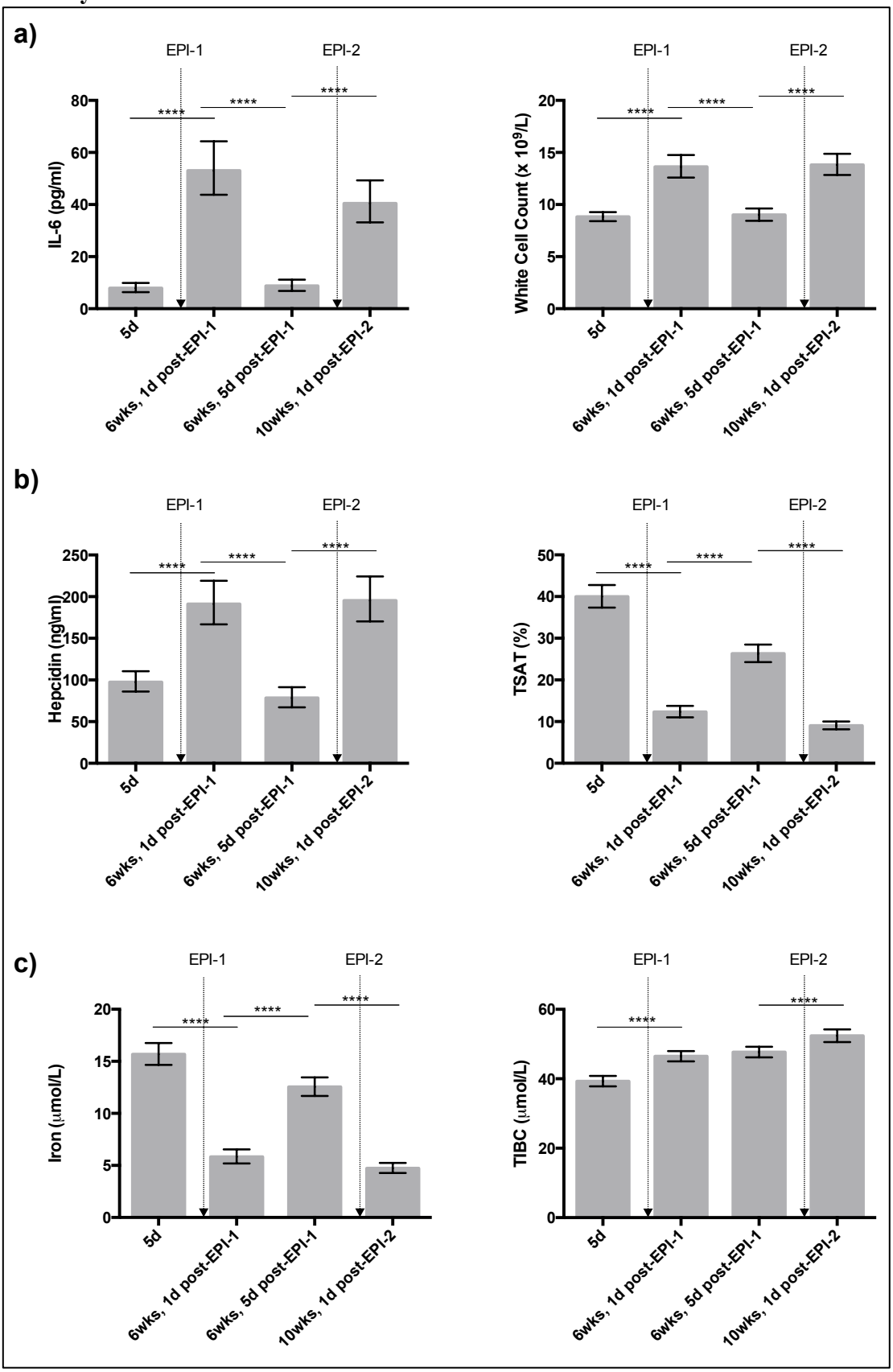
Geometric mean (IL-6, hepcidin, ferritin, TSAT, iron, TIBC) or arithmetic mean (haemoglobin, total leukocytes) and standard error of the mean for parameters in cord blood. BCG vaccination at birth n≥113, BCG vaccination at 6 weeks n≥118. For exact numbers tested for each parameter see Table 3.1.1, Appendix 9. BCG, Bacille Calmette Guerin; IL, interleukin; TSAT, transferrin saturation; TIBC, total iron binding capacity; wks, weeks.

7.4.2 Routine immunisations act as acute *in vivo* inflammatory stimuli and stimulate the inflammatory-iron axis, confirming pilot study findings

Figure 7.26 shows the changes to geometric means (all infants) induced by EPI vaccinations. Routine immunisations at both 6 weeks and 10 weeks of age resulted in a large increase in IL-6 and white blood cell counts (primarily neutrophils) (Figure 7.26a)). These had returned to pre-immunisation levels by 5 days post-routine immunisations. The increased IL-6 was associated with increased hepcidin levels post-EPI vaccinations, and a consequent reduction in serum TSAT (Figure 7.2.6b)). The reduction in TSAT appeared to be mediated by a reduction in serum iron rather than an acute increase in serum transferrin levels, which showed no significant change following EPI-1 but rather showed a general trend to increase over the first 10 weeks of life (Figures 7.26c)).

These findings provide good evidence that EPI vaccinations act as strong pro-inflammatory stimuli with resultant induction of the inflammatory-iron axis. These effects appear to be short-lived, having largely returned to baseline by 5 days post-EPI receipt. These findings confirm the pilot study findings and support the use of EPI-immunisations as an *in vivo* non-specific stimulant in this thesis. A detailed discussion of the white cell count changes following routine immunisations, and its implications for the management of the febrile young infant, can be found in Paper 4 at the end of this chapter.

Figure 7.26 The effect of EPI-vaccinations on inflammation and iron parameters, all study infants



Geometric means and standard errors of inflammatory-iron parameters measured before and after *in vivo* non-specific stimulation with EPI vaccinations. IL-6 and hepcidin were measured by competitive ELISA. Iron, TSAT, TIBC and WBCs were measured by automated analysers. EPI, Expanded Programme of Immunisations; IL, interleukin; TSAT, transferrin saturation; TIBC, total iron binding capacity; d, days; wks, weeks; *=p<0.05; **=p<0.01; ***=p<0.001; ****=p<0.0001

7.4.3 Cross-sectional comparison of geometric mean inflammatory-iron parameters by BCG status

7.4.3.1 IL-6 concentrations were significantly increased at 6 weeks of age (5 days after EPI-1 vaccinations/BCG in the delayed group (S3)) in infants BCG vaccinated at birth compared to infants BCG vaccinated at 6 weeks of age.

The GMR of components of the inflammatory-iron axis, comparing BCG vaccination at birth with vaccination at 6 weeks, are shown in Figure 7.27. At time-point S3 (6 weeks of age, 5 days after EPI-1 and 4 days after BCG vaccination in the delayed group), IL-6 was significantly higher in infants BCG vaccinated at birth (GMR 1.57 (1.02-2.41), p=0.04). There was a similar trend toward higher ferritin and hepcidin levels, although these did not reach statistical significance when male and female infants were analysed together. No associated differences in TSAT, iron or transferrin were seen.

No significant differences in GMR by BCG status, or any clear patterns of changes, were seen at any other blood sampling time-points. Of note, IL-6 at S2 showed a trend toward being lower in infants BCG vaccinated at birth but this did not reach statistical significance (GMR 0.72 (0.50-1.04) p=0.08) and was not reflected by lower hepcidin and ferritin.

Although the difference in IL-6 at S3 was statistically significant, it reflects a small difference in actual geometric mean IL-6 levels (11.41pg/ml in infants with BCG at birth, 7.00pg/ml in infants with BCG at 6 weeks).

7.4.3.2 Higher IL-6 at S3 in infants BCG vaccinated at birth was significant only in male infants, who also showed higher hepcidin and ferritin levels

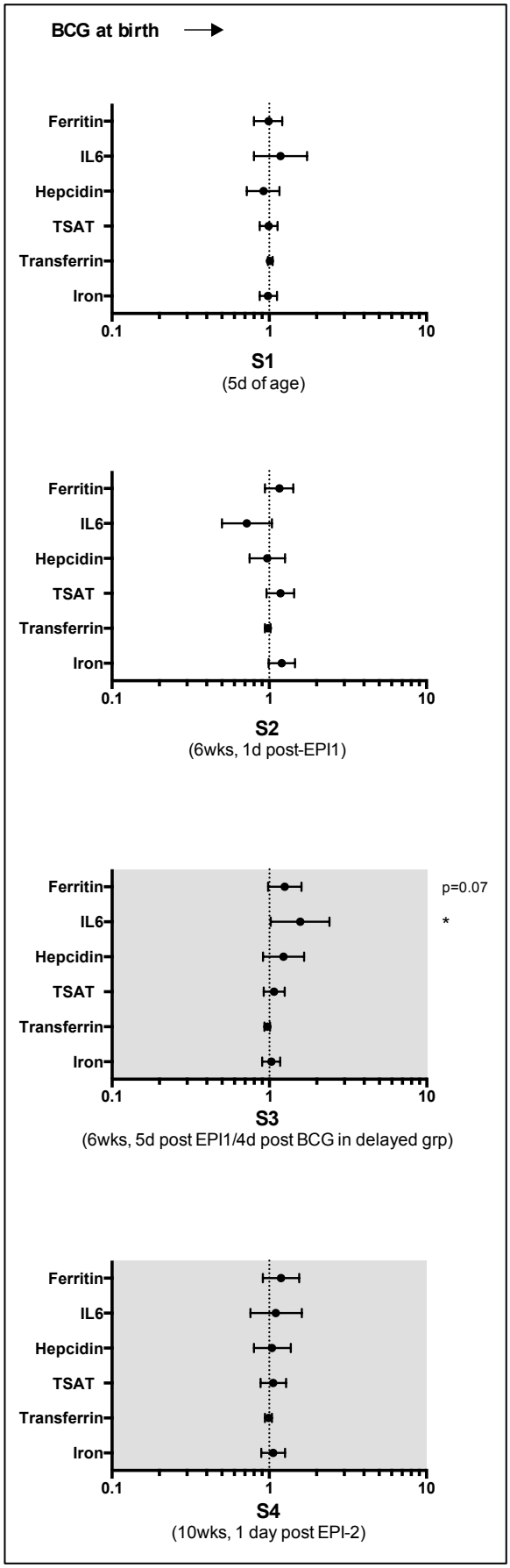
When GMR of components of the inflammatory-iron axis by BCG status were analysed by sex, the effects seen at S3 were more pronounced in male infants (Figure 7.28, Table 3.2.1 Appendix 9).

Male infants BCG vaccinated at birth had significantly higher IL-6, hepcidin and ferritin levels than male infants BCG vaccinated at 6 weeks (GMR 1.93 (1.03-3.63), p=0.04, 1.85 (1.17-2.91), p=0.009 and 1.61 (1.14-2.29), p=0.008 respectively). The expected decreases in TSAT and iron levels with increased hepcidin were not seen, however, with a tendency toward higher levels and significantly lower transferrin. Although at S3 the geometric mean hepcidin level in boys BCG vaccinated at birth is nearly double that

of boys BCG vaccinated at 6 weeks (90.25ng/ml vs. 48.06ng/ml), both values are within the normal range of hepcidin for infants.[273] The difference might not be sufficient to stimulate clinically relevant changes to TSAT and iron and, perhaps, may serve more as a marker of inflammatory state in general, rather than acute induction of the inflammatory-iron axis.

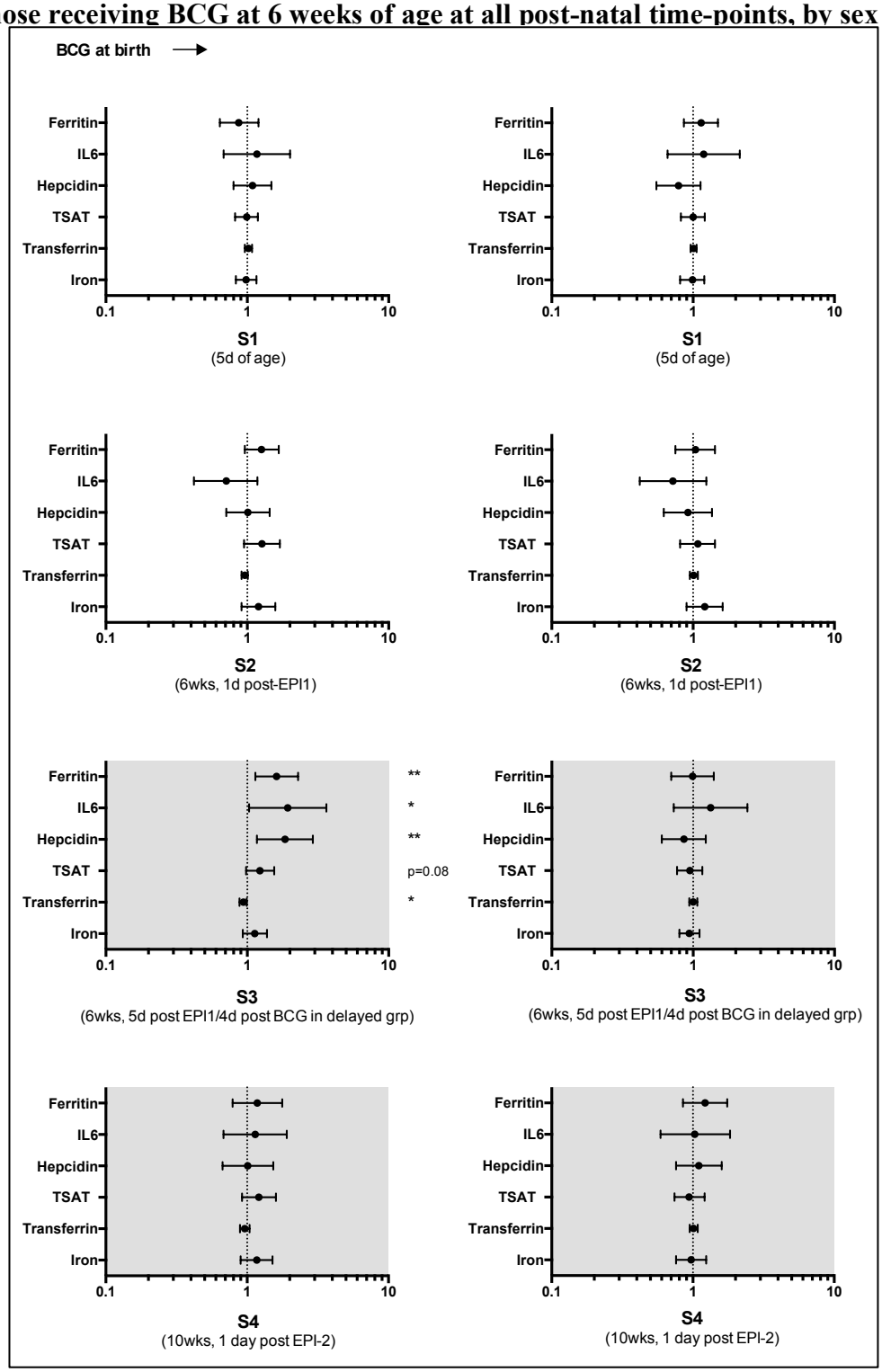
Female infants did not show the same significant differences in GMR of components of the inflammatory-iron axis by BCG status at S3 as males. IL-6 levels showed the same trend toward being lower with BCG vaccination at birth (GMR 1.33 (0.73-2.42), p=0.35), but this did not reach statistical significance. Hepcidin and ferritin levels showed a non-significant trend toward being lower in girls BCG vaccinated at birth. This trend was significantly different to that seen in boys; test for interaction p=0.009 and p=0.05, respectively.

Figure 7.27. Ratios of iron parameters by BCG status, all post-natal time-points



Ratios of geometric mean concentrations of iron parameters, infants BCG vaccinated at birth: infants BCG vaccinated at 6 weeks of age. IL-6 and hepcidin were measured by competitive ELISA. Iron, transferrin TSAT and ferritin were measured using automated Cobas Integra. N≥41 per group. Exact numbers for each parameter and time-point can be found in Table 3.1.1 Appendix 9. A ratio of 1 indicates equivalence. Ratios >1 show higher levels in infants BCG vaccinated at birth, ratios <1 show higher levels in infants BCG vaccinated at 6 weeks. BCG, Bacille Calmette Guerin; EPI, Expanded programme of immunisations; IL, interleukin; TSAT, transferrin saturation. *=p<0.05; **=p<0.01; ***=p<0.001; ****=p<0.0001. Note: the clear background denotes that comparisons for the time-point are between BCG vaccinated infants and BCG unvaccinated infants. Grey backgrounds denote that comparisons at this time-point are between infants BCG vaccinated at birth and infants BCG vaccinated at 6 weeks.

Figure 7.28. Ratios of iron parameters comparing infants receiving BCG at birth to those receiving BCG at 6 weeks of age at all post-natal time-points, by sex



Ratios of geometric mean concentrations of iron parameters, infants BCG vaccinated at birth: infants BCG vaccinated at 6 weeks of age, separated by sex. IL-6 and hepcidin were measured by competitive ELISA. Iron, transferrin TSAT and ferritin were measured using automated Cobas Integra. N≥39 per group. Exact numbers for each parameter and time-point can be found in Table 3.1.1 Appendix 9. A ratio of 1 indicates equivalence. Ratios >1 show higher levels in infants BCG vaccinated at birth, ratios <1 show higher levels in infants BCG vaccinated at 6 weeks. BCG, Bacille Calmette Guerin; EPI, Expanded programme of immunisations; IL, interleukin; TSAT, transferrin saturation. *=p<0.05; **=p<0.01; ***=p<0.001; ****=p<0.0001. Note: the clear background denotes that comparisons for the time-point are between BCG vaccinated infants and BCG unvaccinated infants. Grey backgrounds denote that comparisons at this time-point are between infants BCG vaccinated at birth and infants BCG vaccinated at 6 weeks.

7.4.4 Cross-sectional comparison of geometric mean inflammatory-iron parameters by BCG status

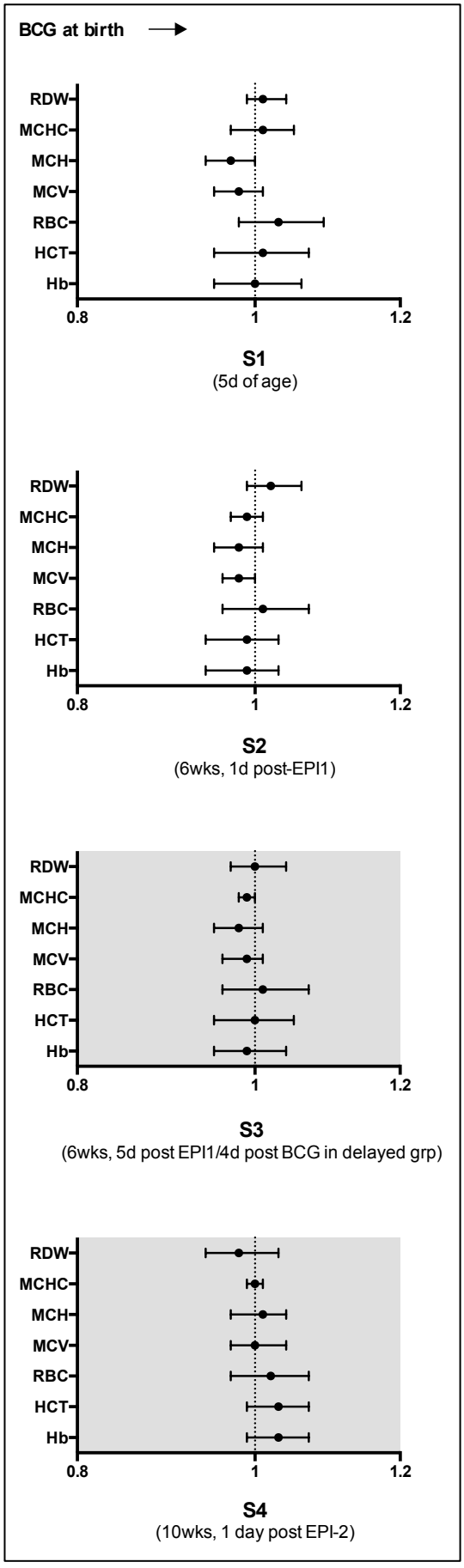
No significant impacts of BCG vaccination were seen on erythrocyte parameters at the various post-natal time points, when both sexes were analysed together (Figure 7.29).

7.4.4.1 An interaction between sex and BCG status on haemoglobin, mean cell volume, mean cell haemoglobin and mean cell haemoglobin concentration was seen at S3 and S4

When analysed by sex, significant differences in the related parameters of MCV, MCH and MCHC by BCG status were seen in female infants at S2 (6 weeks, 1 day post-EPI-1) and in male infants at S3 (6 weeks, 5 days post-EPI-1 and 4 days post BCG in the delayed group) and S4 (10 weeks, 1 days post-EPI-2) (Figure 7.30). MCV and MCH were significantly lower with BCG vaccination at birth in female infants at S2 (GMR 0.96 (0.92-1.0), p=0.02) and male infants at S3 (GMR 0.95 (0.91-0.98), p=0.007). At S4 male infants BCG vaccinated at birth had significantly higher MCH, MCHC and haemoglobin compared to infants BCG vaccinated at 6 weeks (GMRs 1.06 (1.01-1.12), p=0.02, 1.03 (1.01-1.04), p=0.0006 and 1.08 (1.01-1.14), p=0.02 respectively)). The effect of BCG on erythrocyte parameters was significantly different between sexes for MCV and MCH at S3, with tests for interaction: p=0.05 and p=0.02, and for MCV, MCH and MCHC at S4, with tests for interaction: p=0.01, p=0.01 and p<0.0001, respectively.

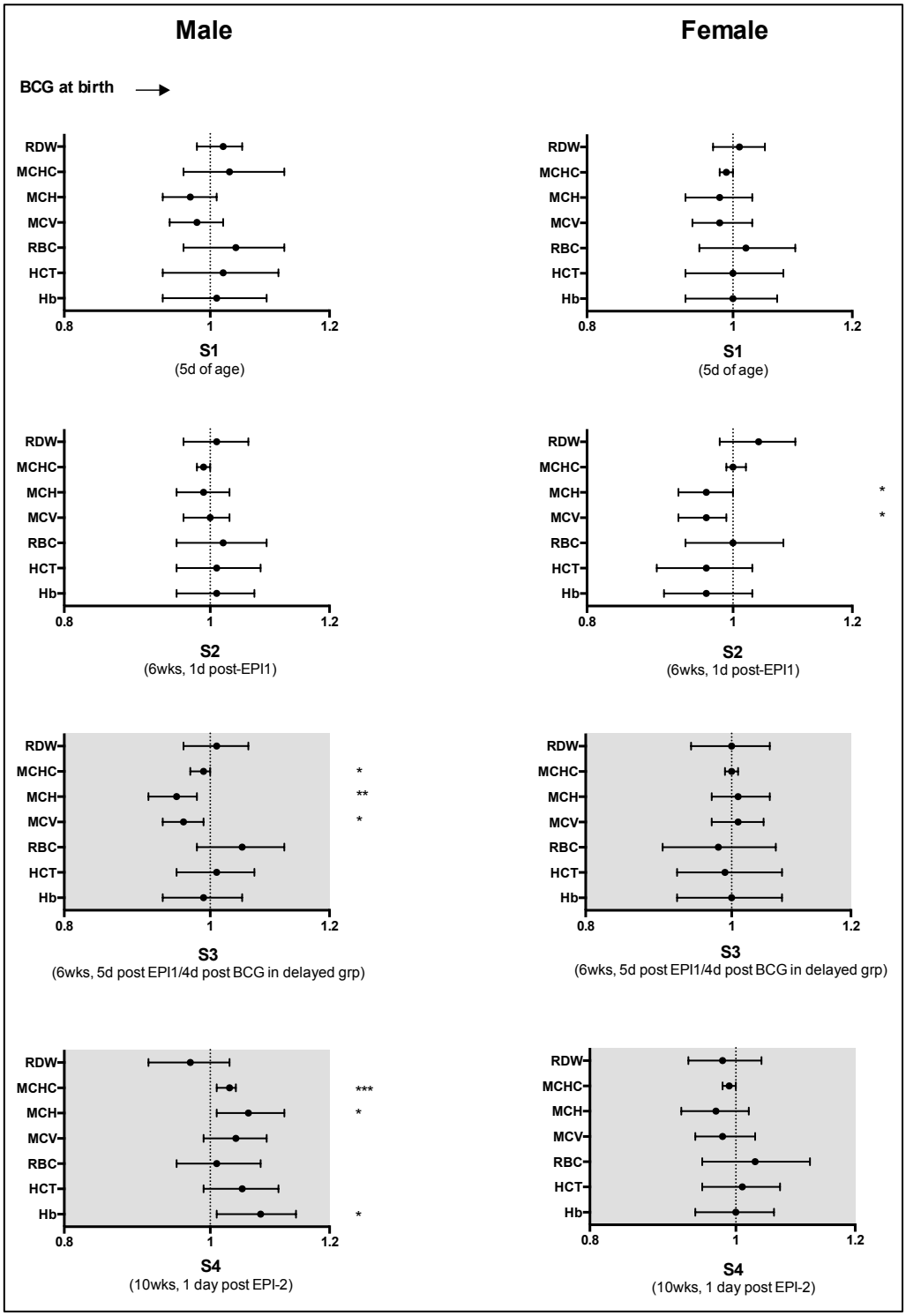
Again, although changes to some erythrocyte parameters reached statistical significance, they represent very small changes in their actual levels. For instance, the geometric mean concentrations of haemoglobin at S4 in males are 10.53g/dl with BCG vaccination at birth, and 9.79g/dl with BCG vaccination at 6 weeks, a difference for which the clinical relevance may be debatable.

Figure 7.29. Geometric mean ratios of erythrocyte parameters comparing infants BCG vaccinated at birth with infants BCG vaccinated at 6 weeks, all post-natal time-points



Ratios of geometric mean concentrations of erythrocyte parameters, measured using automated analyser, infants BCG vaccinated at birth: infants BCG vaccinated at 6 weeks of age. N≥39 per group. Exact numbers for each parameter and time-point can be found in Table 3.1.1 Appendix 9. A ratio of 1 indicates equivalence. Ratios >1 show higher levels in infants BCG vaccinated at birth, ratios <1 show higher levels in infants BCG vaccinated at 6 weeks. BCG, Bacille Calmette Guerin; EPI, Expanded programme of immunisations; Hb, haemoglobin; HCT, haematocrit; RBC, red blood cells; MCV, mean cell volume; MCH, mean cell haemoglobin; MCHC, mean cell haemoglobin concentration; RDW, red-cell distribution width; *=p<0.05; **=p<0.01; ***=p<0.001; ****=p<0.0001. The clear background denotes that comparisons for the time-point are between BCG vaccinated infants and BCG unvaccinated infants. Grey backgrounds denote that comparisons at this time-point are between infants BCG vaccinated at birth and infants BCG vaccinated at 6 weeks. Note, magnified logarithmic scale.

Figure 7.30. Geometric mean ratios of erythrocyte parameters comparing infants BCG vaccinated at birth with infants BCG vaccinated at 6 weeks, all post-natal time points



Ratios of geometric mean concentrations of erythrocyte parameters, measured using automated analyser, comparing infants BCG vaccinated at birth with those BCG vaccinated at 6 weeks of age. N≥39 per group. Exact numbers for each parameter and time-point can be found in Table 3.1.1 Appendix 9. A ratio of 1 indicates equivalence. Ratios >1 show higher levels in infants BCG vaccinated at birth, ratios <1 show higher levels in infants BCG vaccinated at 6 weeks. BCG, Bacille Calmette Guerin; EPI, Expanded programme of immunisations; Hb, haemoglobin; HCT, haematocrit; RBC, red blood cells; MCV, mean cell volume; MCH, mean cell haemoglobin; MCHC, mean cell haemoglobin concentration; RDW, red-cell distribution width; *=p<0.05; **=p<0.01; ***=p<0.001; ****=p<0.0001. The clear background denotes that comparisons for the time-point are between BCG vaccinated infants and BCG unvaccinated infants. Grey backgrounds denote that comparisons at this time-point are between infants BCG vaccinated at birth and infants BCG vaccinated at 6 weeks. Note, magnified logarithmic scale.

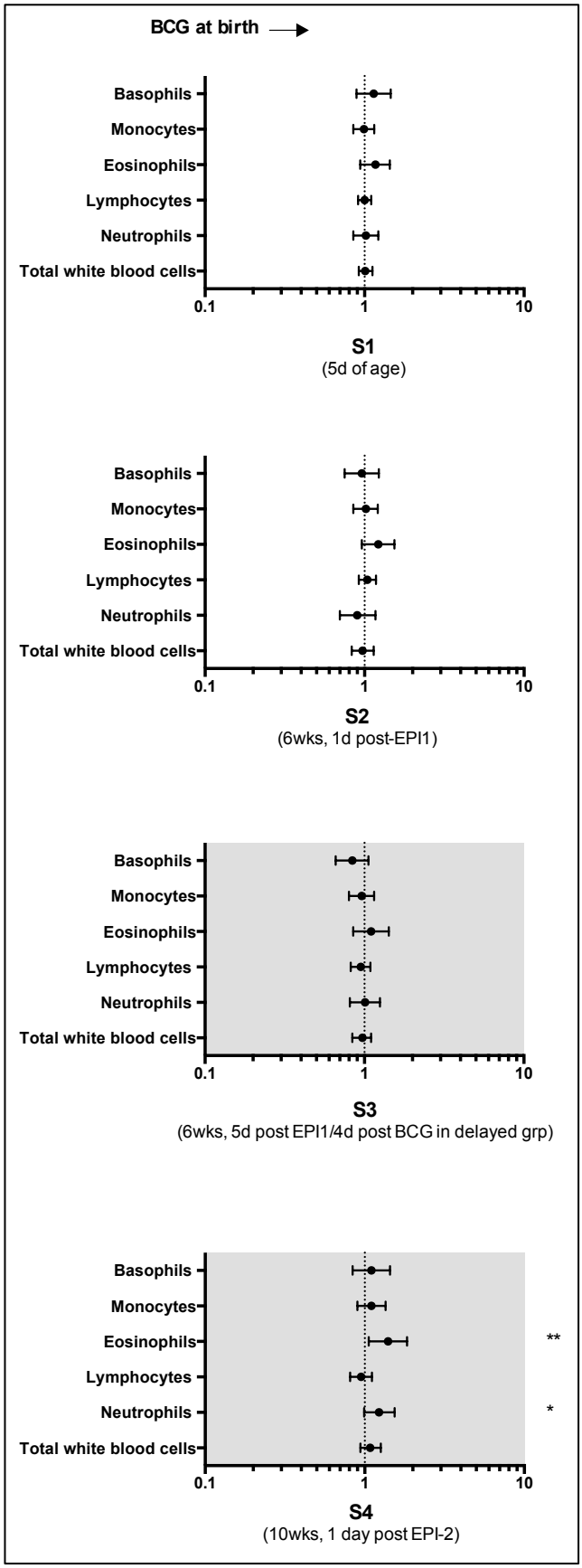
7.4.5 Cross-sectional comparison of geometric mean leucocyte counts by BCG status

The GMRs of leucocyte differentials by BCG status are shown in Figure 7.31 (Table 3.2.2, Appendix 9). Eosinophil numbers were significantly higher at S4 (10 weeks, 1 day post EPI-2) in infants BCG vaccinated at birth compared to 6 weeks of age (GMR 1.40 (1.06-1.84) p=0.007). A similar trend was seen at all other post-natal time-points, although this did not reach statistical significance. Neutrophil counts were also higher in infants BCG vaccinated at birth at S4 (GMR 1.23 (1.0-1.54) p=0.02) but similar trends were not seen at other time-points. No other significant differences in leucocyte differential counts by BCG status were seen.

7.4.5.1 Higher eosinophil counts at S4 in infants BCG vaccinated at birth are more pronounced in male infants.

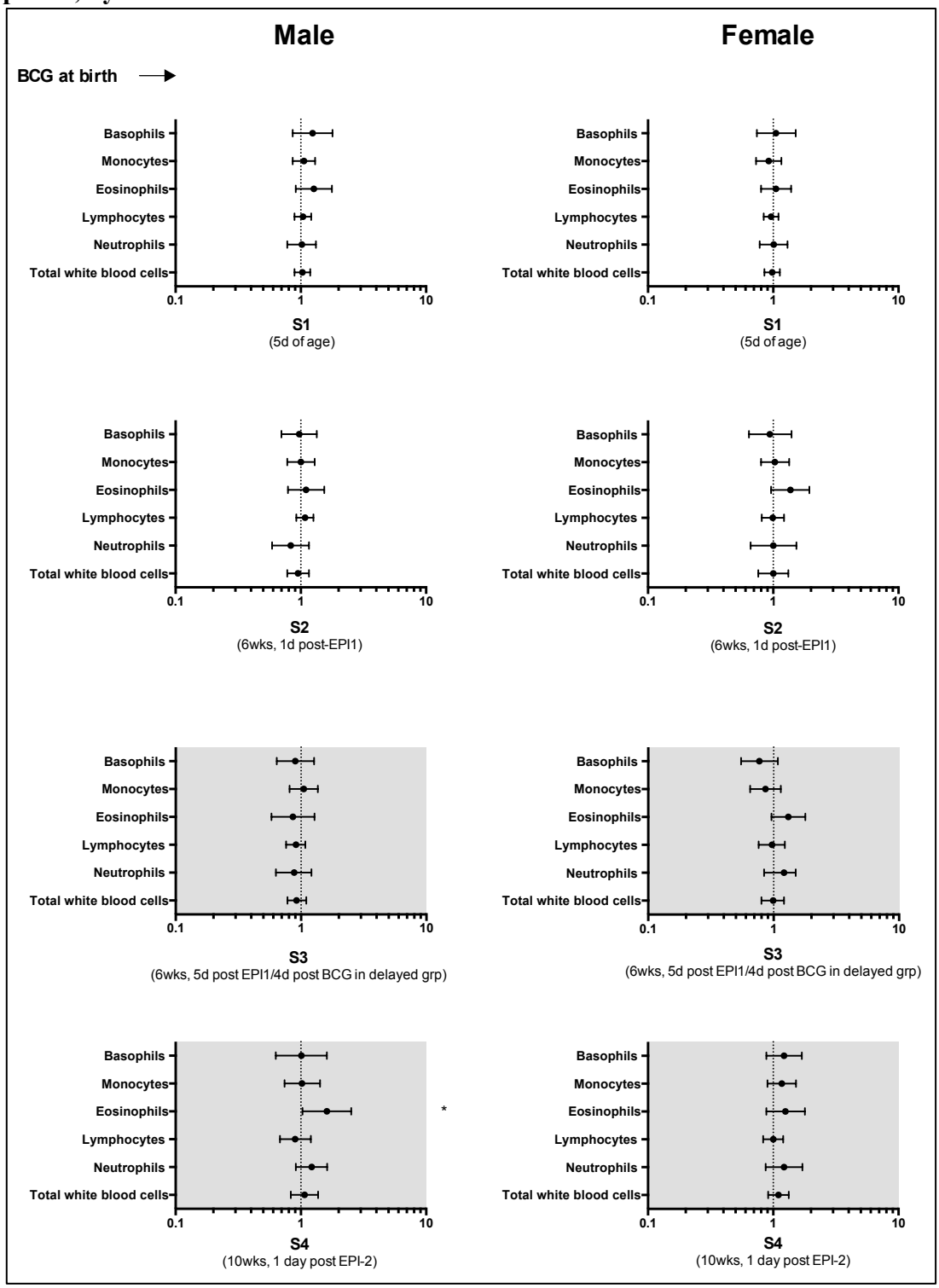
When analysed by sex, the significantly higher eosinophil count at S4 in infants BCG vaccinated at birth was only retained in male infants (GMR 1.61 (1.03-2.52) p=0.05) (Figure 7.32). No significant interactions between sex and BCG group on leucocyte differentials were seen.

Figure 7.31 Ratios of leucocyte differential counts comparing infants BCG vaccinated at birth with infants BCG vaccinated at 6 weeks, at all post-natal time points



Ratios of geometric mean concentrations of leucocyte differential counts, measured using automated analyser, infants BCG vaccinated at birth: infants BCG vaccinated at 6 weeks of age. N≥39 per group. Exact numbers for each parameter and time-point can be found in Table 3.1.1 Appendix 9. A ratio of 1 indicates equivalence. Ratios >1 show higher levels in infants BCG vaccinated at birth, ratios <1 show higher levels in infants BCG vaccinated at 6 weeks. BCG, Bacille Calmette Guerin; EPI, Expanded programme of immunisations; *=p<0.05; **=p<0.01; ***=p<0.001; ****=p<0.0001. The clear background denotes that comparisons for the time-point are between BCG vaccinated infants and BCG unvaccinated infants. Grey backgrounds denote that comparisons at this time-point are between infants BCG vaccinated at birth and infants BCG vaccinated at 6 weeks. Note, magnified logarithmic scale.

Figure 7.32. Ratios of leucocyte differential counts, comparing infants BCG vaccinated at birth with infants BCG vaccinated at 6 weeks, at all post-natal time points, by sex



Ratios of geometric mean concentrations of leucocyte differential counts, measured using automated analyser, infants BCG vaccinated at birth: infants BCG vaccinated at 6 weeks of age, separated by sex. N≥39 per group. Exact numbers for each parameter and time-point can be found in Table 3.1.1 Appendix 9. A ratio of 1 indicates equivalence. Ratios >1 show higher levels in infants BCG vaccinated at birth, ratios <1 show higher levels in infants BCG vaccinated at 6 weeks. BCG, Bacille Calmette Guerin; EPI, Expanded programme of immunisations; *=p<0.05; **=p<0.01; ***=p<0.001; ****=p<0.0001. The clear background denotes that comparisons for the time-point are between BCG vaccinated infants and BCG unvaccinated infants. Grey backgrounds denote that comparisons at this time-point are between infants BCG vaccinated at birth and infants BCG vaccinated at 6 weeks. Note, magnified logarithmic scale.

7.4.6 No clear correlations between inflammatory-iron axis parameters with scar size or episodes of infection were seen

Tables 7.6 and 7.7 display the significant Spearman rank correlations of inflammatory-iron axis parameters, erythrocyte parameters and white blood cell parameters with scar size at 10 weeks and total infection episodes (clinic presentations and parentally reported). When analysed as either all participants together, or divided by BCG status or by sex and BCG status, few significant correlations were seen. Nearly all correlations were only just statistically significant, and did not show logical, consistent trends over time-points, or with associated parameters. These are, therefore, likely to be artefacts of multiple testing. A negative association of TSAT and scar size at S4 in female infants BCG vaccinated at birth was more strongly significant (Rho -0.66, p=0.001), but as there were no corresponding associations with the components of TSAT (iron and transferrin), this is also likely to be artefactual.

These findings corroborate similar findings from the epigenetic and cytokine sub-studies, and suggest that scar size may not be a good indicator of the NSE of BCG.

Table 7.6. Correlations of inflammatory-iron, erythrocyte and leucocyte parameters with scar size and number of infection episodes

	All infants				BCG at birth				BCG at 6 weeks			
Correlations with scar size	**S1**	**S2**	**S3**	**S4**	**S1**	**S2**	**S3**	**S4**	**S1**	**S2**	**S3**	**S4**
IL-6				0.21*	0.38*							
Hepcidin	-0.43*											
Ferritin											-0.38*	
TSAT								-0.31*				
MCH								0.31*				
MCHC		0.40*		0.23*				0.34*				
WBC counts											-0.39*	
Neutrophil counts			0.44*									
Eosinophil counts				0.26*								
Correlations with infections	**S1**	**S2**	**S3**	**S4**	**S1**	**S2**	**S3**	**S4**	**S1**	**S2**	**S3**	**S4**
TSAT			-0.27*									

Spearman rank correlations between inflammatory-iron, erythrocyte and leucocyte parameters, and BCG scar size measured at 10 weeks of age or infections. Statistically significant data only shown with Rho value and significance level. *=p<0.05; **=p<0.01; ***=p<0.001; ****=p<0.0001. S1, 5 days of age; S2, 6 weeks of age, 1 day post-EPI-1; S3, 6 weeks of age, 5 days post-EPI-1 +/- BCG in delayed group; S4, 10 weeks of age, pre-EPI-2. BCG, Bacille Calmette Guerin; IL, interleukin; TSAT, transferrin saturation; MCH, mean cell haemoglobin; MCHC, mean cell haemoglobin concentration; WBC, white blood cell.

Tables 7.7 a) and b). Spearman rank correlations of inflammatory-iron, erythrocyte and leucocyte parameters with scar size and number of infection episodes, by sex

a) Male infants

	BCG at birth				BCG at 6 weeks			
Correlations with scar	**S1**	**S2**	**S3**	**S4**	**S1**	**S2**	**S3**	**S4**
Ferritin						-0.69*		
Correlations with infections	**S1**	**S2**	**S3**	**S4**	**S1**	**S2**	**S3**	**S4**
TSAT	-0.64*							
WBC count		-0.57*	0.61*					

b) Female infants

	BCG at birth				BCG at 6 weeks			
Correlations with scar	**S1**	**S2**	**S3**	**S4**	**S1**	**S2**	**S3**	**S4**
Hepcidin					-0.71*			-0.41*
TSAT				-0.66***				
MCHC				0.49*				
Neutrophil count			0.75**				-0.59*	
Correlations with infections	**S1**	**S2**	**S3**	**S4**	**S1**	**S2**	**S3**	**S4**
TSAT		0.50*					-0.57*	
Iron								
WBC count							-0.48*	

Spearman rank correlations between inflammatory-iron, erythrocyte and leucocyte parameters, and BCG scar size measured at 10 weeks of age or infections. Statistically significant data only shown with Rho value and significance level. *=p<0.05; **=p<0.01; ***=p<0.001; ****=p<0.0001. S1, 5 days of age; S2, 6 weeks of age, 1 day post-EPI-1; S3, 6 weeks of age, 5 days post-EPI-1 +/- BCG in delayed group; S4, 10 weeks of age, pre-EPI-2. BCG, Bacille Calmette Guerin; IL, interleukin; TSAT, transferrin saturation; MCH, mean cell haemoglobin; MCHC, mean cell haemoglobin concentration; WBC, white blood cell.

7.4.7 Within-infant changes over time to inflammatory-iron, erythrocyte and leucocyte parameters

Within-infant changes to inflammatory iron, erythrocyte and leucocyte parameters were used to explore the impact of BCG vaccination on changes to these parameters over time. The numbers of infants available for each comparison were small, particularly when divided by sex, reducing the power to detect significant differences (see Table 3.1, Appendix 9, often n<10). As in the cytokine sub-study, the large numbers of parameters and time-points available greatly increased the risk of significant values resulting from multiple-testing, and makes identification of key trends challenging. Therefore, the results presented below are for those parameters for which significant differences were seen between cross-sectional group means. Full results of within-infant changes between all time-points for all parameters can be found in Tables 3.3.1-3.3.19, Appendix 9, with a summary of statistically significant changes shown in Table 7.8 below. Comparison with changes to cross-sectional geometric mean levels between time-points is made in an attempt to provide corroborative evidence for changes seen, but again, these analyses should be viewed as exploratory only, not conclusive.

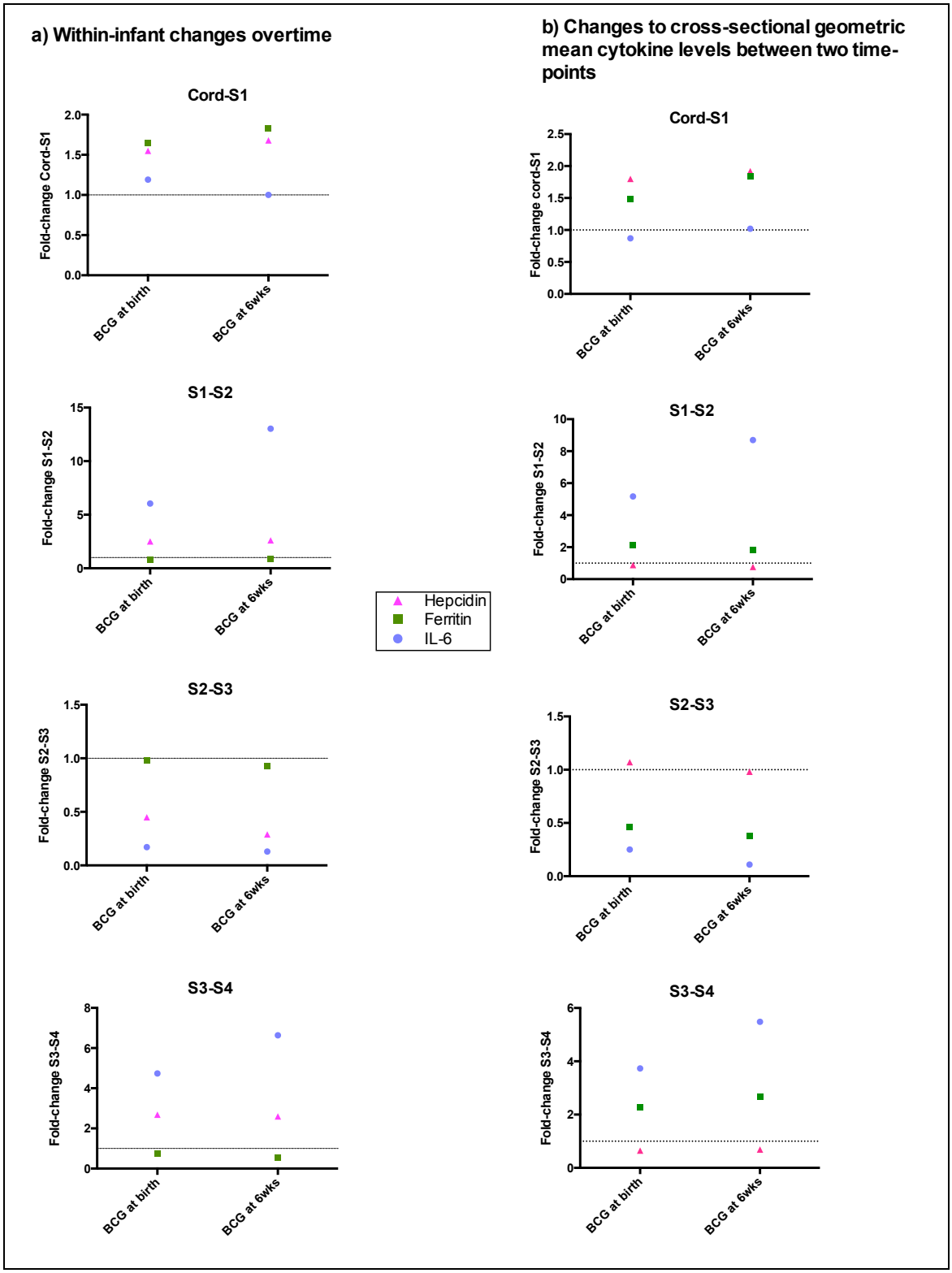
As shown in Figures 7.33-7.35, the changes over time were very similar for most parameters studied, when comparing infant BCG vaccinated at birth with those vaccinated at 6 weeks. Although some of these differences reached statistical significance, these were mainly borderline, and no clear, consistent, logical trends were seen (see Table 7.7).

Changes to IL-6 production over-time, however, did show clear and consistent differences by BCG vaccination status, when analysed either by median within-infant changes over time or by changes in cross-sectional geometric means between time-points (Figure 7.33). These findings are most clearly displayed in composite figures of changes over time (Figure 7.36). In all infants, IL-6 production increased between cord blood and/or 5 days of age (S1) and 6 weeks of age, 1-day post-*in vivo* stimulation from EPI-1 (S2). The increase was higher in infants without BCG vaccination (13 fold vs. 6 fold), particularly boys. By 5 days post-EPI-1, and 4 days post-BCG in the delayed group, (S3) all infants had a reduction in serum IL-6, but this appeared to occur slightly less in infants BCG vaccinated at birth. Between S3 and 10 weeks of age, 1-day post *in vivo* stimulation from EPI-2 (S4), all infants had increased IL-6 production. When both

sexes were analysed together, the IL-6 increase appeared to be more in those BCG vaccinated at birth. However, this differed by sex. In boys, the increase in IL-6 between S3 and S4 was greater in those BCG vaccinated at birth (13 fold vs. 10 fold). In girls, the increase in IL-6 between S3 and S4 was greater in those BCG vaccinated at 6 weeks (5 fold vs. 3 fold), although this was not corroborated when looking at changes to cross-sectional geometric mean levels between the two time-points.

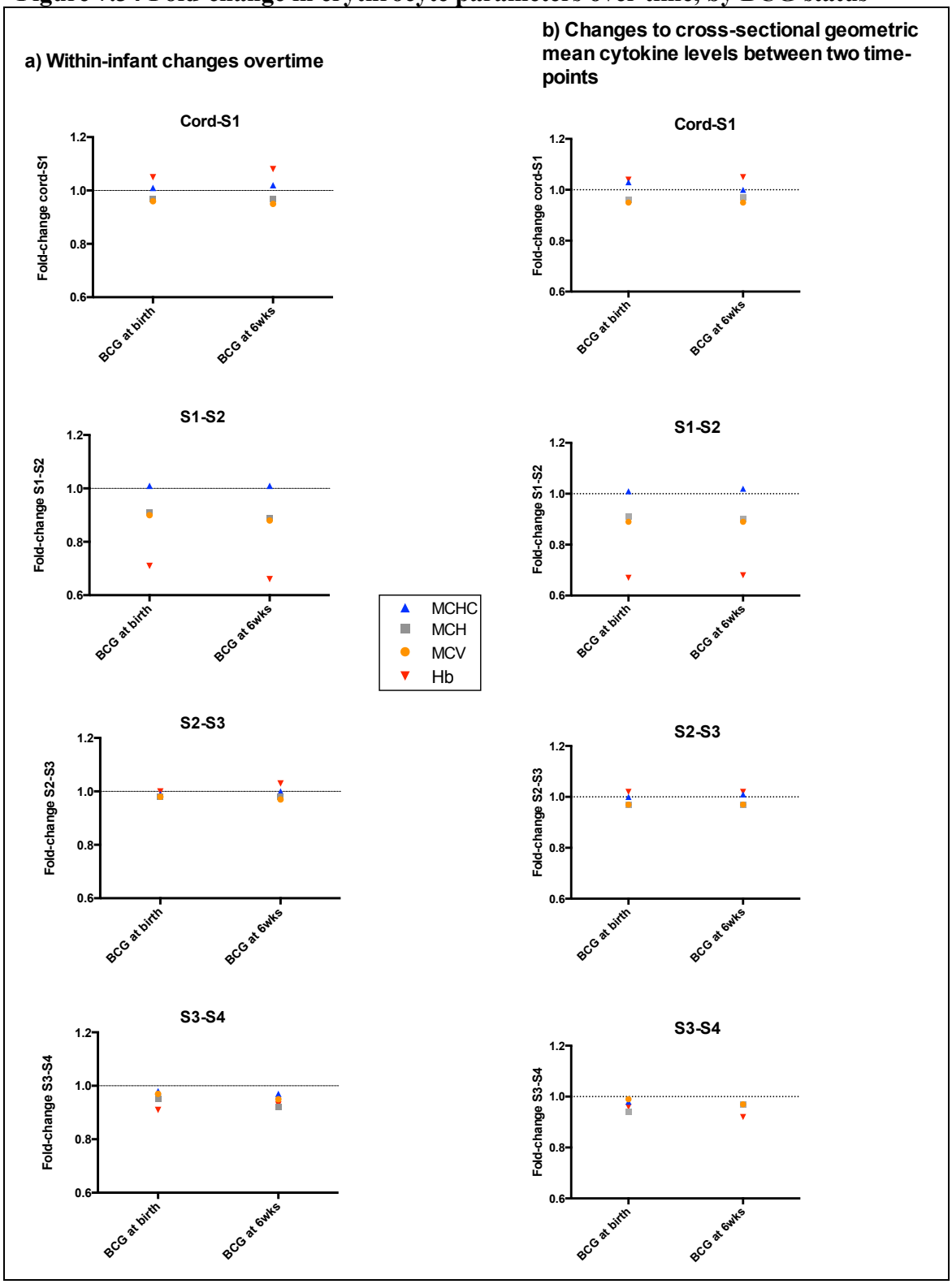
Although these trends over time were largely non-significant findings, and should be interpreted with caution, it is interesting to note that the pattern of changes seen in male infants, following *in vivo* non-specific stimulation, mimicked those shown in the cytokine sub-study, following *in vitro* non-specific stimulation. In both studies, pro-inflammatory cytokine production appeared to be lower in male infants for whom BCG vaccination was the last vaccine received.

Figure 7.33 Fold-change in inflammatory-iron axis parameters over time, by BCG status



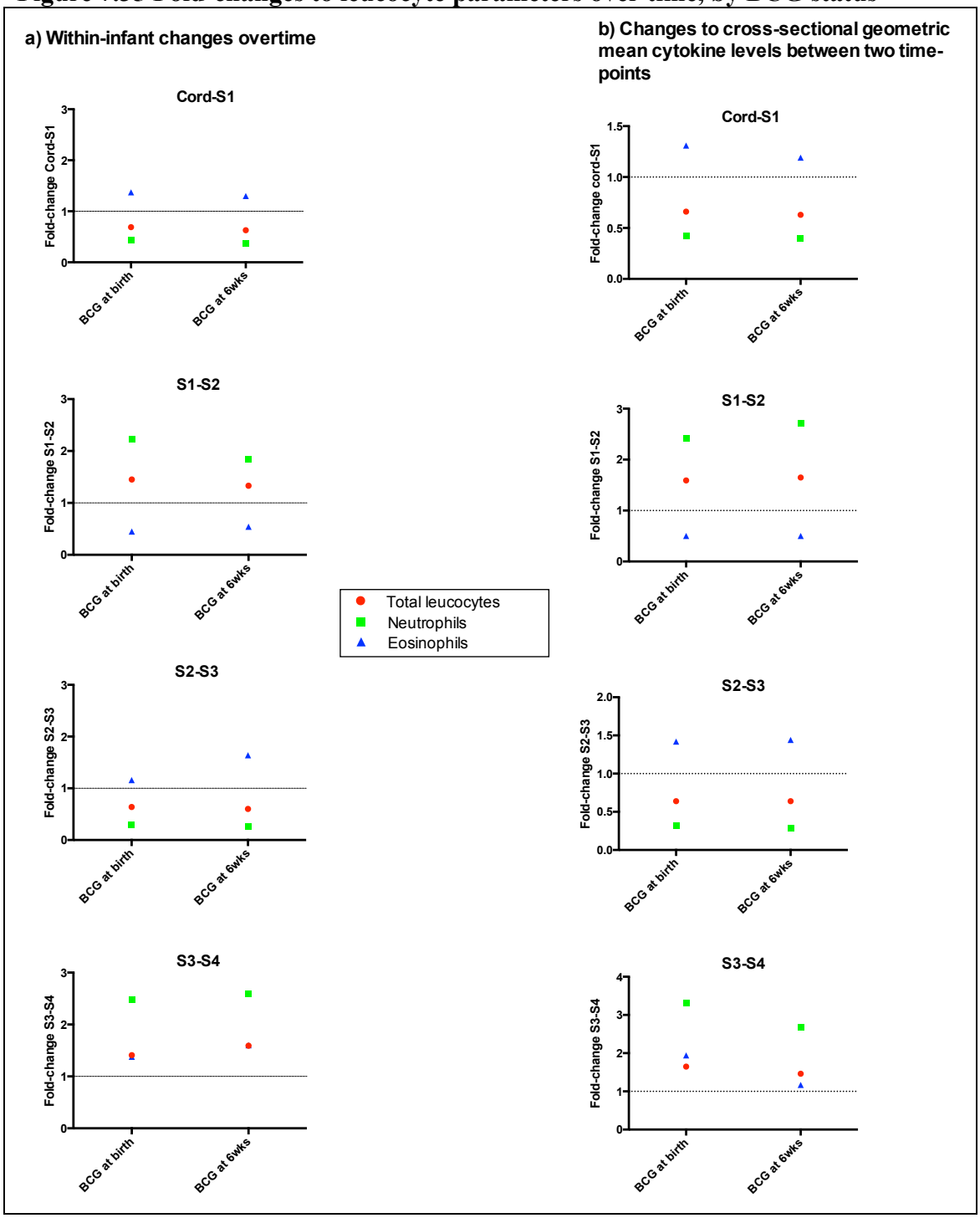
Results are displayed as geometric mean fold-change in within-infant changes over time (Figure 7.33a) and fold-change in the cross-sectional geometric means of the two time-points (Figure 7.33b). Fold-change was calculated as the later time-point/earlier time-point, therefore 1 indicates equivalence. Change <1 shows a reduction in stimulated cytokine production over time, >1 shows an increase in production over time. Within-infant changes n≥12, cross-sectional changes n≥41. S1, 5 days of age; S2, 6 weeks of age, 1 day post-EPI-1 vaccinations; S3, 6 weeks of age 5 days post-EPI-1/4 days post-BCG in the delayed group; S4, 10 weeks of age, 1 day post-EPI-2. EPI, Expanded Programme of Immunisations; BCG, Bacille Calmette Guerin; IL, interleukin.

Figure 7.34 Fold-change in erythrocyte parameters over time, by BCG status



Results are displayed as geometric mean fold-change in within-infant changes over time (Figure 7.34a) and fold-change in the cross-sectional geometric means of the two time-points (Figure 7.34b). Fold-change was calculated as the later time-point/earlier time-point, therefore 1 indicates equivalence. Change <1 shows a reduction in stimulated cytokine production over time, >1 shows an increase in production over time. Within-infant changes n≥11, cross-sectional changes n≥39. S1, 5 days of age; S2, 6 weeks of age, 1 day post-EPI-1 vaccinations; S3, 6 weeks of age 5 days post-EPI-1/4 days post-BCG in the delayed group; S4, 10 weeks of age, 1 day post-EPI-2. EPI, Expanded Programme of Immunisations; BCG, Bacille Calmette Guerin; IL, interleukin; MCH, mean cell haemoglobin; MCHC, mean cell haemoglobin concentration; MCV, mean cell volume.

Figure 7.35 Fold-changes to leucocyte parameters over time, by BCG status



Results are displayed as geometric mean fold-change in within-infant changes over time (Figure 7.34a) and fold-change in the cross-sectional geometric means of the two time-points (Figure 7.34b). Fold-change was calculated as the later time-point/earlier time-point, therefore 1 indicates equivalence. Change <1 shows a reduction in stimulated cytokine production over time, >1 shows an increase in production over time. Within-infant changes n≥11, cross-sectional changes n≥39. S1, 5 days of age; S2, 6 weeks of age, 1 day post-EPI-1 vaccinations; S3, 6 weeks of age 5 days post-EPI-1/4 days post-BCG in the delayed group; S4, 10 weeks of age, 1 day post-EPI-2. EPI, Expanded Programme of Immunisations; BCG, Bacille Calmette Guerin.

Table 7.8. Statistically significant within-infant changes between time points. Blue=median within-infant change over time higher in infants BCG vaccinated at birth. Red=median within-infant change over time higher in infants BCG vaccinated at 6 weeks.

	Cord-S1	S1-S2	S2 – S3	S3 – S4
Hepcidin				Male*
Transferrin				Male*
Eosinophils	Male*			
Basophils				
Hb			All* Male*	
MCV			All** Male***	
MCH				All** Female*
MCHC	All* Male*	Male**		
RBC			Male*	All*

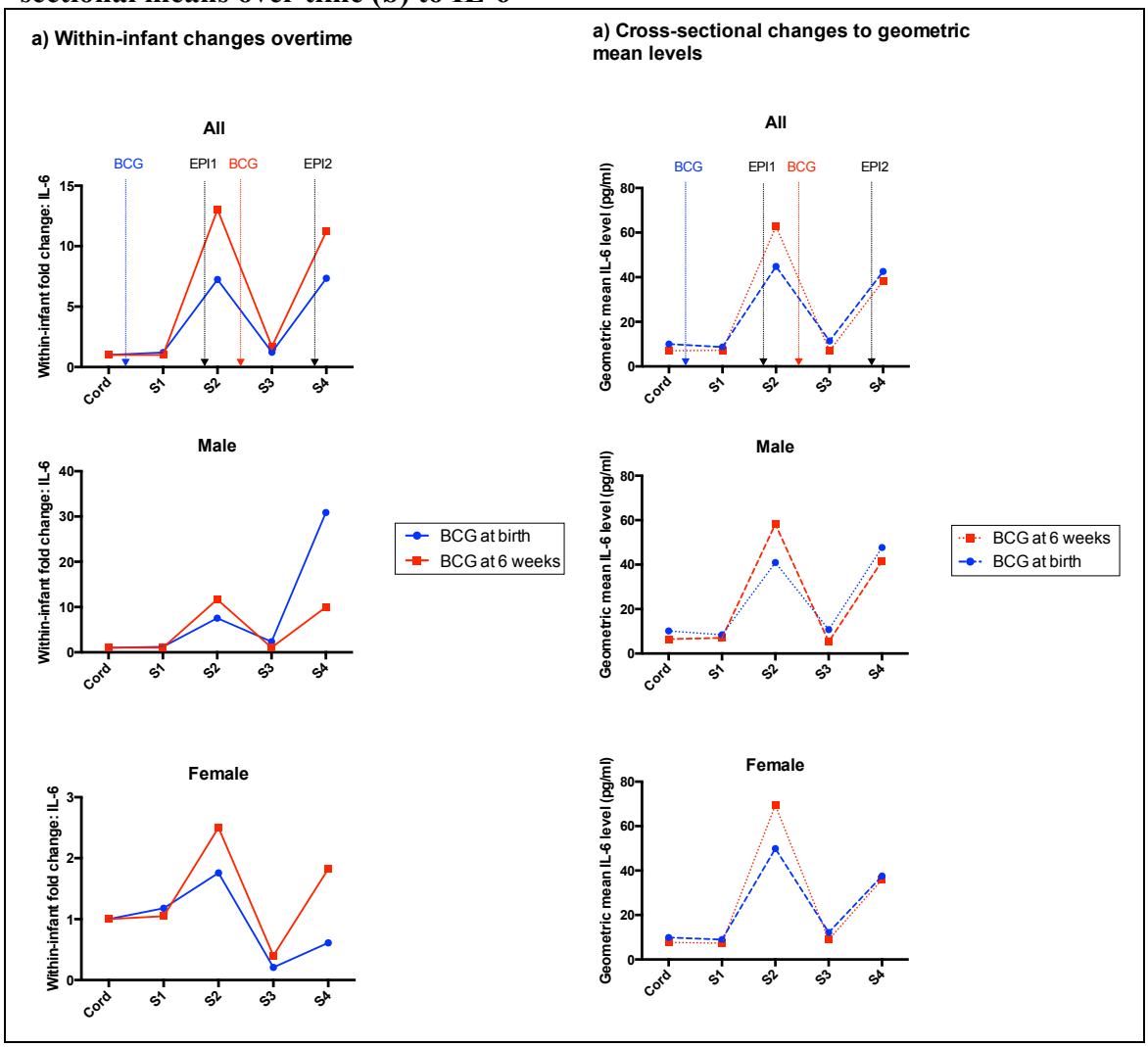
	Cord-S2	S1-S3	S2 – S4
IL-6	All* Male*		
Ferritin	All* Male*		
WBC			Female*
Eosinophils			All*
HCT	Female*		
MCV		Male*	
MCH		Male**	

	Cord-S3	S1-S4
Ferritin	Male*	
MCHC	Male*	

	Cord-S4
Hb	Male*
MCH	Male**
MCHC	Male**

Hb, haemoglobin; MCV, mean cell volume; MCH, mean cell haemoglobin; MCHC, mean cell haemoglobin concentration; RBC, red blood cells; IL, interleukin; HCT, haematocrit; S1, 5 days of age; S2, 6 weeks of age, 1 day post-EPI-1 vaccinations; S3, 6 weeks of age, 5 days post-EPI-1 vaccinations (and 4 days post BCG in the delayed group); S4, 10 weeks of age, 1 day post-EPI-2 vaccinations. *=p<0.05, **=p<0.01, ***=p<0.001, ****=p<0.0001.

Figure 7.36. Median within-infant changes over time (a) and changes to cross-sectional means over time (b) to IL-6



Composite graphs showing the pattern of change to IL-6 concentrations over time, when assessed by median within-infant changes (a) or changes to cross-sectional geometric mean concentrations (b). IL, interleukin; BCG, Bacille Calmette Guerin; EPI, Expanded Programme of Immunisations.

7.5 Clinical outcomes

Clinical illness episodes were assessed for the duration of a participant's involvement in the study. This was accomplished in a number of ways, as described in Methods section 6.1.11, including open access for clinical assessment, routine questioning about interim illnesses at clinic visits and weekly telephone enquiries regarding health status.

A total of 585 clinical illness episodes occurred during the study, comprising 470 presentations to clinic (physician-diagnosed) and 115 parental reports of interim illness for which clinic presentation did not occur. The majority of these illness events were infectious in origin. Presentations to clinic for non-infectious reasons included 56 presentations for expected normal infant variants (such as natal teeth, tongue-tie and cord granulomas). There were 22 presentations at clinic where the infant was deemed to be well by a physician. In total, 16 infants were hospitalised during the study, largely for infections (5 LRTI, 10 neonatal sepsis and 1 for duodenal atresia) and there were 8 deaths. Causes of death determined by verbal autopsy were: duodenal atresia (1), cord accident (1), LRTI (2), suspected sepsis (1), cot-death/suffocation (2) and prematurity/respiratory compromise (1). One infant was found to have died two days following withdrawal from the study. This death was reported as cot-death/suffocation. Inclusion of this death in statistical analysis made little difference to the results.

7.5.1 Infants vaccinated with BCG have significantly fewer infectious illness episodes compared to unvaccinated infants in the first 6 weeks of life

The absolute frequencies and cumulative incidence of illness events occurring during the first 10 weeks of life did not vary significantly by BCG status, when analysed as either physician diagnosed alone (Cumulative incidence (CuI) 85/100 infants receiving BCG at birth vs. 91/100 infants receiving BCG at 6 weeks (Hazard ratio (HR): 0.94 (0.80-1.11)) (Tables 7.9 and 7.10)) or combined analysis of physician diagnosed and parental report of interim illnesses (CuI 106/100 infants receiving BCG at birth vs. 113/100 infants receiving BCG at 6 weeks (HR 0.96 (0.83-1.10)) (Table 7.11)). However, when analysed by time-periods before/after the delayed group received BCG (and all infants received EPI-1 vaccinations), a significant impact of BCG was seen.

In the period prior to BCG vaccination in the delayed group, infants who had received BCG at birth had significantly fewer presentations to clinic than infants who were not BCG vaccinated (CuI 47/100 infants BCG vaccinated at birth vs. 58/100 infants BCG

vaccinated at 6 weeks (HR 0.77 (0.60-1.00) Tables 7.9 and 7.10). When infants in the delayed group had received BCG vaccination, the trend reversed, with a tendency to fewer presentations in the infants BCG vaccinated at 6 weeks (HR 1.12 (0.89-1.39)). Although this did not reach statistical significance, the test for interaction of the time-

Table 7.9. Frequencies of physician-diagnosed illness presentations by BCG vaccination status

		Total frequency			Pre-DTP+/-BCG			Post-DTP+/-BCG		
		BCG at birth	**BCG at 6 weeks**	**p-value**	**BCG at birth**	**BCG at 6 weeks**	**p-value**	**BCG at birth**	**BCG at 6 weeks**	**p-value**
All presentations	Total	228	242	0.14	**128**	**156**	**0.02**	100	86	0.28
	Male	106	115	0.32	59	76	0.06	47	39	0.30
	Female	122	127	0.27	69	80	0.14	53	47	0.61
Infection	Total	186	205	0.09	**98**	**129**	**0.008**	88	76	0.33
	Male	83	95	0.20	**42**	**62**	**0.02**	41	33	0.28
	Female	103	110	0.24	56	67	0.14	47	43	0.76
Fever (reported or recorded)	Total	50	54	0.61	29	42	0.09	21	12	0.12
	Male	18	20	0.75	9	16	0.15	9	4	0.15
	Female	32	34	0.67	20	26	0.28	12	8	0.40
Sepsis	Total	33	31	0.84	22	26	0.51	11	5	0.14
	Male	17	15	0.70	11	11	0.99	6	4	0.51
	Female	16	16	0.92	11	15	0.36	5	1	0.11
Death	Total	6	3	0.33	4	3	0.72	2	0	0.16
	Male	3	2	0.65	2	2	1.0	1	0	0.32
	Female	3	1	0.33	2	1	0.58	1	0	0.33

Table 7.10 Clinical illness event hazard ratios (BCG vaccinated at birth:BCG vaccinated at 6 weeks), physician-diagnosed only

		Total follow-up		Pre-EPI-1 (and BCG in delayed group)		Post-EPI-1 (and BCG in delayed group)		Test for interaction pre/post EPI-1
		Hazard Ratio	p-value	Hazard Ratio	p-value	Hazard ratio	p-value	
Any presentation	Total	0.94 (0.80-1.11)	0.49	**0.77 (0.60-1.00)**	**0.05**	1.12 (0.89-1.39)	0.34	**0.04**
	Male	0.87 (0.68-1.12)	0.29	**0.68 (0.47-0.99)**	**0.04**	1.08 (0.79-1.49)	0.62	0.06
	Female	1.01 (0.81-1.26)	0.90	0.88 (0.62-1.25)	0.48	1.16 (0.85-1.57)	0.36	0.28
Infectious presentation	Total	0.91 (0.76-1.10)	0.33	**0.71 (0.53-0.95)**	**0.02**	1.10 (0.87-1.40)	0.43	**0.02**
	Male	0.84 (0.63-1.11)	0.22	**0.57 (0.36-0.89)**	**0.01**	1.11 (0.78-1.59)	0.56	**0.01**
	Female	0.99 (0.78-1.25)	0.93	0.87 (0.59-1.27)	0.47	1.11 (0.81-1.52)	0.53	0.35
Reported or recorded fever	Total	0.97 (0.70-1.36)	0.88	0.72 (0.44-1.17)	0.19	1.37 (0.84-2.22)	0.20	0.07
	Male	0.88 (0.52-1.51)	0.65	0.55 (0.24-1.25)	0.15	1.39 (0.65-2.97)	0.40	0.11
	Female	1.06 (0.70-1.61)	0.80	0.85 (0.47-1.55)	0.61	1.38 (0.74-2.58)	0.31	0.29
Recorded fever>37.5˚C	Total	0.89 (0.50-1.57)	0.69	0.80 (0.35-1.83)	0.60	1.01 (0.44-2.28)	0.99	0.71
	Male	0.86 (0.34-2.15)	0.75	0.98 (0.26-3.74)	0.97	0.74 (0.20-2.70)	0.65	0.77
	Female	0.92 (0.45-1.91)	0.83	0.72 (0.25-2.08)	0.55	1.27 (0.44-3.68)	0.66	0.47
Reported or recorded fever likely due to infection	Total	0.93 (0.64-1.35)	0.71	0.73 (0.44-1.20)	0.21	1.34 (0.75-2.40)	0.32	0.12
	Male	0.86 (0.45-1.63)	0.65	0.46 (0.18-1.13)	0.09	2.04 (0.73-5.69)	0.17	**0.03**
	Female	0.99 (0.63-1.56)	0.98	0.94 (0.51-1.71)	0.83	1.09 (0.53-2.25)	0.82	0.76
Recorded fever>37.5˚C likely due to infection (i.e. not post-immunisation pyrexia)	Total	0.73 (0.37-1.43)	0.36	0.74 (0.32-1.71)	0.48	0.72 (0.23-2.24)	0.57	0.97
	Male	0.84 (0.29-2.40)	0.74	0.78 (0.19-3.21)	0.73	0.93 (0.19-4.51)	0.93	0.88
	Female	0.68 (0.28-1.64)	0.39	0.72 (0.25-2.08)	0.55	0.54 (0.10-2.93)	0.48	0.78
Post-immunisation pyrexia	Total	1.58 (0.62-4.01)	0.34					
	Male	1.20 (0.33-4.36)	0.79					
	Female	2.11 (0.54-8.28)	0.28					

Serious bacterial infections	Total	1.07 (0.64-1.78)	0.79	0.84 (0.47-1.51)	0.57	2.01 (0.77-5.27)	0.16	0.11
	Male	1.08 (0.50-2.34)	0.84	0.89 (0.37-2.11)	0.79	1.62 (0.43-0.49)	0.43	0.37
	Female	1.06 (0.54-2.07)	0.87	0.81 (0.37-1.80)	0.61	2.72 (0.54-13.85)	0.23	0.20
Hospital admissions	Total	2.12 (0.75-6.49)	0.15	Numbers too small for analysis				
	Male	1.20 (0.33-4.38)	0.79					
	Female	6.34 (0.73-54.94)	0.09					
Death	Total	1.34 (0.30-6.01)	0.70	0.67 (0.11-4.02)	0.66	Numbers too small for analysis		
	Male	0.96 (0.13-6.81)	0.97	0.49 (0.04-5.39)	0.56			
	Female	2.11 (0.19-23.56)	0.54	1.03 (0.06-16.75)	0.98			

BCG, Bacille Calmette Guerin; EPI, Expanded Programme of Immunisation

Table 7.11 Clinical illness event hazard ratios (BCG vaccinated at birth:BCG vaccinated at 6 weeks), physician-diagnosed and parental report of interim illness

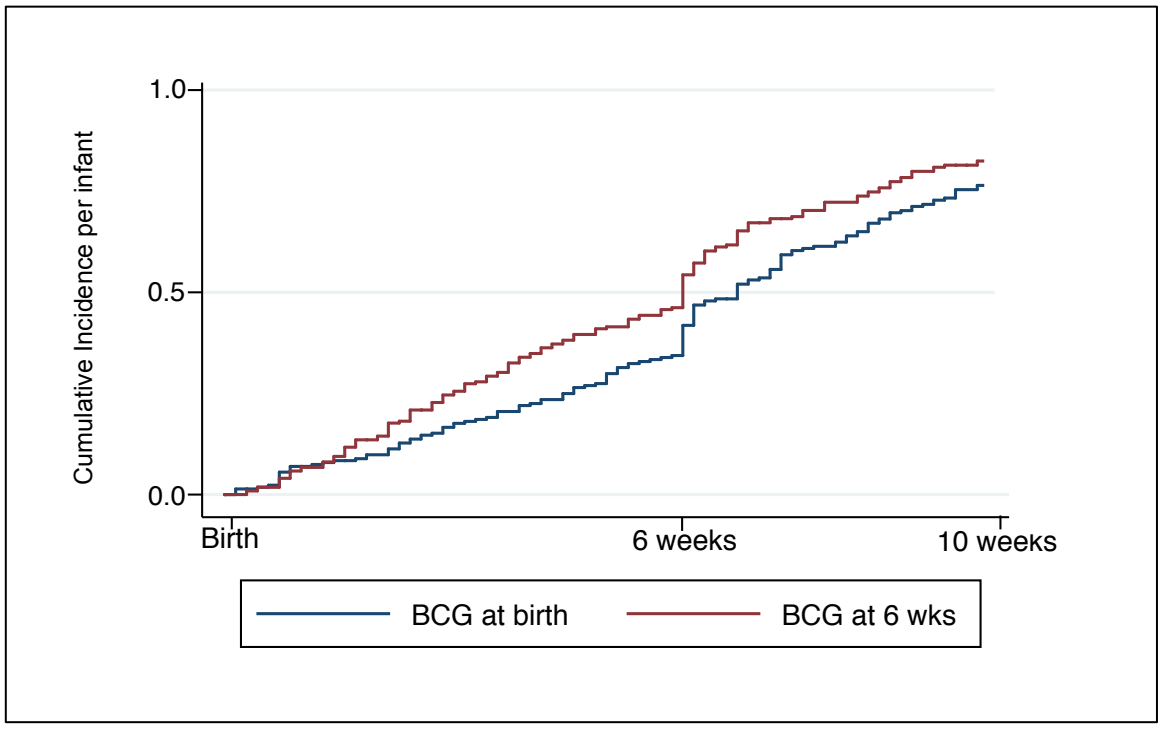
		Total follow-up		Pre-EPI-1 (and BCG in delayed group)		Post-EPI-1 (and BCG in delayed group)		Test for interaction pre vs. post EPI-1
		Hazard Ratio	p-value	Hazard Ratio	p-value	Hazard ratio	p-value	
Any presentation/report	Total	0.96 (0.83-1.10)	0.56	**0.77 (0.59-0.99)**	**0.04**	1.07 (0.92-1.24)	0.38	**0.01**
	Male	0.98 (0.79-1.20)	0.80	0.66 (0.46-0.96)	**0.03**	1.15 (0.93-1.42)	0.21	**0.003**
	Female	0.95 (0.78-1.15)	0.60	0.89 (0.62-1.25)	0.47	1.01 (0.82-1.23)	0.94	0.47
Infectious presentation/report	Total	1.03 (0.88-1.21)	0.68	0.94 (0.80-1.10)	0.45	1.18 (0.96-1.46)	0.12	**0.01**
	Male	1.10 (0.86-1.39)	0.45	0.93 (0.74-1.18)	0.55	1.40 (1.02-1.92)	**0.04**	**0.002**
	Female	0.99 (0.80-1.23)	0.92	0.95 (0.76-1.19)	0.67	1.04 (0.78-1.39)	0.77	0.48
Reported or recorded fever	Total	1.03 (0.75-1.41)	0.16	0.89 (0.64-1.23)	0.48	1.31 (0.87-1.99)	0.20	**0.02**
	Male	1.07 (0.64-1.77)	0.81	0.88 (0.53-1.46)	0.63	1.49 (0.76-2.91)	0.25	**0.05**
	Female	1.02 (0.68-1.53)	0.93	0.91 (0.60-1.38)	0.65	1.24 (0.73-2.11)	0.42	0.17
Reported or recorded fever likely due to infection	Total	0.97 (0.65-1.46)	0.90	0.86 (0.58-1.27)	0.44	1.36 (0.73-2.52)	0.34	0.08
	Male	0.96 (0.49-1.90)	0.92	0.76 (0.39-1.47)	0.42	1.78 (0.63-5.09)	0.28	**0.05**
	Female	1.00 (0.61-1.65)	0.99	0.93 (0.58-1.51)	0.78	1.19 (0.54-2.59)	0.67	0.48

BCG, Bacille Calmette Guerin; EPI, Expanded Programme of Immunisation

period on the effect of BCG was significant (p=0.04), supporting the suggestion that the effect of BCG group on illness presentations is different before and after the delayed group received BCG. These findings were retained when parental reports of interim illness were included in the analysis (Table 7.11).

As shown in Figure 7.37 the effect of BCG on illness events was particularly strong for physician-diagnosed infectious diseases in the period prior to BCG receipt in the delayed group (CuI 36/100 infants BCG vaccinated vs. 46/100 in BCG unvaccinated infants, HR 0.71 (0.53-0.95)). Again, this trend reversed following receipt of BCG in the delayed group, with a significant test for interaction of time-period (p=0.02). As physician diagnosis was preformed blinded to vaccination status, the strengthening of the association when limited to infectious illnesses provides some evidence to suggest that increased clinic presentations in unvaccinated infants was not due to increased parental anxiety about illnesses because of the lack of BCG vaccination.

Figure 7.37. Kaplan-Meier plot of incidence of physician-diagnosed infectious disease by BCG group



The Kaplan-Meier plot of the incidence of infections over the course of the study shows that infants in both BCG groups appear to have an increase in infectious disease events at 6 weeks, around the time of vaccinations. As cases of post-immunisation pyrexia were excluded from this analysis, and no post-immunisation reactions were diagnosed,

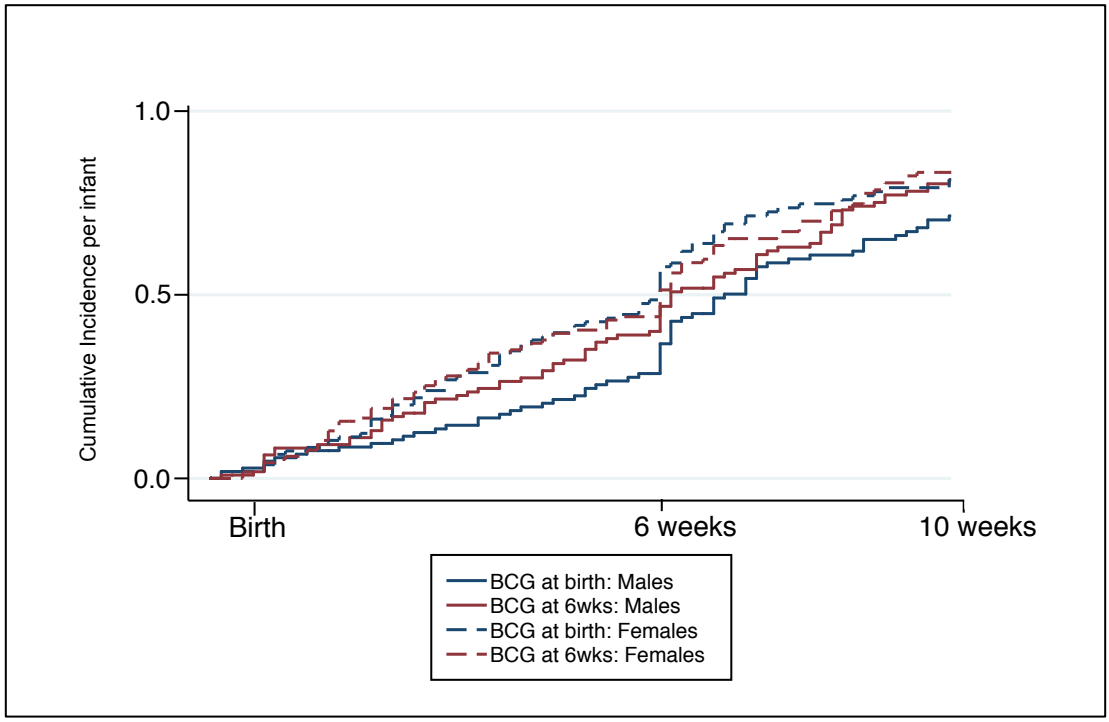
this increase is likely to be a function of the study design. All infants were routinely clinically reviewed at 6 weeks of age, and parents may have delayed presentation at clinic for infants with non-serious illnesses occurring close to this review, making it appear that they all occurred at the time of clinic review.

The same trend of reduced events in infants receiving BCG at birth in the period prior to BCG vaccination in the delayed group, and reduced events in infants vaccinated at 6 weeks thereafter, was consistently seen when other indicators of illness were analysed. These included objective measures of infection, such as reported and recorded pyrexia (not including post-immunisation pyrexia). Of note, despite the very low number of deaths in the study, a trend toward a protective effect of BCG in the first 6 weeks of life was seen. Differences were not significantly different, however, likely due to low event numbers. The same trends were observed when parental reports of illness were analysed together with clinic presentations.

7.5.1 The effects of BCG on illness events were particularly pronounced in male infants

Analysis by sex showed that the effects of BCG on total illness events and infectious illnesses were significant only in male infants (Tables 7.10 and 7.11, Figure 7.38). In general, female infants showed the same pattern of effects, with infants receiving BCG at birth having clinical illness events until the delayed group received BCG after which time the delayed group had fewer illness events, but the between group differences were not statistically significant and there was no significant differences in the effect of BCG by time-period; test for interaction, p=0.28.

Figure 7.38. Kaplan-Meier plot of incidence of physician-diagnosed infectious disease by BCG group and sex.



For most other clinical outcome measures, the trend of the effects of BCG (with reduced cumulative incidence in BCG vaccinated infants pre-BCG at 6 weeks and increased cumulative incidence post-BCG at 6 weeks) was stronger in males than females, with hazard ratios often close to equality for female infants. The exception to this was post-immunisation pyrexia, for which the tendency toward more frequent occurrence in infants in the delayed group was more pronounced in female infants compared to male.

7.5.2 The effects of BCG on illness events were particularly pronounced in LBW infants

Due to previous evidence suggesting that the greatest NSE of BCG may be in the smallest neonates, analysis was performed according to birth-weight (LBW≤2.5kg, normal birthweight >2.5kg). A total of 28 infants (5%) were LBW in the study. Despite small numbers, LBW infants who were BCG vaccinated at birth had significantly fewer infection episodes during the study, when measured by absolute frequency (1 vs. 18, p=<0.0001) or cumulative incidence (HR 0.07 (0.01-0.45)) (Table 7.11). Again, reduction in infectious presentations was most pronounced in the period prior to receipt

7.12 Frequencies and hazard ratios of physician diagnosed illness episodes in LBW infants (≤2.5kg)

		Total frequency			Pre-EPI1+/-BCG			Post-EPI1+/-BCG		
		BCG at birth	**BCG at 6wks**	**p-value**	**BCG at birth**	**BCG at 6wks**	**p-value**	**BCG at birth**	**BCG at 6wks**	**p-value**
All presentations	Total	**5**	**22**	**<0.0001**	**4**	**17**	**0.01**	1	5	0.26
	Male	**0**	**10**	**<0.0001**	**0**	**8**	**0.007**	0	2	0.31
	Female	**5**	**12**	**0.04**	4	9	0.25	1	3	0.47
Infection	Total	**1**	**18**	**<0.0001**	**1**	**14**	**0.002**	0	4	0.11
	Male	**0**	**9**	**0.002**	**0**	**7**	**0.02**	0	2	0.31
	Female	**1**	**9**	**0.007**	**1**	**7**	**0.04**	0	2	0.22
Fever (reported or recorded)	Total	1	7	0.11	1	6	0.17	0	1	0.44
	Male	0	5	0.07	0	4	0.12	0	1	0.49
	Female	1	2	0.77	1	2	0.77	0	0	
Serious Bacterial Infection	Total	1	4	0.40	1	4	0.40	0	0	
	Male	0	1	0.49	0	1	0.49	0	0	
	Female	1	3	0.47	1	3	0.47	0	0	
Death	Total	1	1	0.69	1	1	0.69	0	0	
	Male	0	0		0	0		0	0	
	Female	1	1	0.78	1	1	0.78	0	0	

		Total follow-up		Pre-EPI-1 (and BCG in delayed group)		Post-EPI-1 (and BCG in delayed group)		Test for interaction pre/post EPI-1
		Hazard Ratio	**p-value**	**Hazard Ratio**	**p-value**	**Hazard ratio**	**p-value**	
Any presentation	Total	**0.27 (0.08-0.87)**	**0.03**	0.36 (0.10-1.23)	0.10	**0.14 (0.02-0.99)**	**0.05**	0.34
	Male	**1.91^{-8} (6.01^{-9}-6.04^{-8})**	**<0.0001**	**1.35^{-8} (4.12^{-9}-4.45^{-9})**	**<0.001**	**2.76^{-9} (7.66^{-10}-9.93^{-9})**	**<0.0001**	0.56
	Female	0.43 (0.13-1.36)	0.15	0.60 (0.16-2.30)	0.46	0.20 (0.03-1.50)	0.12	0.33
Infectious presentation	Total	**0.07 (0.01-0.45)**	**0.006**	**0.10 (0.01-0.75)**	**0.03**	**1.31^{-8} (5.64^{-9}-3.03^{-8})**	**<0.0001**	**<0.0001**
	Male	**1.91^{-9} (5.98^{-9}-6.07^{-8})**	**<0.0001**	**1.35^{-8} (4.08^{-9}-4.49^{-8})**	**<0.0001**	**2.76^{-9} (7.66^{-10}-9.93^{-9})**	**<0.0001**	**0.82**
	Female	**0.11 (0.02-0.78)**	**0.03**	0.18 (0.02-1.40)	0.10	**2.05^{-8} (6.27^{-9}-6.68^{-8})**	**<0.0001**	**<0.001**
Fever likely due to infection	Total	0.18 (0.02-1.31)	0.09	0.25 (0.03-1.91)	0.18	**4.79^{-8} (1.13^{-8}-2.04^{-7})**	**<0.0001**	**<0.0001**
	Male	**1.91^{-9} (5.29^{-9}-6.87^{-8})**	**<0.0001**	**1.35^{-8} (3.35^{-9}-5.47^{-8})**	**<0.0001**	**4.89^{-8} (1.04^{-8}-2.29^{-7})**	**<0.0001**	0.78
	Female	0.55 (0.06-4.97)	0.60	0.53 (0.06-4.77)	0.57	Too few to analyse		0.62
Serious bacterial infection	Total	0.31 (0.04-2.56)	0.28	0.31 (0.04-2.58)	0.28	Too few to analyse		0.31
	Male	**7.54^{-8} (7.23^{-9}-7.86^{-7})**	**<0.0001**	**8.83^{-8} (9.01^{-9}-8.66^{-7})**	**<0.0001**	Too few to analyse		**<0.0001**
	Female	0.37 (0.04-3.06)	0.35	0.35 (0.04-2.91)	0.33	Too few to analyse		0.38
Death	Total	1.18 (0.07-21.34)	0.91	1.25 (0.07-21.64)	0.88	Too few to analyse		0.87

BCG, Bacille Calmette Guerin; EPI, Expanded Programme of Immunisation

of EPI vaccinations and BCG in the delayed group, although the trend remained the same in the post-vaccination period. Reductions in fever and serious bacterial infections were also seen with BCG receipt at birth, although there were low event numbers and did not reach statistical significance.

When normal birth-weight infants were analysed alone, the pattern of reduction in infectious episodes in the period prior to receipt of EPI1/BCG in the delayed group remained (Table 7.13). However, both the point-estimate of effects and the significance of the findings were weakened compared to analysis of the entire data-set, reflecting the marked weighting of effects in LBW infants.

7.5.3 No clear differences in spectrum of infectious diseases were seen by BCG vaccination status.

Figure 7.39 shows the aetiology of infectious illness presentation by BCG vaccination status overall (Figure 7.39a) and in the first 6 weeks of life, prior to BCG vaccination in the delayed group (Figure 7.39b). The spectrum of infections was remarkably similar between the two groups. There was no clear evidence for a reduction in serious bacterial infections such as sepsis and LRTIs with BCG vaccination at birth, although frequency of these outcomes was small.

7.13 Frequencies and hazard ratios of physician diagnosed illness episodes in normal birth-weight infants (>2.5kg)

		Total frequency			Pre-EPI1+/-BCG			Post-EPI1+/-BCG		
		BCG at birth	BCG at 6wks	p-value	BCG at birth	BCG at 6wks	p-value	BCG at birth	BCG at 6wks	p-value
All presentations	Total	223	220	0.52	124	139	0.09	99	81	0.23
	Male	106	105	0.77	59	68	0.20	47	37	0.27
	Female	117	115	0.53	65	71	0.25	52	44	0.54
Infection	Total	185	187	0.39	**97**	**115**	**0.05**	88	72	0.26
	Male	83	86	0.51	42	55	0.07	41	31	0.23
	Female	102	101	0.55	55	60	0.32	47	41	0.68
Fever (reported or recorded)	Total	49	47	0.98	28	36	0.21	21	11	0.09
	Male	18	15	0.65	9	12	0.45	9	3	0.09
	Female	31	32	0.68	19	24	0.30	12	8	0.43
Serious Bacterial Infection	Total	32	27	0.62	21	22	0.75	11	5	0.15
	Male	17	14	0.64	11	10	0.88	6	4	0.56
	Female	15	13	0.82	10	12	0.56	5	1	0.12
Death	Total	5	2	0.28	3	2	0.69	2	0	0.17
	Male	3	2	0.68	2	2	0.97	1	0	0.32
	Female	2	0	0.17	1	0	0.33	1	0	0.33

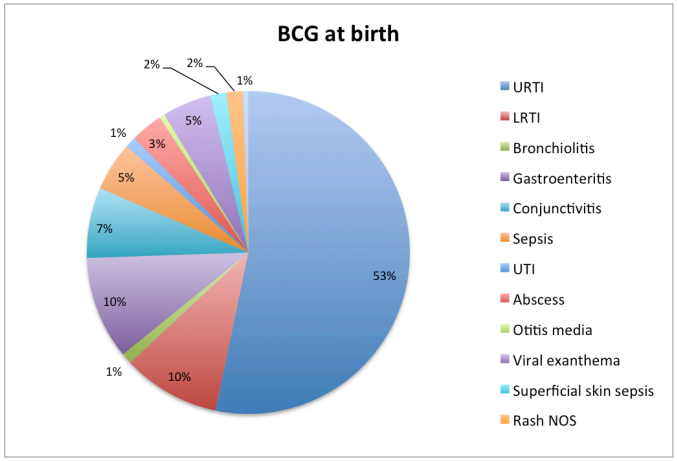
		Total follow-up		Pre-EPI-1 (and BCG in delayed group)		Post-EPI-1 (and BCG in delayed group)		Test for interaction pre/post EPI-1
		Hazard Ratio	p-value	Hazard Ratio	p-value	Hazard ratio	p-value	
Any presentation	Total	1.01 (0.85-1.19)	0.94	0.83 (0.64-1.08)	0.17	1.18 (0.94-1.49)	0.15	**0.05**
	Male	0.94 (0.73-1.21)	0.63	0.75 (0.51-1.10)	0.14	1.14 (0.82-1.57)	0.45	0.11
	Female	1.08 (0.86-1.34)	0.52	0.92 (0.64-1.32)	0.65	1.23 (0.90-1.70)	0.18	0.25
Infectious presentation	Total	0.99 (0.82-1.19)	0.89	0.79 (0.59-1.07)	0.12	1.16 (0.91-1.48)	0.22	**0.05**
	Male	0.91 (0.68-1.21)	0.51	**0.63 (0.40-1.00)**	**0.05**	1.18 (0.82-1.69)	0.38	**0.03**
	Female	1.07 (0.84-1.36)	0.58	0.96 (0.66-1.41)	0.85	1.17 (0.85-1.62)	0.33	0.45
Fever likely due to infection	Total	1.04 (0.70-1.53)	0.85	0.80 (0.47-1.36)	0.42	1.49 (0.82-2.73)	0.19	0.14
	Male	1.13 (0.56-2.25)	0.73	0.56 (0.21-1.46)	0.24	3.36 (0.96-11.72)	0.06	**0.03**
	Female	1.02 (0.64-1.62)	0.93	0.98 (0.52-1.84)	0.95	1.08 (0.53-2.23)	0.82	0.84
Serious bacterial infection	Total	1.17 (0.69-2.01)	0.55	0.95 (0.51-1.76)	0.86	1.99 (0.76-5.22)	0.16	0.18
	Male	1.14 (0.52-2.51)	0.74	0.96 (0.39-2.33)	0.93	1.60 (0.48-5.32)	0.44	0.44
	Female	1.22 (0.59-2.50)	0.60	0.93 (0.40-2.23)	0.89	Too few to analyse		0.27
Death	Total	1.99 (0.36-10.92)	0.43	0.99 (0.14-7.07)	0.99	Too few to analyse		

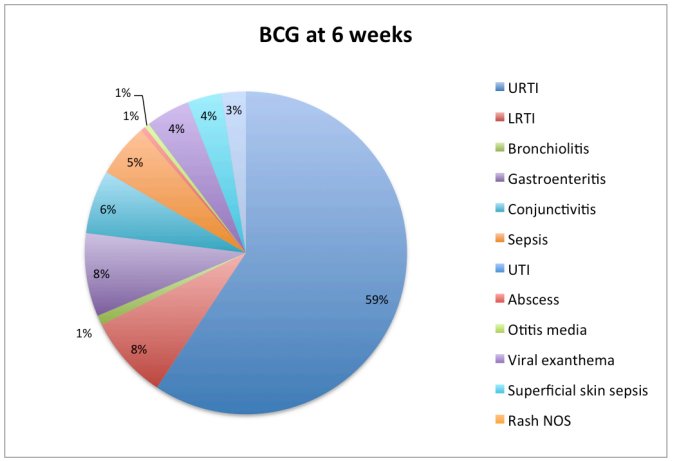
	Male	0.94 (0.13-6.70)	0.95	0.48 (0.04-5.28)	0.55	
	Female	Too few to analyse		Too few to analyse		Too few to analyse

BCG, Bacille Calmette Guerin; EPI, Expanded Programme of Immunisation

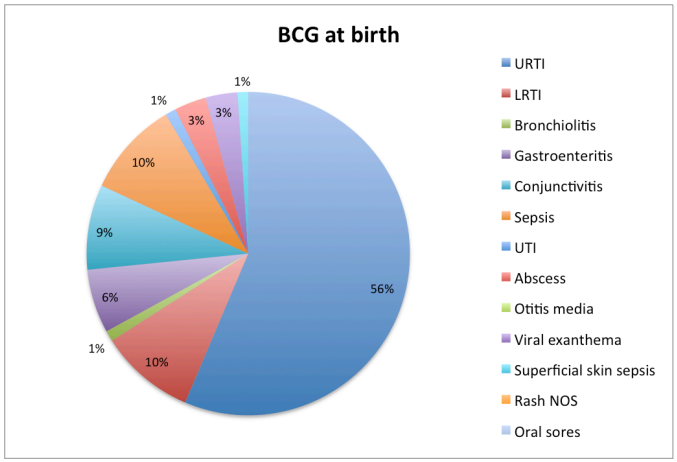
Figure 7.39. Aetiologies of infections by BCG status, a) for the total duration of study follow-up (early vs. delayed BCG) or b) for the first 6 weeks of life (BCG vaccinated vs. BCG unvaccinated infants)

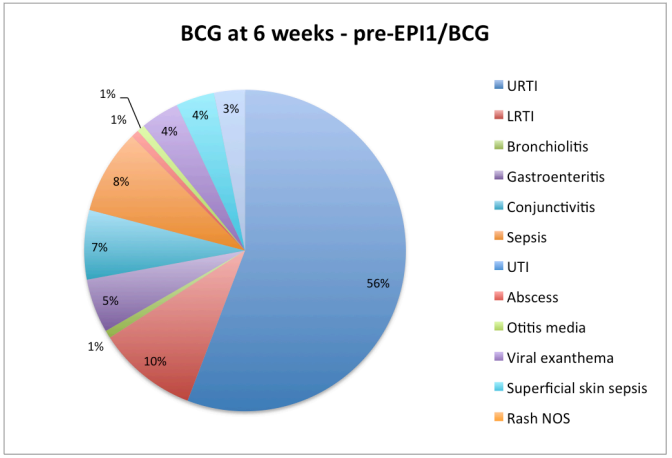
a)





b)





URTI, upper respiratory tract infection; LRTI, lower respiratory tract infection; UTI, urinary tract infection; NOS, not otherwise specified

7.5.3 No significant correlations between infection rates and scar size at 10 weeks were found

There were no significant correlations between total clinical illness events, infections or any other markers of illness and scar size measured at 10 weeks, when analysed together, by BCG status, or by BCG status and sex. These findings agree with the lack of clear correlations between scar size and non-specific immunological changes induced by BCG vaccination.

7.6 Paper 4: Post-immunization leucocytosis and its implications for the management of febrile infants.

London School of Hygiene & Tropical Medicine
Keppel Street, London WC1E 7HT
www.lshtm.ac.uk

Registry
T: +44(0)20 7299 4646
F: +44(0)20 7299 4656
E: registry@lshtm.ac.uk

LONDON SCHOOL of HYGIENE &TROPICAL MEDICINE

RESEARCH PAPER COVER SHEET

PLEASE NOTE THAT A COVER SHEET MUST BE COMPLETED FOR EACH RESEARCH PAPER INCLUDED IN A THESIS.

SECTION A – Student Details

Student	Sarah Prentice
Principal Supervisor	Stephen Cose
Thesis Title	Investigating the Non-Specific Effects of BCG in Neonates

If the Research Paper has previously been published please complete Section B, if not please move to Section C

SECTION B – Paper already published

Where was the work published?			
When was the work published?			
If the work was published prior to registration for your research degree, give a brief rationale for its inclusion			
Have you retained the copyright for the work?*		Was the work subject to academic peer review?	

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SECTION C – Prepared for publication, but not yet published

Where is the work intended to be published?	Vaccine
Please list the paper's authors in the intended authorship order:	Prentice S, Kamushaaga Z, Nash SB, Elliott AM, Dockrell HM, Cose S
Stage of publication	Accepted

SECTION D – Multi-authored work

For multi-authored work, give full details of your role in the research included in the paper and in the preparation of the paper. (Attach a further sheet if necessary)	I was responsible for the concept, design of the study, data analysis and manuscript preparation, with advice from SBN, HMD, AME and SC. I conducted all laboratory analyses other than automated erythrocyte counts which were conducted by ZK

Student Signature: [redacted] **Date:** 25/03/2018

Supervisor Signature: [redacted] **Date:** 25/03/2018

Post-immunization leucocytosis and its implications for the management of febrile infants

Dr Sarah Prentice[a,b*] sarah.prentice@lshtm.ac.uk

Mr Zephyrian Kamushaaga[b] zephyrian.kamushaaga@mrcuganda.org

Mr Stephen B Nash[c] stephen.nash@lshtm.ac.uk

Professor Alison M Elliott[a,b] alison.elliott@lshtm.ac.uk

Professor Hazel M Dockrell[d] hazel.dockrell@lshtm.ac.uk

Dr Stephen Cose[b,d] stephen.cose@lsthm.ac.uk

[a] Department of Clinical Research. London School of Hygiene and Tropical Medicine, Keppel Street, London, WC1E 7HT. Tel: +44 (0) 20 7636 8636

[b] MRC/UVRI Uganda Research Unit, 51-59 Nakiwogo Road, Entebbe, Uganda, PO Box 49

[c] Department of Infectious Disease Epidemiology. London School of Hygiene and Tropical Medicine, Keppel Street, London, WC1E 7HT. Tel: +44 (0) 20 7636 8636

[d] Department of Immunology and Infection. London School of Hygiene and Tropical Medicine, Keppel Street, London, WC1E 7HT. Tel: +44 (0) 20 79272466

*Corresponding author. Email sarah.prentice@lshtm.ac.uk.

Key words: Immunization, leucocytosis, fever, clinical management, infant.

Word count: 2686

Abstract

Aims: Clinical guidelines for management of infants with fever but no evident focus of infection recommend that those aged 1-3 months with a white cell count >$15x10^9$/litre have a full septic screen and be admitted for parenteral antibiotics. However, there is limited information about leucocyte changes following routine immunization, a common cause of fever. We investigated white cell counts shortly after routine immunization in Ugandan infants under 3 months of age.

Methods: White cell counts were measured in 212 healthy infants following routine immunizations (DTwP-HepB-Hib, oral polio and pneumococcal conjugate 7 vaccines) received prior to 3 months of age.

Results: Mean leucocyte counts increased from $9.03x10^9$/l (95% confidence interval 8.59-$9.47x10^9$/l) pre-immunizations to $16.46x10^9$/l (15.4-$17.52x10^9$/l) at one-day post-immunizations at 6 weeks of age, and $15.21x10^9$/l (14.07-$16.36x10^9$/l) at one-day post-immunizations at 10 weeks of age. The leucocytosis was primarily a neutrophilia, with neutrophil percentages one-day post-immunization of 49% at 6 weeks of age and 46% at 10 weeks of age. White cell parameters returned to baseline by two-days post-immunization. No participant received antibiotics when presenting with isolated fever post-immunization and all remained well at follow-up.

Conclusions: In our study almost half the children <3 months old presenting with fever but no evident focus of infection at one-day post-immunization met commonly used criteria for full septic screen and admission for parenteral antibiotics, despite having no serious bacterial infection. These findings add to the growing body of literature that questions the utility of

white blood cell measurement in identification of young infants at risk of serious bacterial infections, particularly in the context of recent immunizations, and suggest that further exploration of the effect of different immunization regimes on white cell counts is needed.

This observational work was nested within a clinical trial, registration number ISRCTN59683017

Introduction

Fever is one of the most common reasons for presentation of children to medical professionals [1]. Children presenting with no obvious focus for their infection can pose a diagnostic challenge to clinicians. Algorithms exist to assist in the identification of children who would benefit from investigation and admission to hospital for treatment. These guidelines are particularly stringent for febrile infants less than 3 months old, due to the increased risk of occult serious bacterial infections [2]. Guidelines used in the UK [3], and in adapted forms worldwide, advise that a full blood count and partial septic screen should be performed on any infant presenting with a fever >38˚C without focus when less than 3 months of age, even if otherwise well-looking and regardless of recent immunization history. Infants who have a white cell count of $>15x10^9$/l are then admitted to hospital for a full septic screen, including lumbar puncture, and parenteral antibiotics whilst culture results are pending (usually a minimum of 48 hours).

Infants worldwide commonly receive a number of vaccinations in the first few months of life, generally with multiple antigens administered on one day [4]. These vaccines are highly immunostimulatory and the occurrence of fever >38˚C following routine vaccinations is well recognised. However, the effect on white cell counts of the co-administration of multiple vaccine antigens, such as those received during primary immunizations, is unknown. Studies conducted in the 1980s in Finland and the USA in a small number of older infants, showed an increase in white cell counts post administration of the combined Diptheria-Tetanus- whole cell Pertussis (DTwP) vaccination [5]. However, few similar studies have been published looking at younger infants and using the enhanced combination of vaccine antigens currently in use.

Lack of knowledge regarding alterations to white cell count levels following routine immunization could severely impede clinical decision making during the assessment of a feverish child. This may have negative consequences for the child due to unnecessary invasive investigations and antibiotic administration. This study investigated alterations to white cell counts during the period immediately following routine immunization, in the first 3 months of life.

Methods

Post-immunization blood samples were collected from 212 Ugandan infants as part of a randomised controlled trial investigating the impact of BCG vaccination on the innate immune system (described elsewhere [6]). In brief, infants were randomised to receive BCG either at birth or at 6 weeks of age. All other routine immunizations were given as per Ugandan national guidelines: oral polio vaccine (OPV) at birth and pentavalent vaccine (diptheria-tetanus-whole-cell pertussis/*Haemophilus influenzae* B/Hepatitis B), OPV and pneumococcal vaccine (PCV10) at 6 weeks, 10 weeks and 14 weeks of age (hereafter referred to as 'primary immunization'). Infants were then randomly assigned to have venous blood samples taken on two out of four possible time points: 1) 5 days of age, 2) 6 weeks of age, 1 day following immunization, 3) 6 weeks of age, 5 days following routine immunization and 4) 10 weeks of age, 1 day following routine immunization. In practice, blood samples were taken at a range of times post-routine immunization, due to delayed attendance at clinic for some participants. Infants with blood samples taken more than 15 days following immunization were excluded from analysis (n=1). BCG vaccination in the delayed group was given after blood sample 2 but prior to blood sample 3. However, upon analysis, no significant impact of the different BCG schedules on white blood cell count was shown and data were analysed together.

Anthropometry, vital sign measurement and clinician review of participants occurred at each appointment. Temperatures were measured using a digital axillary thermometer, following current best practice recommendations. Active follow-up of participants occurred for the duration of the trial with open access to clinician review and treatment, as well as weekly telephone follow-up, to confirm health status.

Full blood counts were obtained using the automated Coulter AcT 5diff CP (Beckman-Coulter, California, USA), from 0.5ml of venous blood drawn from the dorsum of the hands or feet into an EDTA containing microtainer (Becton-Dickson).

Data were analysed using STATA version 14.1 (StataCorp, Texas, USA) and graphs produced using Prism 6 (GraphPad Software, Inc. California, USA). Results were normally distributed so means with 95% confidence intervals are reported, with Student's t-test used for comparison of means pre- and post-immunization. Changes in mean values over time were analysed using a random effects model to account for repeated measurements and including both linear and quadratic terms for time to allow for a non-linear relationship.

Ethical approval for the trial was obtained from the Uganda Virus Research Institute Research and Ethics Committee (Ref: GC/127/13/11/432), the Uganda National Council for Science and Technology (Ref: HS 1524), The Office of the President of Uganda and the London School of Hygiene & Tropical Medicine (Ref: 6545). The study was conducted according to the principles of the Declaration of Helsinki. Written, informed consent of mothers was obtained by trained study nurses prior to any procedures.

Results

Two hundred and twelve infants provided blood samples for this study, 49% of them male. The background of the population was East African, primarily of the Buganda tribe and participants came from a mixture of urban, semi-urban and rural fishing communities. No participant was severely malnourished at the time of blood sample collection.

Average white cell counts were significantly increased at one-day post receipt of primary immunizations at both 6 weeks of age ($16.46x10^9$/l (95% confidence interval 15.40-$17.52x10^9$/l) and 10 weeks of age ($15.21x10^9$/l (14.07 -$16.36x10^9$/l)), compared to pre-immunization values (9.03 $x10^9$/l (8.59-9.47 $x10^9$/l), p-values for difference with post-immunization levels <0.0001, see Table 1 and Figure 1).

This rise in mean total leucocytes was short-lived, returning to levels not significantly different from baseline by two days post-immunization, but continuing to decline up to six-days post-immunization ($p<0.0001$) (Figure 2). Although mean white cell counts at one day post-immunization fell within the normal range expected for age (5.0-19.5 $x10^9$/l) [7], there was a wide range of values (8.00-32.90 $x10^9$/l at one-day post 6-week immunization and 6.20-29.80 $x10^9$/l at one day post 10-week immunization). At both time-points an average of 22% of white cell counts measured fell outside of the normal range for age. At one day post-immunization, on average 53% of measured white cell counts were above the $15x10^9$/l cut-off for further intervention when managing a febrile child <3 months old (Figure 1).

The leucocytosis observed at one-day post immunization was primarily a neutrophilia (Table 1 and Figure 3). Little change occurred to total lymphocyte levels, other than an expected increase with age (see Figure 3). As a result at one-day post-primary immunization, the percentage of the white cell count made up by lymphocytes dropped as the percentage

accounted for by neutrophils increased (Table 1 and Figure 3). The average percentage of neutrophils was above the normal range for age (up to 32% neutrophils [7]) at one-day post-primary immunization at both 6 weeks of age (49%) and 10 weeks of age (46%). Total monocyte and basophil levels mimicked changes to neutrophils post-immunization, though to a much smaller extent (Table 1). The reverse occurred with eosinophils, with total eosinophils dropping at 1-day post-immunization and rising by day 2. The changes to monocyte, basophil and eosinophil count were only significant at the 6-week time-point. There was little change to the percentage of monocytes, eosinophils and basophils by immunization status.

Linear regression analysis provided good evidence ($p<0.0001$) of a weak, positive association of temperature and white cell counts, with each one degree Celsius increase in temperature associated with a $0.04x10^9$/l increase in white cell count (Figure 4). Of all children studied that presented with a fever >38˚C when the blood sample was taken, 5 out of 11 (45%) had a white cell count above the currently recommended threshold for further investigation and inpatient management with IV antibiotics. A further 17 mothers reported that their children had been pyrexial prior to presentation. Of these, 3 (18%) had white cell counts above 15 $x10^9$/l. All children presenting with either fever >38˚C or with parental report of fever were clinically assessed as being well and treated conservatively as outpatients without antibiotics. All remained well at follow-up and no cases of serious bacterial infection occurred. Eighty-five infants had white cell counts >15 $x10^9$/l, but were afebrile, with 28 of these having white cell counts above the normal reference range for age.

These data provided no evidence that either BCG immunization status or gender had any impact on results. There was also no evidence of a difference in mean haemoglobin and

platelet counts comparing pre- and post-immunization levels, other than an expected decrease in haemoglobin with increasing infant age (see Table 1).

Discussion

This study shows a rapid and large increase in white cells, primarily neutrophils, occurring in infants < 3 months old immediately following primary immunizations. This increase is above current guideline thresholds for further investigation and treatment in nearly half of febrile infants studied and above the normal white cell count range for age in more than a quarter of infants studied. These infants all remained well during the post-immunization period, in the absence of intervention, and mean white cell counts returned to baseline by two-days post-immunization. These infants therefore represent a group that may cause diagnostic confusion and undergo unnecessary investigations and interventions if they present to a clinician febrile, or if they have a blood test taken for an unrelated condition, at one-day post-immunization. The development of new post-immunization reference ranges could help to mitigate this. In the absence of other data for our population, our study would suggest a reference range of total leucocytes: $7.76x10^9$/l - $27.25x10^9$/l, percentage neutrophils: 29%-65% (2.5^{th}-97.5^{th} centiles [8]) as appropriate for infants less than 3 months old, one-day following routine immunizations.

This study's strengths lie in its comparatively large study numbers, giving robust results, and the presence of blood samples from a variety of time-points post-primary immunizations, allowing the timing of changes in white cell counts post-primary immunizations to be investigated. The close follow-up of participants during the post-immunization period provides reassuring evidence that children with fevers and high white cell counts immediately following immunization can remain well without further intervention.

The study has a number of limitations. Firstly, it is a secondary analysis conducted as part of a larger randomized controlled trial that was not specifically designed to look at white cell counts in post-immunization pyrexia. As a result, the number of febrile infants in the study was limited. However, the correlation between temperature and white cell count seen in our study suggests that these results can be extrapolated to febrile infants more generally, with higher white cell counts expected in those infants that have post-immunization pyrexia. Supporting this theory, a study investigating serious bacterial infections in recently immunized infants in the USA similarly showed an increase in white cell counts in recently immunized febrile infants with no serious bacterial infection [9]. The finding of increased white cell counts in afebrile infants post-immunization is also important, as nearly a quarter of cases in our study fell outside the normal range. These cases might cause diagnostic confusion if blood is sampled following immunizations for another reason.

Another limitation of this study is that the time course of changes to white cell counts post-immunizations could be examined only because some participants did not attend their per-protocol appointments at the correct time (24 hours or 5 days post-immunizations). It may be argued that these participants represent a different sub-set of the population, for instance infants who had fewer post-immunization symptoms, and may therefore have falsely lower white cell counts than the population as a whole. However, the time-course of white cell count changes followed a logical pattern with average levels declining until day 7 post-immunization (which encompassed the per-protocol appointment day 5 post-immunizations) and mirrored the time-course of changes to IL-6 and CRP that have been shown post-DTwP immunization in another study [10]. Also, the timing of blood samples used to obtain pre-immunization average white cell counts was at an average of 10 days of post-natal age, rather than immediately prior to the receipt of primary immunizations, due to the design

requirements of the parent trial. This comparison was deemed to be acceptable, however, as white cell counts are known to be high at birth, falling to adult levels by approximately 2 weeks of age [11]. Samples taken at an average of 10 days of post-natal age would therefore be more likely to under-estimate the degree of change in white cell counts following primary immunizations, rather than falsely over-estimate it.

The generalizability of this study's findings may be limited due to its restricted study population and the choice of vaccine combination used for primary immunization. As the study was conducted in Uganda, the ethnicity of infants was solely black African. White cell counts in black Africans, however, tend to be lower than in other ethnic groups [12-14]. It is therefore possible that white cell count changes post-primary immunization would be at least as marked, if not more, in other populations. Previous studies conducted in white European and mixed American populations have also shown white cell count increases at one-day post-immunization [5, 9], though to a lesser extent than with the combination of vaccines used in this study.

The combination of vaccines used as primary immunizations is not the same throughout the world and this may limit the global applicability of these findings. Most primary immunization regimes include components against diptheria, tetanus, pertussis, *Haemophilus influenzae* type B and pneumococcus (as were included in this study) [4]. However, the use of oral polio vaccine has been replaced in high-income countries with an inactivated vaccine [15], and immunization against hepatitis B is often only given to those deemed at high risk. Additional vaccines, not used in this study, such as meningococcal and rotavirus vaccine are also commonplace in many other areas of the world. The differences in vaccine components used may cause variations in the degree of post-immunization leukocytosis. Of these, the replacement of whole cell pertussis (used in this study and in many low income countries as part of the 5-in-1 vaccine) with acellular pertussis (used in many European and North

American countries) may have the most impact on post-immunization leukocytosis [10, 16], though a study into serious bacterial infections in the context of post-immunization pyrexia used DTaP and also revealed a raised white cell count post-immunizations [9]. A previous study conducted in Gambian neonates [17] showed no increase in white cell counts following oral polio and hepatitis B vaccination (as well as BCG), suggesting that it was not these components of primary immunizations that were responsible for post-immunization leukocytosis (unpublished findings), and thus the discontinuation of their use in high-income countries might not affect results. The addition of further antigens/adjuvants/vaccines to the basic vaccine combination used in this study may be hypothesized to further increase immunostimulation and white cell counts, rather than diminish them. Thus, the recommendations of this study may be a conservative estimation of changes occurring in other areas of the world. However, further studies in different settings would be necessary for the development of a robust global reference range for post-immunization white cell counts. The timing of primary immunizations also varies globally, which may affect a child's post-immunization white cell count response. However, this study showed similar increases in white cell counts at 6 weeks and 10 weeks of age, suggesting that small variations in immunization timing are unlikely to affect overall responses.

This study adds to the current debate regarding the utility of white cell counts in the assessment of children who present febrile with no clear focus for infection. Since the introduction of immunizations against *Streptococcus pneumonia* and *Haemophilus influenza*, the incidence of serious bacterial infections in young febrile infants has reduced [18]. Several studies have subsequently found that a cut-off of $15x10^9$/litre white cells is neither sensitive nor specific for the identification of serious bacterial infections in febrile children [19-27]. Newer proposed algorithms for assessment of fever with no focus have tended to relegate this

parameter in favour of other markers of infection, such as CRP and procalcitonin [28, 29]. However, these new algorithms have not been widely adopted at present. We suggest that, particularly in the context of immunization within the previous 24 hours, white cell count should not be used as a discriminatory factor when deciding whether to admit and treat children under the age of 3 months old who present with fever and no source of infection. If the use of white cell counts is continued, we suggest that policymakers consider introducing either a higher white cell count threshold for further investigation and management in an otherwise well child <3 months old presenting one day post-immunizations, or a provision for a 24-hour observation period with repeat white cell count, into the current guidelines for the treatment of febrile infants. This would reduce harm to patients by avoiding unnecessary invasive procedures and antibiotics, and reduce the burden on paediatric healthcare systems.

Declaration of competing interests All authors have completed the Unified Competing Interest form at www.icmje.org/coi_disclosure.pdf (available on request from the corresponding author) and declare that SP, ZK, HMD, AME and SC have no financial or non-financial interests that may be relevant to the submitted work.

Details of contributors SP was responsible for the study design, conduct, data collection, data analysis and manuscript preparation. ZK performed the white cell counts using the automated Coulter Counter. SN provided statistical support. HMD, AME and SC provided advice and support for all aspects of the above work including manuscript preparation. All authors read and approved the final manuscript.

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and sponsor had no input in the study design; in the collection, analysis, and interpretation of data; in the writing of the report; and in the decision to submit the article for publication. The researchers and funder remain independent. AME was supported by Wellcome Trust grant number 095778, SC by Wellcome Trust grant number 084344 and MRC grant number MR/K019708.

SP and SN had full access to the data (including statistical reports and tables) and can take responsibility for the integrity of the data and the accuracy of the data analysis.

Transparency Declaration SP affirms that the manuscript is an honest, accurate, and transparent account of the study being reported; that no important aspects of the study have been omitted; and that any discrepancies from the study as planned and registered have been explained. Some of these results were presented at the Royal College of Paediatrics and Child Health Conference, April 2016.

Data sharing statement Data is available upon request from the principal author.

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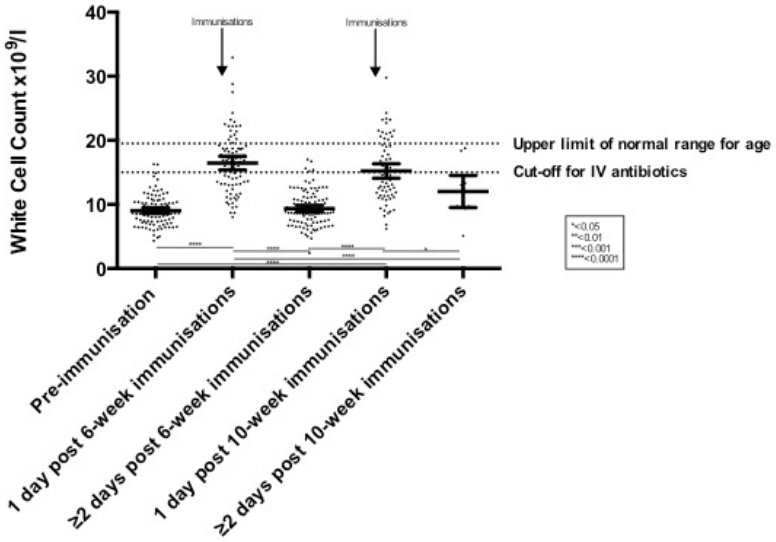
Tables and Figures, Titles and Legends

Table 1. Blood count parameters in relation to primary immunizations

CI: confidence interval

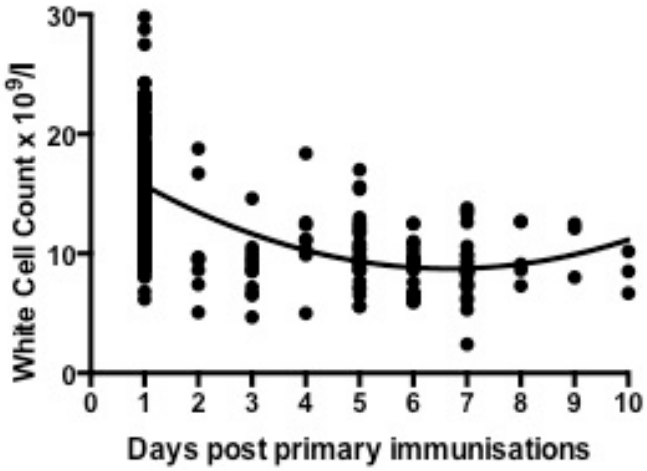
	Pre-immunizations.	**6 weeks of age**		**10 weeks of age**	
	Mean age 10 days (range 2-19 days) **n=106**	**1-day post-primary immunizations** **n=81**	**≥2-days post-primary immunization (mean 5.7 days)** **n=111**	**1-day post-primary immunization** **n=70**	**≥2-days post primary immunization (mean 3.4 days)** **n=12**
Total White Cell Count x10^9/l (95% CI)	9.03 (8.59-9.47)	16.46 (15.40-17.52)	9.34 (8.84-9.84)	15.21 (14.07-16.36)	12.02 (9.51-14.52)
Subset Counts x10^9/l (95% CI)					
Neutrophils	2.65 (2.42-2.88)	8.58 (7.64-9.52)	2.21 (2.02-2.41)	7.00 (6.36-7.64)	3.07 (1.94-4.20)
Lymphocytes	4.64 (4.40-4.87)	6.15 (5.78-6.51)	5.81 (5.48-6.14)	6.24 (5.73-6.76)	7.22 (5.80-8.64)
Monocytes	1.13 (1.04-1.22)	1.70 (1.55-1.85)	0.92 (0.85-0.99)	1.43 (1.27-1.58)	1.06 (0.74-1.38)
Eosinophils	0.35 (0.31-0.38)	0.14 (0.12-0.16)	0.27 (0.23-0.30)	0.33 (0.28-0.38)	0.46 (0.26-0.66)
Basophils	0.26 (0.22-0.31)	0.26 (0.23-0.29)	0.14 (0.12-0.15)	0.22 (0.19-0.25)	0.21 (0.15-0.27)
Percentage (95% CI)					
Neutrophils	28.6 (27.08-30.12)	48.89 (46.98-50.80)	23.54 (22.12-24.97)	45.87 (43.93-47.81)	24.96 (19.54-30.37)
Lymphocytes	52.19 (50.36-54.03)	38.42 (36.58-40.27)	62.28 (60.68-63.88)	41.41 (39.42-43.39)	61.05 (54.72-67.37)
Monocytes	12.44 (11.79-13.09)	10.30 (9.77-10.82)	9.86 (9.33-10.38)	9.22 (8.71-9.73)	8.40 (7.08-9.72)
Eosinophils	3.96 (3.52-4.39)	0.88 (0.78-0.97)	2.92 (2.52-3.32)	2.17 (1.90-2.44)	3.94 (2.35-5.53)
Basophils	2.80 (2.38-3.21)	1.48 (1.39-1.58)	1.41 (1.33-1.49)	1.32 (1.22-1.43)	1.67 (1.35-1.99)
Haemoglobin g/dl (95% CI)	15.98 (15.57-16.38)	10.68 (10.39-10.96)	11.09 (10.87-11.31)	10.30 (10.07-10.54)	10.79 (10.24-11.34)
Platelet counts x10^9/l (95% CI)	362.10 (337.08-387.11)	524.52 (493.86-555.17)	575.62 (547.11-604.13)	443.26 (416.87-469.64)	520.42 (455.12-585.71)

Figure 1. Total white cell counts by immunization status



Individual data points are represented by dots. Error bars display the 95% confidence interval.

Figure 2. White cell count by time post-immunizations



Individual data points are represented by dots. The line represents results of the random effects regression model.

Figure 3. Total and percentage neutrophils and leucocytes by immunization status

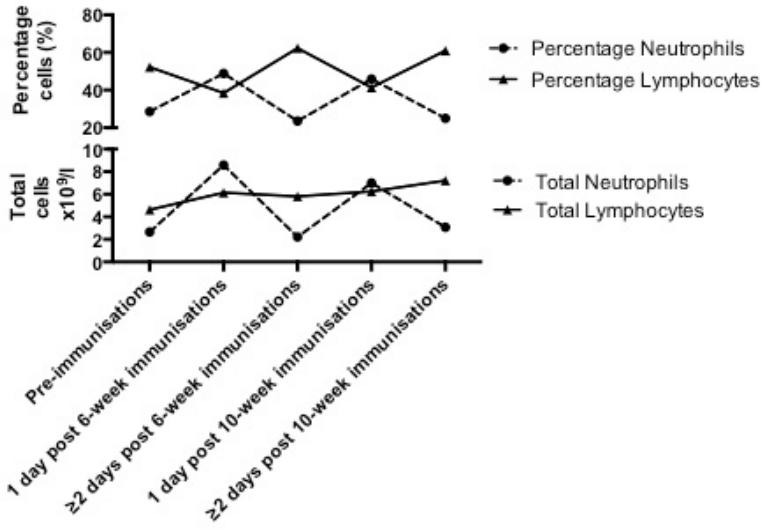
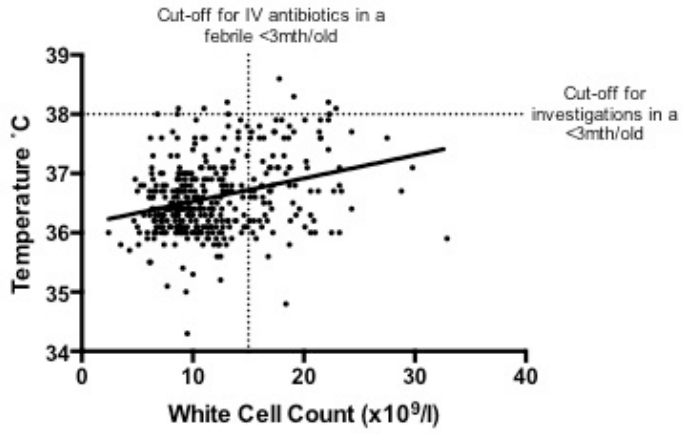


Figure 4. Axillary temperature of children in relation to their white cell count



Individual data points are represented by dots. The line represents results of the linear regression model

8. Discussion, summary and conclusions

A number of epidemiological studies and clinical trials have suggested that BCG has beneficial effects against non-tuberculous infectious disease in high mortality settings. The work described in this thesis aimed to answer some outstanding questions that have hitherto limited the acceptance of the NSE theory, namely:

1. Are the clinical beneficial NSE of BCG vaccination present in high-mortality settings other than Guinea-Bissau and in normal term neonates?
2. If BCG vaccination induces NSE in neonates, what immunological mechanisms underlie this?

For both of these questions, the work also aimed to interrogate whether the clinical and immunological NSE of BCG were different in males and females, and whether altering the timing of BCG vaccination impacted any NSE.

8.1. Are the beneficial NSE of BCG globally applicable?

This study, carried out in healthy neonates in Uganda, showed a 29% reduction in episodes of infection in the first 6 weeks of life in infants who had been BCG vaccinated at birth compared to infants who had not. Consistent similar trends towards reductions in febrile illness, serious bacterial infections and death were also seen, although these were not statistically significant. Reductions were in all-cause infectious disease with no clear pronounced effects on specific aetiologies such as sepsis or ALRI. As with the trials conducted in Guinea-Bissau, reductions in all-cause infectious outcomes were particularly pronounced in low birthweight infants.

These findings support previous clinical trial and epidemiological work conducted in high mortality settings. The WHO-commissioned meta-analysis of trials conducted up to March 2013, suggested a 30% reduction in all-cause mortality with BCG vaccination compared to none,[38] as did a trial from Guinea-Bissau in low birthweight infants that was published subsequently.[53] The point-estimate for reduction in all-cause infection in this study is strikingly similar. The reduction in all-cause infection rate seen in this study may not necessarily lead to comparable changes in all-cause mortality, though, for instance if the reduction occurs solely in low-grade, self-limiting infections. However, it may be reasonably extrapolated to have some effect on mortality due to its consistency with other studies and the finding of similar, though non-significant, reductions in serious bacterial infections and deaths. The small sample size and lower infant mortality rate in this study setting, compared to other trials, produced limited power to detect significant differences in these parameters.

The previous trials, conducted in Guinea-Bissau, suggested that reduction in all-cause mortality were most pronounced for sepsis and ALRI.[51-53] This study did not confirm these findings, although this may have been due to the small numbers of these outcomes. As sepsis and ALRI are the most common causes of infectious death in early infancy, the reductions seen in previous studies may also simply have reflected the increased power to detect significant differences in these causes of death, due to the increased event numbers, even if BCG has a broad impact on all-causes of infectious disease as suggested by this study.

8.1.1 Does the timing of BCG alter its beneficial NSE?

The hazard rate ratio for infection episodes was reversed in this study after the delayed group had received BCG vaccination at 6 weeks of age. The point-estimate for reduction in infection episodes in infants receiving BCG at 6 weeks of age, compared to those who had received BCG at birth was 21%. Although this was not statistically significant in itself, the difference in the hazard ratios before and after BCG vaccination of the delayed group was significant. The power to detect significant differences in illness event outcomes during the later time-period would have been limited compared to the earlier time-period due to the slightly shorter follow-up time (4 weeks compared to 6 weeks) and the reduction in incidence of infections occurring in later infancy compared to the neonatal period. Again, the trend toward reduced hazard rate ratios with BCG as the most recent vaccination was seen consistently when more objective measures of infection, such as fever, were assessed and when restricted to serious bacterial infections.

The finding of varying hazard rate ratios for infectious diseases by time may be interpreted in a number of different ways:

Clinical interpretation 1: BCG vaccination has beneficial NSE when given at any time during infancy, the benefits are greatest immediately after vaccination (e.g. infants receiving BCG at 6 weeks show a reduction in infection episodes acutely, compared to those receiving it at birth) but with time the effects of BCG converge so that protection against non-tuberculous disease is equivalent no matter when BCG was received. If this is the case, then BCG at birth would have the greatest effect on infant morbidity and mortality overall, due to high rates in the neonatal period, but receipt of BCG at any time would be beneficial.

Clinical interpretation 2: The NSE of BCG vaccination are greater with BCG given later in infancy compared to at birth, leading to durably enhanced protection against heterologous disease. If this is the case, then the benefits of delaying BCG beyond the neonatal period would depend on the duration of the enhanced NSE. If the non-specific protection against infectious disease is prolonged, then this may have a greater overall beneficial effect than the protection afforded in the early neonatal period. However, with short durations of effects, BCG in the neonatal period is still likely to have the greatest overall impact on infectious disease incidence and mortality, due to the higher risks in early life.

Clinical interpretation 3: Receipt of EPI-1 vaccinations reduces the beneficial NSE of BCG given at birth and this is countered by receipt of BCG concurrently with EPI-1. If this is the case, then receipt of BCG at 6 weeks would be likely to produce better protection against infectious disease only until receipt of the next dose of EPI vaccinations, at 10 weeks. This short duration of enhanced protection with delayed BCG would be unlikely to lead to overall reductions in infectious disease incidence compared to BCG at birth, due to the lower rates of infections. Thus, if this is the case, BCG at birth would still be the most effective vaccination regime for non-specific protection against infectious disease incidence and mortality, although it might suggest that a booster vaccination with BCG at the end of the EPI course could be beneficial.

The short duration of follow-up in this study makes it impossible to determine which of the above interpretations regarding the effects of delaying BCG vaccination is correct. Follow-up from the three clinical trials conducted in low birthweight infants in Guinea-Bissau showed that BCG at birth was associated with a 16% non-significant reduction in all-cause mortality at 12 months, compared to infants receiving BCG at 6 weeks of age.[51-53] The majority of benefit from early BCG was confined to the period prior to when BCG was received in the delayed group. These findings would favour Clinical Interpretations 1 or 3 above, suggesting that the NSE of BCG are not confined to BCG in the neonatal period but that BCG vaccination at birth produces the greatest overall benefit due to protection in the high-risk early neonatal period. Conversely, meta-analysis of several cohort studies, comparing BCG before DTP with BCG/DTP co-administration or BCG after DTP, suggests that reductions in all-cause mortality are more pronounced with the later regimes (relative risk 0.60 (0.42-0.86) with BCG co-administered with or after DTP, compared to BCG before DTP, with follow-up ranging from 5 months to 2 years).[10, 11, 14, 15] On face value, these findings would support interpretation 2 or 3 above. However, follow-up in each of these studies commenced from timing of DTP administration (i.e. 6 weeks of age), and hence the impact of NSE of BCG in the neonatal period was not accounted for. Therefore, the findings of these epidemiological studies are similar to the findings in this study (reduced infectious disease incidence with BCG as the most recent vaccination) but they do not provide good evidence as to the overall benefit of different regimes on total infant infectious disease rates. On-balance, the pronounced beneficial effects of early BCG shown in this study, and other clinical trials, support the assertion that the greatest overall benefit in terms of infant infectious disease incidence is likely to be obtained with BCG at birth.

The findings of benefits with, or after EPI vaccinations may suggest that a booster dose of BCG at the end of the EPI course would provide extra benefit. However, the one study to investigate this thus far, did not show a significant beneficial effect.[33]

8.1.2 Do the NSE of BCG occur differently in male and female infants?

The non-specific beneficial effect of BCG on reducing all-cause infectious disease in this study was consistently more pronounced in male infants. In the first 6 weeks of life, BCG vaccinated male infants had a 43% reduced risk of infections compared to unvaccinated infants. Male infants receiving BCG at 6 weeks of age then had a non-significantly reduced risk of infections compared to male infants receiving BCG at birth, during the following 4 weeks. Although similar trends were seen in female infants, the point-estimates were lower and not statistically significant. The opposing infection risk ratios before and after BCG vaccination of the delayed group were statistically significant only in male infants (p=0.01). Thus, although this study provides evidence to suggest beneficial NSE in all infants, they appear to be particularly marked in boys.

The evidence surrounding a differential heterologous effect of BCG by sex from previous studies has been equivocal. Epidemiological studies have tended to suggest that the beneficial effects of live vaccines, and the detrimental effects of inactive vaccines, both occur to a greater extent in female infants.[11, 16, 22, 23, 31, 62] This study did not provide good evidence to support either assertion, with beneficial effects of BCG being less pronounced in female than male infants, and no clear impact of inactive vaccines shown when BCG was given either before or concurrently with EPI vaccination. A true negative effect of inactive vaccinations in girls may not have been detected in this study because all infants received OPV (a live vaccination) at birth and concurrently with EPI vaccinations, which may have modified any negative effects. Also, no infant received EPI vaccinations in the absence of BCG (given either prior to EPI or concurrently). Thus, if negative NSE of inactive vaccinations are only seen in the absence of any BCG or live vaccination, this study would not have been able to detect them. Although vaccinations are often delayed in low-income countries, catch-up immunisations generally involve a combination of inactive and live vaccinations. The study provides reassuring evidence that, in the context of these mixed vaccination

regimes, inactive vaccinations do not appear to have negative non-specific effects during the first 10 weeks of life.

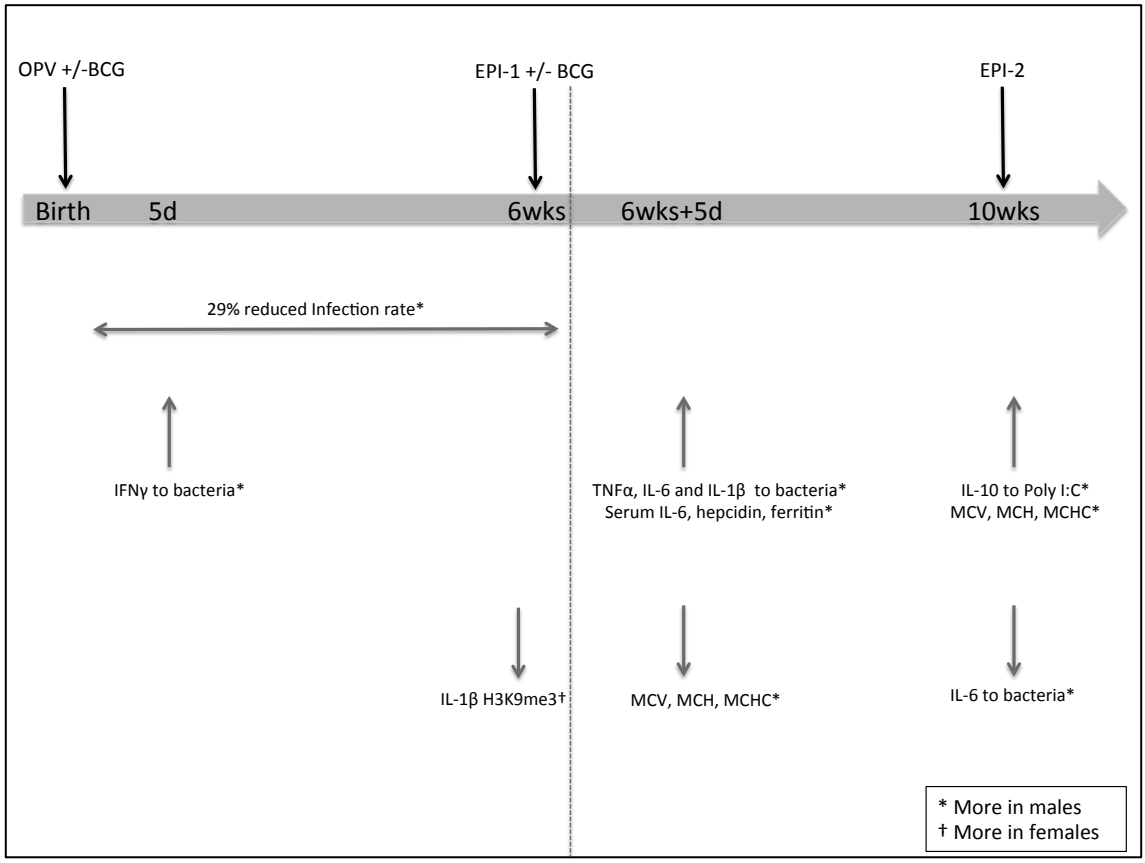
Although no previous single clinical trial investigating the NSE of early vs. delayed BCG has shown significant sex-differential effects, a meta-analysis of the three Guinea-Bissau trials conducted in low birthweight infants suggested a marked beneficial effect of BCG at birth in male infants during the first week of life (64% reduction in all-cause mortality), with beneficial effects in female infants seen from weeks 2-4 (44% reduction in all-cause mortality).[35] This Ugandan study did not wholly confirm these findings, with incidence of infectious disease throughout the first 6 weeks of life being very similar in female infants who were BCG vaccinated compared to BCG naïve. Indeed, even in male infants, the beneficial effects of BCG on reduction in infectious disease incidence appeared to occur after the first week of life (see Results Graph 7.38), a finding that is corroborated by the lack of significant differences within the first 96 hours of life seen in the associated Gambian pilot study.[259]

8.2. What are the immunological mechanisms responsible for the NSE of BCG in neonates?

Studies conducted in adults have suggested that BCG mediates its beneficial NSE by epigenetic modifications at the promoter region of pro-inflammatory cytokines, resulting in increased production of these cytokines upon heterologous pathogen challenge. This study investigated whether comparable epigenetic modifications, and their associated downstream effects, occur in infants following BCG vaccination.

The main significant findings, when comparing group geometric mean outcomes at individual time-points in this study, were seen at 6 weeks of age, 5 days after EPI vaccinations in all infants, and BCG vaccination in the delayed group (S3). At this time-point, *in vitro* bacterial stimulated pro-inflammatory cytokine production was higher in infants who had received BCG at birth, than in infants who had received BCG at 6 weeks, as were serum levels of IL-6. These differences were only significant for male infants. Male infants who were BCG vaccinated at birth also had significantly increased IFNγ production following *in vitro* bacterial stimulation at 5 days of age (S1), increased IL-10 production following *in vitro* stimulation with Poly I:C at 10 weeks of age (S4) and decreased IL-6 production following *in vitro* bacterial stimulation at 10 weeks of age (S4), compared to male infants BCG vaccinated at 6 weeks. MCV, MCH and MCHC in male infants BCG vaccinated at birth, were lower at 6 weeks, 5 days after EPI+/-BCG (S3) and higher at 10 weeks (S4) than in male infants BCG vaccinated at 6 weeks. There was a clear trend toward reduced H3K4me3 (stimulatory) and H3K9me3 (inhibitory) epigenetic modifications at the promoter regions of pro-inflammatory cytokines at 6 weeks of age, pre-immunisations (S2) in BCG vaccinated infants, compared to those who had not been vaccinated. This was statistically significant for H3K9me3 at the IL-1b promoter region. However, when analysed by sex, the decreased H3K9me3 appeared to be limited to female infants and the decreased H3K4me3 limited to male infants (though non-significantly). The significant changes are summarised in Figure 8.1.

Figure 8.2. Significant effects of BCG at birth, compared to BCG at 6 weeks of age, on measured outcomes



OPV ,oral polio vaccine; BCG, Bacille Calmette Guerin; EPI, Expanded Programme of Immunisations; d, days; wks, weeks; IFN, interferon; TNF, tumour necrosis factor; IL, interleukin; Poly I:C, polyinosinic:polycytidylic acid; H3K9me3, histone-3 lysine-9 trimethylation; MCV, mean cell volume; MCH, mean cell haemoglobin; MCHC, mean cell haemoglobin concentration.

When the cross-sectional changes are viewed in isolation, the effect of BCG could be interpreted thus:

***Immunological interpretation 1:* BCG mediates its NSE by increasing pro-inflammatory cytokine production in response to heterologous bacteria, particularly in male infants.**

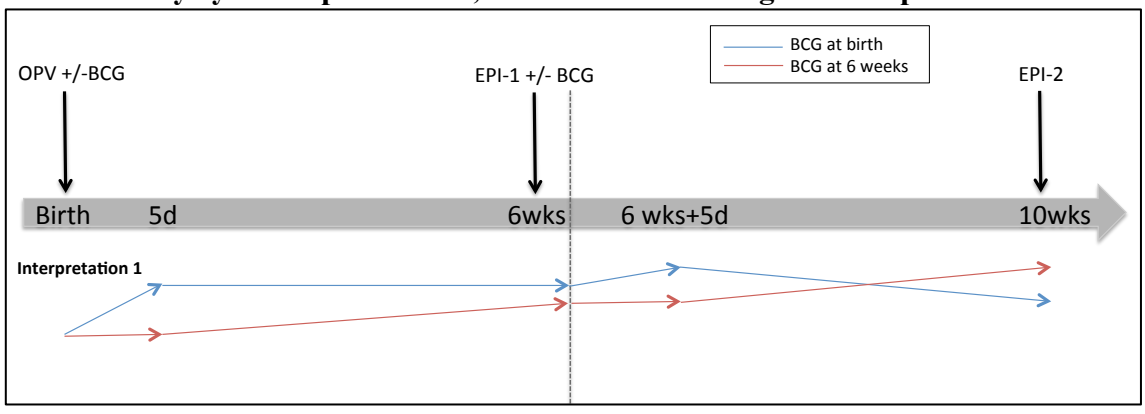
This interpretation agrees with the finding of increased IFNγ production to bacterial stimulation at 5 days of age in male infant BCG vaccinated at birth. It could also fit with the findings of increased *in vitro* and serum pro-inflammatory cytokines in male infants BCG vaccinated at birth at the 6 week time-point, 5 days after EPI +/- BCG (S3), but only if either:

a) the increase in pro-inflammatory cytokines following BCG at 6 weeks takes longer than 5 days to occur, in contrast to the effect of BCG at birth on IFNγ, and/or

b) the increased pro-inflammatory cytokine production associated with BCG at birth is boosted by the receipt of EPI immunisations.

Either or both of these interpretations would result in pro-inflammatory cytokine levels remaining higher in male infants BCG vaccinated at birth despite the delayed group having also received BCG by the S3 time-point. Increased pro-inflammatory cytokine production following BCG at 6 weeks, but taking longer than 5 days to occur, would then explain the lower IL-6 levels at 10 weeks of age in male infants BCG vaccinated at birth compared to at 6 weeks of age. The reduction in the inhibitory H3K9me3 epigenetic modification at the 6 week, pre-immunisation time-point (S2), seen in all infants who were BCG vaccinated at birth compared to infants BCG vaccinated at 6 weeks, would support increased pro-inflammatory cytokine production as the immunological mechanism underlying the NSE of BCG. The reduction in MCV, MCH and MCHC at 6 weeks of age, 5 days post-EPI immunisations (S3), and increased MCV, MCH, MCHC and haemoglobin at 10 weeks of age (S4), in male infants BCG vaccinated at birth could also fit with this interpretation of the immunological results, with increased IL-6 and hepcidin production resulting in decreased iron absorption and a tendency toward lower MCV, MCH and MCHC levels. An example schema for the changes to pro-inflammatory cytokine production occurring if this interpretation were correct is found in Figure 8.2.

Figure 8.3. Example schema of the effects of BCG vaccination on pro-inflammatory cytokine production, based on Immunological Interpretation 1.



OPV, Oral Polio Vaccine; BCG, Bacille Calmette Guerin; EPI, Expanded Programme of Immunisations; d, days; wks, weeks.

This interpretation of the immunological findings is consistent with studies conducted in adults that showed BCG increases TNFα, IFNγ and IL-1β in response to *S.aureus* for at least 2 months post-vaccination, although notably the epigenetic mechanism

responsible for the increase would be different (increased stimulatory H3K4me3 in the Dutch adult studies, decreased inhibitory H3K9me3 in this study).[168, 201] However, H3K9me3 was not investigated in the Dutch studies so changes to this may also have occurred. Interestingly, in contrast to the Dutch studies, increased pro-inflammatory cytokine production with BCG at birth was not seen in this study at 6 weeks pre-immunisations, but only after, suggesting (if this interpretation of the results is correct), that the NSE of BCG wane over time in infants in the absence of subsequent heterologous stimuli which act as amplifiers to the effects.

Other studies conducted in infant populations investigating the impact of BCG on cytokine production following heterologous stimulation also largely support the NSE of BCG being mediated by increased pro-inflammatory cytokine production. Studies in 4-week old infants, prior to EPI vaccinations, in Guinea-Bissau reported increased TNFα and IFNγ in unstimulated samples, increased IL-6, TNFα, IFNγ and TNFα:IL-10 ratio following stimulation with Pam3CSK4, and increased IL-6 and IFNγ production following PMA/ionomycin stimulation, in infants receiving BCG at birth compared to BCG unvaccinated infants.[193] In the Guinea-Bissau study, the effects of BCG on pro-inflammatory cytokine production were stronger in girls than in boys, in contrast to the findings from the Ugandan study described in this thesis. A study in Australian infants at 7 days of age also suggested that BCG at birth resulted overall in an increased pro-inflammatory:anti-inflammatory ratio of cytokines following heterologous stimulation, although notably decreased IL-6 and IL-1ra levels were shown following TLR2 and TLR7/8 stimulation.[92] Lastly, a UK-based study comparing infants BCG vaccinated at 6 weeks with unvaccinated infants also suggested increased pro-inflammatory cytokine production at 4 months following BCG vaccination, although in different combinations and for different stimuli than the above studies.[200] No changes to TNFα or IL-1β were noted.

Thus, overall, previous studies in adults and infants support the assertion that the NSE of BCG are mediated by increased pro-inflammatory cytokine production in response to heterologous stimuli. However, there is a lack of consistency in the results in terms of changes to specific cytokines and stimuli. Possible explanations for this are:

1. *Use of specific TLR-agonists compared to whole pathogens*. Most of the significant findings in previous infant studies have been seen following stimulation with specific TLR-agonists (e.g. TLR-2 and TLR-7/8).[92, 193, 200] This study mainly used whole pathogens for better consistency with the adult studies of epigenetic

changes,[168, 201] and to better replicate the heterologous stimuli that infants would be exposed to. Although arguably more reflective of the *in vivo* infant response to heterologous infections, use of whole pathogens may make specific changes to individual cytokines harder to assess and may have limited the significant findings in this study.

2. *Differing blood sample time-points.* The above-mentioned studies have variously investigated the impacts of BCG at 7 days (Freyne *et al.),* 4 weeks (Jensen *et al.),* 2-weeks, 3 months and 1 year (Kleinnijenhuis *et al.),* and 4 months (Smith *et al.)* post-vaccination.[92, 168, 193, 200] Notably, whilst the Guinea-Bissau studies suggested increased production of pro-inflammatory cytokines at 4 weeks of age with BCG vaccination at birth, this was not seen in this study at 6 weeks of age (pre-EPI). Although this may be accounted for by the different heterologous stimuli assessed, it may also suggest a waning of the NSE of BCG overtime, in the absence of amplification of responses from EPI vaccinations (as suggested by increased pro-inflammatory cytokine production 5 days following EPI-vaccination at 6 weeks, in infants BCG vaccinated at birth).
3. *Different sample handling conditions.* Time from blood collection to processing may impact on both epigenetic modifications and cytokine production, as may sample storage conditions.[292] Although in the design of this study, consistency with previous work was attempted as far as possible, some variations between the studies occurred. For further discussion of this, please see the 'Limitations' section below.
4. *Differing BCG vaccination timings.* Previous studies have suggested that the immunological NSE of BCG may be greater when infants receive BCG after 48 hours of age compared to at birth.[92, 198] All infants in this study received BCG within the first 24 hours of age, which may, therefore, have limited the ability to detect significant immunological differences if these are more pronounced with later vaccination. Countering this argument, however, is the fact that clinical differences were still detectable with early BCG in this study, in contrast to the Danish study which showed more pronounced immunological differences with later BCG but no detectable differences in clinical outcome.
5. *Genetic/population differences.* Just as MTB-specific protection from BCG vaccination may vary between populations,[225] so might non-specific protection. Also, if exposure to pathogens acts to amplify the NSE of BCG, as suggested in this study by enhanced effects measurable 5 days following EPI-vaccinations, then

varying background rates of infections would also modify the measurable immunological NSE of BCG.

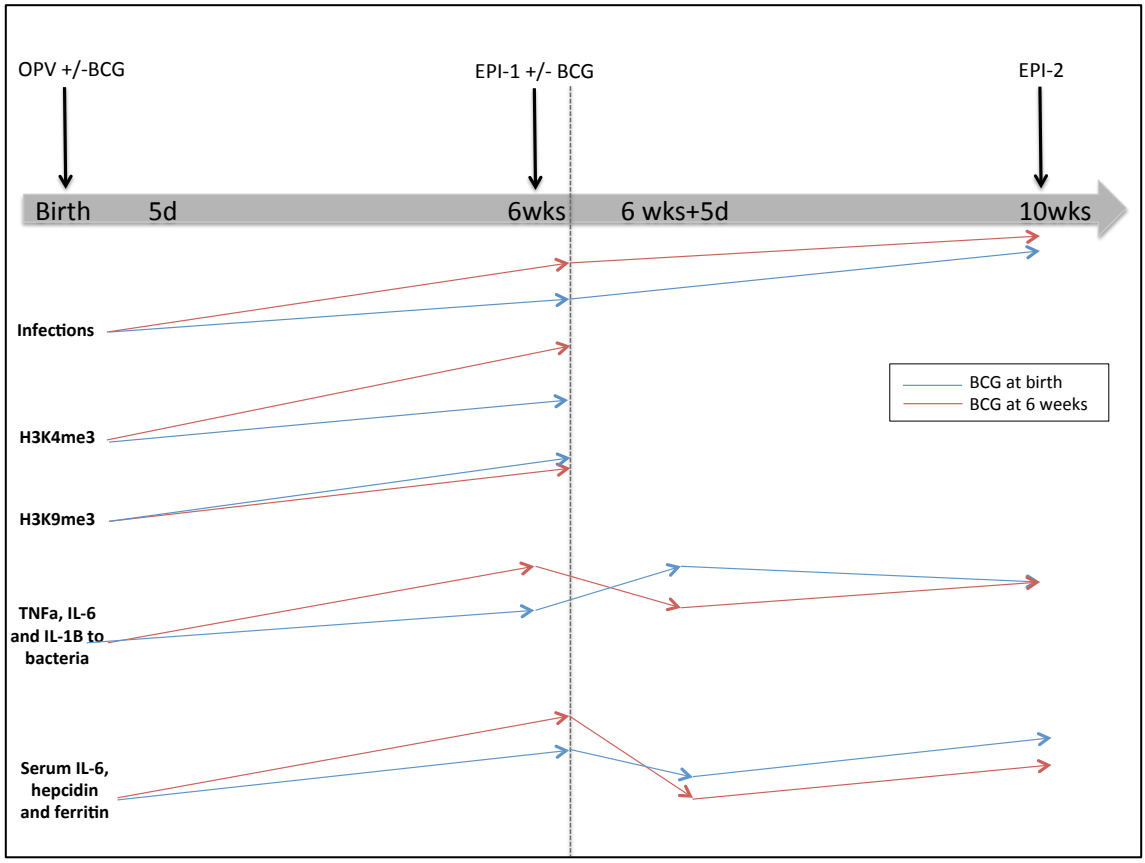
A number of the findings from this study, however, do not fit exactly with the interpretation that BCG mediates its NSE by increasing pro-inflammatory cytokine production to heterologous stimuli, with effects not seen at 5 days post BCG vaccination at 6 weeks of age due to development of NSE taking longer than 5 days and/or boosting of the NSE of BCG at birth occurring following EPI vaccinations:

1. *Sex-differential epigenetic modifications.* Although overall, levels of inhibitory H3K9me3 at the promoter region of pro-inflammatory cytokines were lower at 6 weeks of age in infants BCG vaccinated at birth (which would be consistent with a BCG-induced tendency toward increase pro-inflammatory cytokine production upon heterologous pathogen challenge), these findings were significant only in female infants. In male infants, for whom the changes in cytokine production and clinical outcomes were more pronounced, epigenetic modifications were the reverse, i.e. a consistent trend toward reduced stimulatory H3K4me3 at 6 weeks of age in infants BCG vaccinated at birth, and no clear differences to H3K9me3. These changes would imply that the impact of BCG-induced epigenetic modification in male infants would result in lower cytokine production, not raised concentrations. As epigenetic modifications were not studied at 5 days of age, it is not clear how quickly these changes might occur. Supporting this interpretation was the finding that although stimulatory H3K4me3 levels tended to be higher in infants who had had interim infections, regardless of BCG status, infants who had received BCG at birth also had increased inhibitory H3K9me3, whereas unvaccinated infants had reduced tri-methylation levels compared to infants without interim infections. This could be interpreted that exposure to pathogens in the absence of BCG vaccination leads to an overall increased pro-inflammatory cytokine response upon subsequent pathogen challenge, whereas similar exposure in the presence of BCG vaccination leads to a more balanced cytokine response (due to increased stimulatory and inhibitory epigenetic modifications). Reduction in pro-inflammatory cytokine responses to pathogens may reduce neonatal morbidity related to hyper-reactive immune responses.[293] However, as the epigenetic modifications were only assessed at one post-natal time-point, it is impossible to determine cause and effect of the differing changes to epigenetic modifications. An alternative interpretation of these

findings could, therefore, be that in BCG vaccinated infants, infections occurred more frequently in infants who had higher pre-existing H3K9me3. The small participant numbers contributing data to these sub-studies also cautions against over-interpretation of the, mainly non-significant, results.

2. *Lack of significant differences in NSE of BCG at 1-day post-EPI immunisations at 6 weeks of age (S2i).* If a BCG-mediated increase in pro-inflammatory cytokine production in response to heterologous pathogens wanes by 6 weeks of age (S2), but is amplified in response to EPI-vaccinations, it might be expected that a significant difference in pro-inflammatory cytokines at one day post-EPI vaccinations would have been seen in the iron sub-study (S2i). This was not seen, with male infants showing a non-significant tendency toward reduced IL-6 levels, and no clear difference in hepcidin levels, with BCG vaccination at birth (see Figure 8.3). The subsequent reversal of the trend in cytokine responses toward higher pro-inflammatory cytokine production with BCG at birth occurred only after the delayed group had received BCG, suggesting that this is the result of an acute effect of BCG at 6 weeks. It could still be consistent with Immunological Interpretation 1, however, if the amplifying effects of EPI-1 vaccinations on the heterologous effects of BCG at birth take several days to develop.
3. *Differential dynamics of timing of effects with BCG at birth and at 6 weeks.* Increased IFNγ responses to heterologous pathogens were seen within 5 days post-vaccination with BCG at birth, but similar responses were not seen at 5 days post-vaccination with BCG at 6 weeks of age. This difference may be due to a) the different ages of the participants when BCG was administered, b) an interaction with EPI vaccinations received at the same time, or c) because infants receiving BCG vaccination at birth also had increased pro-inflammatory cytokine production in response to heterologous pathogens, meaning that differences between the two groups would not be discernible even if there were differences between infants BCG vaccinated at 6 weeks and BCG unvaccinated infants.[200]
4. *Within-infant changes over time suggest the NSE of BCG may be mediated by reduction in pro-inflammatory cytokine production.* Although the cross-sectional differences in geometric mean cytokine production at different time-points suggest that BCG mediates its NSE via increased pro-inflammatory cytokine production in response to heterologous stimuli, the observed changes to immunological parameters over time do not support this interpretation (see Figure 8.2).

Figure 8.4. Indicative patterns of within-infant changes to measured outcomes in male infants.



OPV, oral polio vaccine; BCG, Bacille Calmette Guerin; EPI, Expanded Programme of Immunisations; d, days; wks, weeks; IFN, interferon; TNF, tumour necrosis factor; IL, interleukin; Poly I:C, polyinosinic:polycytidylic acid; H3K4me3, histone 3 lysine 4 trimethylation; H3K9me3, histone 3 lysine 9 trimethylation; MCV, mean cell volume; MCH, mean cell haemoglobin; MCHC, mean cell haemoglobin concentration.

Individual within-infant changes over time, and changes to cross-sectional geometric mean parameters at different time-points, are more consistent with the following interpretation of the immunological data:

***Immunological interpretation 2*: BCG mediates its NSE via reductions in pro-inflammatory cytokine production in response to heterologous stimuli, particularly in male infants.**

As can be seen in Figure 8.3, BCG at birth appears to result in reduced cytokine production following *in vitro* heterologous stimulation and *in vivo* stimulation from EPI vaccinations in male infants. This persists until the delayed group receives their BCG vaccination at 6 weeks of age, when the tendency reverses, with reduced pro-inflammatory cytokine production in infants with BCG as their last vaccination. This interpretation fits better with the reduced H3K4me3 but similar H3K9me3 seen in BCG vaccinated male infants at 6 weeks of age compared to unvaccinated infants. It also

correlates well with the timings of clinical changes, as it suggests that BCG at 6 weeks mediates its effects rapidly. This would be needed for the reversal in hazard rate ratios to be seen in the short subsequent period of follow-up, as opposed to the slower development of NSE that would be needed for Immunological Interpretation 1 to be correct. However, the significantly increased IFNγ production seen at 5 days of age in this study does not fit well with this interpretation. Although an acute increase in pro-inflammatory cytokines followed by a longer-term inhibition in production is not impossible, similar findings were not seen following BCG vaccination at 6 weeks, and it is difficult to rationalise which of these changes is most important for the clinical non-specific benefits of BCG.

A reduction in pro-inflammatory cytokine production to heterologous stimuli with prior BCG vaccination may be theorised to reduce clinical morbidity and mortality from infectious disease by limiting hyper-reactive immune responses and reducing immune-mediated pathology.[294] Both excessively high and profoundly low levels of pro-inflammatory cytokines have been associated with mortality from sepsis. However, the previously published studies investigating the immunological mechanisms responsible for the NSE of BCG do not provide evidence to support this interpretation. Studies also show that premature infants have attenuated innate immune cytokine responses compared to older infants and adults, and this has been suggested to contribute to their enhanced susceptibility to infectious diseases.[295] As the NSE of BCG have been shown to be particularly beneficial in this population of infants, this argues that the mechanism of action is more likely to be via increased innate immune cytokine responses to heterologous pathogens rather than a further suppression of responses.

Thus, the immunological studies presented in this thesis suggest that BCG vaccination does have NSE on the innate immune system, possibly mediated through changes to epigenetic modification. Whether the lower increase in both H3K4me3 and H3K9me3 occurring in BCG vaccinated infants compared to BCG unvaccinated infants, seen in this study, results in increased or decreased pro-inflammatory cytokine production is not definitively answered. On balance, an interpretation based on increased pro-inflammatory cytokine production in the short-term, with further amplification of production after subsequent heterologous stimulation in the longer-term, is most consistent with previously published studies.[92, 193, 200] However, as most results are close to conventional statistical significance in the context of multiple comparisons, the

possibility that the NSE of BCG are mediated through different, untested immunological mechanisms should also be considered.

8.3 Other significant study findings

Whilst not directly addressing the primary aims of this thesis, the main Ugandan study and associated Gambian pilot study have revealed a number of other interesting results:

8.3.1 BCG scar size at 10 weeks of age correlates poorly with the non-specific beneficial effects of BCG, but well with PPD-induced immunological outcomes.

There was no evidence of an association between the size of the BCG scar at 10 weeks of age and non-tuberculous illness rates. Similarly, correlations between scar size and immunological parameters following both *in vitro* and *in vivo* heterologous stimulation were poor when analysed by BCG status. In fact, the significant reduction in H3K4me3 at 6 weeks of age in BCG vaccinated infants compared to unvaccinated infants, appeared to be limited to infants who ultimately went on to have small BCG scars at 10 weeks of age. This contrasts with strong correlations between BCG scar size at 10 weeks and mycobacteria-specific BCG responses, as assessed by PPD-induced IFNγ production at 6 weeks and 10 weeks of age. These findings suggest that assessment of BCG scar size is not a valid measure of the magnitude of the NSE of BCG. Although an epidemiological study conducted in Guinea-Bissau showed that larger scars were associated with reduced clinic attendance, particularly in girls, this was likely due to a differential effect of vaccinating strain (BCG Danish was shown to produce larger scars than BCG Russia, and was associated with reduced NSE of BCG), rather than acting as an independent measure of the NSE of BCG.[240] Thus, the presence or absence of a BCG scar might still be useful as an indicator of BCG vaccination status, as used in a number of epidemiological studies,[18, 296] but not as a quantitative measure of the NSE of BCG. It should be noted that as with other studies 37 infants (8%) in this study failed to develop a scar by 10 weeks of age, despite definitely receiving BCG. However, the measurement of scar size in this study was limited by the short duration of follow-up. It could be argued that measurement of an established scar, for instance at 1 year post-immunisation, might provide better correlation with the TB-specific and non-specific protection afforded by BCG.

8.3.2 All infants have an increase in stimulatory and inhibitory epigenetic modifications during the first 6 weeks of life

This study is the first to describe changes to histone-protein epigenetic modifications at the promoter region of pro-inflammatory cytokines in the early neonatal period. A highly significant increase in both stimulatory H3K4me3 and inhibitory H3K9me3 epigenetic modifications at the promoter region of TNFα, IL-6 and IL-1β in all neonates between birth and 6 weeks of age was seen. This presumably reflects developmental changes occurring to the innate immune system in the early neonatal period. The study also suggests that infants with clinically diagnosed infectious disease in the first 6 weeks of life have increased H3K4me3 epigenetic modification at the promoter region of pro-inflammatory cytokines compared to infants who did not have infections in the first 6 weeks of life. Whether this increase is a cause or effect of the infectious episodes is not clear.

8.3.3 The inflammatory-iron axis is active and highly stimulated in the first few days of life, in healthy term neonates.

Previous studies investigating iron parameters in neonatal life have used cord blood as a proxy for neonatal blood, suggesting that TSAT and iron levels are high in the neonatal period.[268, 273] The Gambian study described in this thesis revealed a rapid and profound decrease in serum TSAT and iron, with corresponding increases in IL-6 and hepcidin, occurring in the first 12 hours of life.[259] This reduction is associated with reduced *in vitro* growth of common neonatal pathogens (see Paper 2, Chapter 5), and may represent an evolutionary mechanism to protect neonates against pathogenicity during early-microbial colonisation. If stimulation of the inflammatory-iron axis is limited in certain populations, such as premature infants, this could increase their susceptibility to invasive infectious disease. Studies are currently on-going in The Gambia to assess whether gestational age, intra-uterine growth restriction and delivery method influence the stimulation of the inflammatory-iron axis in early neonatal life.

8.3.4 EPI vaccinations produce a rapid, transient, but profound stimulation of the inflammatory-iron axis.

EPI vaccinations stimulated a significant increase in geometric mean IL-6, hepcidin and ferritin (2-5 fold), with corresponding decreases in TSAT and iron levels (3-fold), by 24 hours post-vaccination in all infants. An almost 2-fold increase in the average total white cell count at 24 hours post-vaccination was also seen, which was predominantly a neutrophilia. These changes had returned to baseline by 5 days post-immunisation. These findings suggest that EPI vaccinations could be investigated for their utility as a therapeutic intervention to provide non-specific protection against infections by stimulating the inflammatory-iron axis in infants who have a sub-optimal endogenous response (see above), or as an adjunct to antibiotics in the early stages of infection. The findings also highlight that care should be taken with the interpretation of immunological and haematological parameters in unwell infants presenting shortly after EPI vaccinations (see Paper 4).

Equivalent induction of the inflammatory-iron axis at 24 hours post-BCG vaccination was not seen to occur in the small Ugandan pilot study of timing of changes post-BCG vaccination at 6 weeks (Chapter 6, Figure 6.2). However, this could not have been looked at in the main study because there was no blood sample time-point at 1-day post BCG vaccination, and consequently similar changes post-BCG cannot be ruled out.

8.4 Strengths and limitations

8.4.1 Strengths

This study, and the associated pilot study, are the first randomised controlled trials investigating the NSE of BCG in infants in high mortality settings to have been performed independently from the group carrying out much of the previous NSE work. They provide information from geographically distinct locations and from general neonatal populations, rather than sub-populations at high-risk of mortality. The fact that this work corroborates these previous studies, therefore, provides strong supportive evidence for the NSE of BCG, and shows that the beneficial effects are not limited to a distinct population of infants in one geographical area.

The design of the main study underlies a number of its other strengths:

1. Its randomised design reduces the likelihood of confounding and bias that epidemiological studies are at risk of. Balanced baseline data suggest that randomisation occurred effectively and in an unbiased manner. BCG-specific immunological data suggest that intervention allocation occurred as per intention-to-treat.
2. The study was explicitly designed and powered to investigate whether there were sex-differential effects of BCG rather than an analysis by sex being carried out as a post-hoc investigation. This adds greater weight to the findings that the NSE of BCG are more pronounced in male infants.
3. In this study, the research team administered all vaccinations. This differs from the other randomised controlled trials conducted in high-mortality settings,[51-53] where BCG vaccination at birth was given by the study team but the timing of vaccination in the delayed group was determined by when the infant was given it in the community. This was usually at 6 weeks of age but may have been earlier. Thus, the impacts of differing BCG timings, and interactions with EPI vaccinations, may have been more clearly determined in the study described in this thesis.
4. The active clinical follow-up of participants, with weekly telephone reviews, regular routine clinic reviews and open access for physician review, provides reassurance that data regarding clinical illness events was complete. These methods of clinical follow-up are enhanced compared to previously published randomised controlled trials in high mortality areas, which tended to rely on

more passive detection methods and measurement of mortality only. The comparatively low loss to follow-up, with balanced losses in each vaccination group, also provides reassurance that the clinical data are an accurate representation of the NSE of BCG.

8.4.2 Limitations

As described in the study design section (Chapter 4), parents were not blinded to BCG vaccination status. This was partly for ethical reasons, so that unvaccinated infants lost to follow-up would be more likely to receive BCG in the community, and partly for logistical reasons because BCG vaccination produces a visible scar. It could be argued that the increased rates of clinic presentation seen in unvaccinated infants prior to 6 weeks of age may have resulted from parental anxiety resulting from lack of vaccination. However, there are several reasons to believe that this was not the case, and that the reduction in clinical events seen in BCG vaccinated infants prior to 6 weeks of age is a true NSE of BCG:

1. Although parents were not blinded to vaccination status, physician-blinding occurred effectively. Un-blinding only occurred in 11 cases, and these were excluded from analysis of clinical outcomes. If presentation to clinic resulted from conscious or sub-conscious parental anxiety regarding the lack of BCG, it would be expected that the increase in presentations would be reflected in an increased diagnosis of 'normal infant'. This did not occur in the study, with only 15% of diagnoses in infants with BCG at birth and 14% of diagnoses in infants with BCG at 6 weeks being deemed normal infant variants, in the period prior to BCG vaccination of the delayed group. Instead, the association between BCG status and clinical outcomes was strengthened when restricted only to presentations of physician-diagnosed infectious disease. The similar, though not significant, impacts of BCG at birth on more objective measures of infection, such as fever and death, support the assertion that the observed beneficial NSE of BCG are due to a true effect, not a result of parental anxiety.
2. The beneficial NSE of BCG on clinical outcomes are more pronounced in males, a finding that is consistent with the significant findings in the immunological sub-studies. Although it could be argued that societal factors lead to increased parental anxiety for male infants, and therefore health seeking behaviour, which would lead

to an exaggerated finding of increased presentations for BCG unvaccinated male infants compared to females, this would not lead to the immunological differences seen. Immunological investigations and analysis were conducted according to anonymous, blinded study-number, meaning that a comparable bias in immunological investigators could not have occurred.

This study was designed purposely to investigate a potential biological mechanism underlying the NSE of BCG. Multiple immunological parameters were tested, based on the best available mechanistic evidence in adult populations (epigenetic 'training' of the innate immune system) and to maximise the rare opportunity provided by a randomised controlled trial of BCG to investigate other untested hypotheses (e.g. the effects on the inflammatory-iron axis). As parameters were investigated at multiple time-points and by sex, this resulted in 920 comparisons being conducted overall. It would, therefore, be expected that 46 of these comparisons would reach statistical significance at p=0.05 due to chance alone. In total, 63 significant differences in cross-sectional comparison were found in the study. Within-infant changes to parameters over time were also explored, as were correlations between immunological outcomes, clinical outcomes and BCG-specific responses, increasing the likelihood of multiple testing, although these were conducted as exploratory analyses only and highlighted as such. Additionally, many of the significant immunological findings in this study were close to conventional statistical significance, increasing the likelihood that these were chance findings. Therefore, this thesis focused on interpretation of results that showed clear and consistent trends rather than solely focused on significance levels. There are a number of reasons why this study may have had limited power to detect significant immunological NSE of BCG:

1. **High inter-individual and inter-sample variability:** Cytokine production, as well as inflammatory-iron parameters, have high inter-individual variability.[297] This is likely to be particularly pronounced in the early neonatal period due to rapid immunological development in early infancy, and may limit the ability to detect small differences in outcome by BCG status. Inter-individual variability is also likely to be increased in areas of high-infection rates, such as the study area, as other exposures may also impact on the outcome of interest.[298] Attempts to control for variability of baseline responses, by using medium-subtracted stimulated cytokine responses, including un-stimulated cytokine production as a co-variate in calculation

of geometric mean ratios, and by adjusting for the baseline parameter level when calculating within-infant changes over time, had little impact on results, however. Alterations to sample handling conditions are also known to have marked impacts on the assessment of immunological parameters.[292] Standardised operating procedures were used throughout the study and conditions were kept as consistent as possible. The area of greatest variance was in the time from collection to processing. This was <4 hours for all post-natal blood samples, but for logistical reasons was up to 16 hours for cord blood samples. Even a processing time of up to 4 hours is likely to introduce a degree of unwanted variability in outcomes,[292] but shorter limits were not feasible in the study setting. Multiple freeze-thaw cycles may also have introduced variability in certain parameters, but laboratory analyses were kept within the acceptable limits of freeze-thaw stability as defined by the pilot studies (Chapter 6.3.1.2). Lastly, variability in cytokine response may have been introduced by differences in the day of follow-up that the child presented to the study clinic (Chapter 7, Table 7.1). Although this may have introduced a degree of variability in individual cytokine responses, a non-specific immunological effect of BCG would have to be durable in order to produce the clinical effects measured in this study, and therefore it could be argued that small changes in the age at blood-sampling should not have unduly affected the results. Also, the average day of presentation for each blood sampling time-point was the same for both randomisation groups, so this should not have influenced the ability to detect differences by BCG status.

2. **Assays not sensitive enough:** As described previously, this study favoured the use of whole bacterial pathogens rather than specific TLR-ligands. Also, whole blood rather than PBMCs was used for the *in vitro* cytokine studies and PBMCs rather than monocytes were used in the epigenetic studies. These conditions were used to a) better replicate the true *in vivo* situation in infants following exposure to infectious pathogens, and b) due to limited blood volume availability from neonates. However, they may have made small changes induced by BCG harder to detect. For instance, previous work conducted in Dutch adults showing increased H3K4me3 and associated increases in pro-inflammatory cytokine production to heterologous pathogens 3 months after BCG vaccination showed changes in monocytes and NK cells.[168, 201] The production of *in vitro* stimulated cytokines in the whole blood assay used in this study is likely to have been biased toward production from neutrophils, diluting the ability to detect significant differences in monocyte-derived cytokines. Similarly, the use of PBMCs rather than monocytes for the epigenetic sub-study in

this thesis may have resulted in detection of epigenetic modifications in lymphocytes predominantly, diluting the ability to detect significant changes in monocytes.

3. **Study number too small to detect major effects in healthy infants:** Previous randomised controlled trials, as well as this one, have suggested that the beneficial effects of BCG are more pronounced in low birthweight infants. Immunological effects may be less pronounced in healthy term infants, and therefore require greater numbers of participants to detect. Numbers of low birthweight infants were too few to allow separate analysis of immunological outcomes in this study.
4. **The NSE of BCG not being mediated through simple increased or decreased responses to heterologous stimuli, but more balanced responses:** As suggested by the reduction in both H3K4me3 and H3K9me3 seen in the epigenetic sub-study, the NSE of BCG may not be mediated by a simple increase or decrease in immunological parameters, but rather the production of more balanced, effective response, which are more difficult to detect. In fact, studies in neonatal populations suggest that although innate cytokine production in response to pathogenic stimuli is attenuated compared to adults,[295] they may also show sustained high levels of systemic inflammation and immune dysregulation which may lead to worse clinical outcomes.[299] Thus, a NSE of BCG biasing the innate immune system toward more regulated responses to pathogenic stimuli may have the most beneficial impacts on neonatal morbidity. The impact of BCG on immunological outcomes such as cytokine production is also likely to be different for different pathogens rather than being detectable as a single unifying outcome. A systems-based approach is likely to be more informative in these situations.
5. **NSE of BCG acting via different, untested mechanisms:** It is possible that BCG in neonates mediates its non-specific effects via different mechanisms not tested in this study. It could also mediate its effects via extensions of the mechanisms tested in this study, e.g. training of NK cells,[201] which had not been identified as a putative mechanism in adults prior to the commencement of this study and was therefore not tested, or via different epigenetic modifications or effects on different cytokines. As described above, a systems-based approach is likely to be the most informative for elucidation of the mechanisms underlying the NSE of BCG. Samples from this study are currently being investigated for RNA expression biosignatures and genome wide epigenetic modification in an attempt to provide more holistic information regarding the NSE of BCG. Nasal swab samples are also being

examined for pathogen carriage, and stool microbiome analysis is planned, to provide information about any potential NSE of BCG at mucosal surfaces.

It could be argued that the strict design used in this study makes it difficult to determine the real-world impact of the NSE of BCG. In this study, all infants receiving BCG at birth were vaccinated within the first 24 hours of age. This is extremely unlikely to occur in reality, even if early vaccination was prioritised. It is impossible to determine from this study whether receipt of BCG outside the first 24 hours of life would be similarly beneficial. However, there are a number of reasons to suppose that this would be the case, even if some advantage may be lost due to lack of beneficial effects during the high-mortality early neonatal period. All other randomised controlled trials in high mortality settings that have shown beneficial NSE of BCG have given BCG on discharge from hospital, not within 24 hours of delivery.[51-53] The median age of BCG receipt in these studies was 3 days of age, and similar beneficial NSE of BCG were seen prior to receipt of BCG in the delayed group. Also, the reversal of the trends of non-specific protection subsequent to BCG receipt at 6 weeks in the delayed group seen in this study, argues that BCG given at this time-point produces similar beneficial effects, although the magnitude of these findings may be reduced due to the lower mortality rates at this time-point.

The use of one particular strain and batch of BCG in the study, whilst necessary for accurate assessment of clinical and immunological outcomes, also limits the real-world applicability of the study findings. Many different strains, and batches, of BCG are used around the world and the clinical and immunological effects of BCG may not be induced by all strains, as discussed earlier. The majority of evidence for beneficial NSE of BCG comes from BCG SSI, the strain used in this study. The consistency of these findings in other strains is yet to be determined, though there is some evidence to suggest that findings may not be as pronounced.[42] It is reassuring, however, that clinical benefits of BCG at birth were seen in this study, which used a batch of BCG SSI with normal growth characteristics, not a batch with slow-growth such as was used in the first Aaby study.[51] This suggests that batch growth effects may not have as significant an impact on the NSE of BCG as previously feared.[199]

Studies conducted in Uganda in parallel to this study have provided evidence that maternal response to BCG, as measured by presence of a BCG scar, also affected pro-inflammatory cytokine production to heterologous stimuli.[245] It is likely, therefore, that

maternal BCG response would have had a similar impact in our study and would have been interesting to measure as a co-variate. However, the lack of measurement of maternal BCG scar should not have influenced the differences seen by infant BCG status in this study, as randomisation produced balanced groups for all other demographic variables.

The short duration of follow-up in this study prevents the assessment of the longer-term impacts of different BCG vaccination timings on non-specific clinical or immunological outcomes. Although this fails to forward the scientific evidence regarding the durability of the beneficial effects of BCG, this does not negate the importance of the early beneficial NSE of BCG. As infant mortality is concentrated in the neonatal period, even short-lasting beneficial NSE of BCG in this period may have significant impacts on infant mortality.

8.5 Implications

The finding that early BCG reduces the incidence of all-cause infectious disease in babies, possibly mediated through epigenetic training of the innate immune system, corroborates previous findings in low birthweight babies.[35] It suggests that the non-specific beneficial effects of BCG are widely applicable in different high mortality settings and in babies born healthy and at term. Although the exact immunological mechanisms underlying the NSE of BCG have not been determined, on balance the results from this study support previous studies suggesting that BCG mediates its NSE, at least in part, by epigenetic training of the innate immune system to react in an enhanced manner upon heterologous pathogen challenge. Despite full elucidation of the immunological basis for the NSE of BCG remaining elusive, mechanisms underlying TB-specific protection from BCG are also not entirely known. Thus, this should not limit the recommendations based on the clinical results. Further investigations using a systems-based approach, and on mucosal immunity, are currently on-going.

In areas of high TB-incidence, BCG is recommended as soon as possible after birth or at first health-worker contact.[250] This work strongly supports the continuation of this policy. In reality, BCG administration is often delayed beyond the neonatal period for a variety of reasons.[251] These include delays in receipt of BCG in children born outside of a healthcare setting, reluctance of healthcare professionals to open a multi-dose vial of

BCG if there are limited numbers of infants to vaccinate, and problems with BCG supply. The results presented in this thesis imply that BCG vaccination on the day of birth should be prioritised as an effective intervention against heterologous infections in the neonatal period, a time of high infectious disease mortality. Re-formulation of BCG in single dose vials, rather than multi-dose vials, and distribution to trained village health workers may aid this. As the majority of studies that have shown beneficial NSE of BCG have used BCG SSI, consideration should be made to wider distribution of this BCG strain, particularly in high mortality settings. Challenges with the variable protection given by BCG vaccination against pulmonary TB in different settings also means that alternative vaccinations against TB are actively being sought.[300] If a superior vaccination against TB is found, this work strongly suggests that BCG at birth should be maintained in routine immunisation regimes for its non-specific effects. New vaccines based on recombinant versions of BCG, such as VPM1002,[301] should be compared with BCG in terms of their protective effects against TB and heterologous invasive infectious diseases, before replacing it in immunisation regimes. New vaccination regimes based on prime-boost mechanisms with prior BCG vaccination,[302] may ultimately prove to be favourable in terms of overall benefit to the child.

In areas of low TB-incidence, BCG use is generally limited to specific populations.[250] This study suggests that early BCG, particularly in boys, may have additional benefits. Further work, for instance investigating the use of BCG as an immune-therapeutic in high-risk premature infant populations, would be required before policy changes can be recommended, however. Investigations into the utility of BCG as a broad-spectrum immuno-stimulant in the early stages of outbreaks of novel infections whilst disease-specific vaccinations are being developed, such as during influenza pandemics and viral haemorrhagic fever outbreaks, would also be worthwhile.

8.6 Conclusion

The World Health Organization-commissioned reviews of the clinical and immunological evidence surrounding the non-specific effects of vaccinations concluded that not enough evidence was available to support changes to immunisation policy.[38, 205] This work substantially adds to the existing body of evidence, and suggests that BCG at birth should be prioritised in high-mortality settings as an intervention against all-cause infectious morbidity and mortality, particularly in low birthweight infants and boys.

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Appendices

1. Journal Article A1

   Maternal BCG scar is associated with increased infant proinflammatory immune responses. Mawa PA, Webb EL, Filali-Mouhim A, Nkurunungi G, Sekaly, R-P, Lule SA, Prentice S, Nash S, Dockrell HM, Elliott AM, Cose S. Vaccine 2017, 35(2):273-282

2. Journal Article A2

   They are what you eat: Can nutritional factor during gestation and early infancy modulate the neonatal immune response? Prentice S. Frontiers in Immunology 2017, 8:1641

3. Case Report Forms

   a) First eligibility form

   b) Second eligibility form

   c) Maternal and infant demographic forms

   d) Routine study visit form

   e) Routine phlebotomy/vaccination forms

   f) Routine visit checklist

   g) Illness form

   h) Illness follow-up form

   i) Personal participant plan

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   k) Final status form

4. Information and Consent Forms

   a) Study information sheet

   b) Consent forms

   c) Signs of illness in children information sheet

5. BCG Product Information Sheet

6. Serious Adverse Event Reporting Forms

7. Ethical Committees Approval Letters

   a) LSHTM

   b) MRC/UVRI

   c) UNCST

   d) NDA

8. Reagent List

9. Results Tables

London School of Hygiene & Tropical Medicine
Keppel Street, London WC1E 7HT
www.lshtm.ac.uk

Registry
T: +44(0)20 7299 4646
F: +44(0)20 7299 4656
E: registry@lshtm.ac.uk

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SECTION A – Student Details

Student	Sarah Prentice
Principal Supervisor	Stephen Cose
Thesis Title	Investigating the Non-Specific Effects of BCG in Neonates

If the Research Paper has previously been published please complete Section B, if not please move to Section C

SECTION B – Paper already published

Where was the work published?	Vaccine		
When was the work published?	2017		
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For multi-authored work, give full details of your role in the research included in the paper and in the preparation of the paper. (Attach a further sheet if necessary)	I provided advice to PW regarding the non-specific component of his findings and helped with the manuscript preparation for this component.

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Maternal BCG scar is associated with increased infant proinflammatory immune responses

Patrice Akusa Mawa[a,b,*], **Emily L. Webb**[b], **Abdelali Filali-Mouhim**[c], **Gyaviira Nkurunungi**[a], **Rafick-Pierre Sekaly**[c], **Swaib Abubaker Lule**[a], **Sarah Prentice**[b], **Stephen Nash**[b], **Hazel M. Dockrell**[b], **Alison M. Elliott**[a,b], and **Stephen Cose**[a,b]

[a]MRC/UVRI Uganda Research Unit on AIDS, P.O. Box 49, Entebbe, Uganda [b]London School of Hygiene & Tropical Medicine, Keppel Street, London WC1E 7HT, UK [c]Case Western Reserve University School of Medicine, 10900 Euclid Ave., LC4960, Wood Bldg. W200, Cleveland, OH 44106, United States

Abstract

Introduction—Prenatal exposures such as infections and immunisation may influence infant responses. We had an opportunity to undertake an analysis of innate responses in infants within the context of a study investigating the effects of maternal mycobacterial exposures and infection on BCG vaccine-induced responses in Ugandan infants.

Material and methods—Maternal and cord blood samples from 29 mother-infant pairs were stimulated with innate stimuli for 24 h and cytokines and chemokines in supernatants were measured using the Luminex® assay. The associations between maternal latent *Mycobacterium tuberculosis* infection (LTBI), maternal BCG scar (adjusted for each other's effect) and infant responses were examined using linear regression. Principal Component Analysis (PCA) was used to assess patterns of cytokine and chemokine responses. Gene expression profiles for pathways associated with maternal LTBI and with maternal BCG scar were examined using samples collected at one (n = 42) and six (n = 51) weeks after BCG immunisation using microarray.

Results—Maternal LTBI was positively associated with infant IP-10 responses with an adjusted geometric mean ratio (aGMR) [95% confidence interval (CI)] of 5.10 [1.21, 21.48]. Maternal BCG scar showed strong and consistent associations with IFN-γ (aGMR 2.69 [1.15, 6.17]), IL-12p70 (1.95 [1.10, 3.55]), IL-10 (1.82 [1.07, 3.09]), VEGF (3.55 [1.07, 11.48]) and IP-10 (6.76

*Corresponding author at: MRC/UVRI Uganda Research Unit on AIDS, P.O. Box 49, Entebbe, Uganda. pmawa@uvri.go.ug (P.A. Mawa).

Author contributions

A.M.E. conceived the study. P.A.M., S.C. and A.M.E. designed the study. P.A.M. coordinated the study (together with S.A.L.), carried out the immunoassays under the supervision of S.C., performed the data analysis under the supervision of E.L.W. and S.N, drafted the manuscript and coordinated the writing of the manuscript. A. F. conducted the microarray assays. G.N. performed the T-SPOT. TB assays. P.A.M., E.L.W., H.M.D., A.M.E., S.C., A.F., G.N., R.P.S., S.P., S.N., and S.C. contributed to discussion and interpretation of the results. P.A.M., A.F., H.M.D., A.M.E. and S.C. participated in writing the manuscript. All the authors read and approved the final manuscript.

Conflict of interest statement

The authors have no associations that might pose a conflict of interest.

Appendix 1

[1.17, 38.02]). Further assessment of the associations using PCA showed no differences for maternal LTBI, but maternal BCG scar was associated with higher scores for principal component (PC) 1 (median level of scores: 1.44 in scar-positive versus –0.94 in scar-negative, p = 0.020) in the infants. PC1 represented a controlled proinflammatory response. Interferon and inflammation response pathways were up-regulated in infants of mothers with LTBI at six weeks, and in infants of mothers with a BCG scar at one and six weeks after BCG immunisation.

Conclusions—Maternal BCG scar had a stronger association with infant responses than maternal LTBI, with an increased proinflammatory immune profile.

Keywords

Maternal infections; Latent *Mycobacterium tuberculosis* infection; Maternal BCG scar; Infant innate responses; BCG immunisation; Tuberculosis; Heterologous effects

1 Introduction

The bacillus Calmette-Guérin (BCG) vaccine protects against tuberculous meningitis and miliary tuberculosis (TB) in the infant [1–3], and also protects against leprosy [4]. However, the protective efficacy of BCG against pulmonary TB varies between populations, with latitude highlighted as an important factor for responses in adolescents and adults [1,5,6]. We recently investigated the effect of maternal latent *Mycobacterium tuberculosis* infection (LTBI) on the infant response to BCG immunisation [7], with results suggesting that maternal M. tuberculosis infection may impair adaptive immune responses in the infants, although a study in South Africa showed no such effect [8]. The associations with innate immune responses were not assessed.

Evidence that BCG immunisation may influence innate responses includes findings in both observational studies and randomized controlled trials that have highlighted the heterologous effects of BCG on childhood survival in both low- and high-income countries [9–13]. This has been suggested to be due to BCG-induced increases in function of the innate immune system, a phenomenon termed ‘trained immunity’ [14–18]. This is an observation of great global health significance, since mortality due to infectious agents other than TB is high in developing tropical countries [19].

One of the indicators of previous immunisation with BCG, in place of or in addition to vaccination records, is the presence or absence of a scar [20–22]. It has been shown that 52–97% of newborns administered BCG vaccine develop a scar, with differences depending on the strain of BCG vaccine used, the administrator and age of administration [20,23–26]. However, not all BCG vaccinated babies will scar. There are reports of a correlation between the presence of a scar and protection against TB [27,28], as well as studies showing better survival with fewer respiratory infections [24,29,30], fewer skin infections and sepsis [31] in infants with a BCG scar.

Little is known about the link between the development of a BCG scar in mothers and immune responses in infants. We have previously observed that maternal BCG scar was associated with lower T helper (Th) 2 responses to crude culture filtrate proteins of

Appendix 1

mycobacteria in the infants [32]. In the context of a study designed to investigate the effects of maternal infections, including LTBI, on infant immune responses [7], we had the opportunity to also evaluate associations between maternal BCG scar and immune response profiles in the offspring.

2 Materials And Methods

2.1 Study design, setting and ethical approval

The study design, settings, laboratory and clinical procedures have been described elsewhere [7]. Briefly, women residing within the study area and delivering in Entebbe General Hospital were eligible for inclusion. They were approached for consent, on admission in early labour, if they were willing to participate in the study, had a normal singleton pregnancy and were HIV negative. Cord blood was obtained at delivery, following consent. A questionnaire was completed to assess eligibility after delivery. The tuberculin skin test (TST, Statens Serum Institut, Copenhagen, Denmark) and T-SPOT.TB assay (Oxford Immunotec, Abingdon, UK) were used to test mothers for LTBI at approximately one week after delivery. Infants were then followed up to six weeks of life. This was an exploratory observational study in a relatively small number of subjects. The number of infants included in the study was chosen to be feasible within the time frame and resources available. The study was approved by the Uganda Virus Research Institute-Research and Ethics Committee, the Uganda National Council for Science and Technology and the London School of Hygiene & Tropical Medicine. Written, informed consent was obtained from participating women for themselves and their infant.

2.2 Immunological assays

Innate immune responses were measured in 29 mother-infant pairs using a whole blood assay (WBA) with supernatant analytes measured by Luminex®, and gene expression profiles were measured in infant samples obtained at one (n = 42) and six (n = 51) weeks after BCG immunisation using microarray.

2.3 Innate stimulation and measurement of responses using luminex® assay

Heparinized maternal and cord blood samples were diluted 1:1 with RPMI 1640 medium (Life Technologies Corporation, NY, USA) and stimulated with lipopolysaccharide (LPS) (toll-like receptor (TLR) 4 agonist, 100 ng/ml), FSL-1 (TLR2/6 agonist, 50 ng/ml), CpG-ODN2006 (TLR9 agonist, 5 μg/ml), CL097 (TLR7/8 agonist, 1 μg/ml) all from InvivoGen, San Diego, CA, USA, PAM3Cys-Ser (TLR1/2 agonist; ECM Microcollections GmbH, Tubingen, Germany; 100 ng/ml), Mannan (DC-SIGN agonist; Sigma-Aldrich; 100 μg/ml) and Curdlan (Dectin-1 agonist; Wako Chemicals GmbH, Neuss, Germany; 100 μg/ml). An unstimulated well was included to act as a negative control. After 24 h of incubation at 37 °C in 5% CO_2, culture supernatants were harvested and stored at -80 °C for analysis of cytokines and chemokines. The concentrations of analytes in the culture supernatants were measured using a Bioplex multiplex cytokine assay system (Bio-Rad Laboratories, Hercules, CA, USA), following instructions from the manufacturer. A Bio-Plex 200 System (Bio-Rad Laboratories, Hercules, CA, USA) and the Bio-Plex Manager software (version 6.0; Bio-Rad Laboratories, Hercules, CA, USA) were used to run the samples. According to the

manufacturer's instructions, a curve fit was applied to standard curves, which were then used to extract sample concentrations. Limits of the assay working range (lower limit of quantification (LLOQ) and upper limit of quantification (ULOQ)) quoted by the manufacturer for each cytokine/chemokine were used to clean the data. For values below the acceptable range, half of the LLOQ was used and for values above the ULOQ, the ULOQ value for that particular analyte was used. The cytokines and chemokines analysed were IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12p70, IL-13, IL-17A, IFN-γ, IP-10, MCP-1, MIP-1α, MIP-1β, RANTES, TNF-α, GM-CSF and VEGF.

2.4 RNA amplification and microarray

Gene expression microarrays were undertaken using unstimulated whole blood samples obtained from 42 and 51 infants at one and six weeks, respectively, to assess gene expression profiles after BCG immunisation. The Illumina RNA Amplification Kit (Ambion, Austin, TX, USA) was used to amplify a median of 124 ng (range 63–174 ng) of the extracted RNA. A Biotin-16-UTP label was incorporated into amplified RNA during the *in vitro* transcription process (Perkin Elmer Life and Analytical Sciences, Woodbridge, Ontario, Canada). Amplification gave yields ranging from 1 μg to 25 μg. Amplified RNA (1000 ng per array) was hybridized to the IlluminaHumanHT-12_V4 BeadChip according to the manufacturer's instructions (Illumina, San Diego, CA, USA). The IlluminaHumanHT-12_V4 bead chip comprises 42,000 sequences representing 31,000 annotated genes from the curated portion of the NIH Reference Sequence Database (http://www.ncbi.nlm.nih.gov/RefSeq/). Each sequence is represented at least 30 times on the array. Arrays were scanned with an Illumina bead array confocal scanner, according to the manufacturer's instructions. Array data processing and analysis was performed using Illumina BeadStudio software.

2.5 Statistical analysis

The objective of this analysis was to investigate the effects of maternal latent TB and helminth infection on infant innate immune responses. In the event, helminth infections were rare in this study group [7], so the principal exposures considered were maternal LTBI and maternal BCG scar. In the multivariate analysis, the effects of maternal LTBI and maternal BCG scar were adjusted for. Maternal and infant factors such as maternal age, gravidity status, infant birth weight and gender were not crudely associated with infant responses and were not adjusted for, and the numbers involved were generally small.

Cytokine and chemokine concentrations showed skewed distributions. Results were transformed to $\log_{10}$ (cytokine concentration + 1) for graphical representation using GraphPad Prism v6.0c (GraphPad software, Inc., La Jolla, CA, USA) and for analysis by linear regression using bootstrapping [33] using STATA v. 13.1 (College Station, TX, USA). Results from regression analyses are presented as adjusted geometric mean ratios (aGMR) [95% confidence interval (CI)]. Multiplex data values below the lowest concentration were assigned as 1.6 pg/ml. Unstimulated responses were subtracted from antigen-stimulated results and negative values were set to zero. The Mann–Whitney *U* test was used to compare responses between infants of mothers with and without LTBI and those with and without a BCG scar and correlation between two continuous variables was assessed using the

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spearman rho test. For the different stimuli, the median maternal and cord blood responses, as well as the associations of infant responses with maternal LTBI and maternal BCG scar were analysed. In addition to looking at single cytokines and chemokines, Principal Component Analysis (PCA) [34] was performed on the cytokine and chemokine variables to summarize them. For this, an average cytokine or chemokine response was worked out for each infant by calculating the mean concentration obtained from the seven different stimuli (after subtracting unstimulated responses). The R programme (v3.2.2. R Foundation for Statistical Computing, Vienna, Austria) was used for further assessment of the associations.

For microarray, raw Illumina probe data were exported from BeadStudio and screened for quality. Pre-processing and statistical analysis was conducted using the R statistical language and various software packages from Bioconductor [35]. Quantile normalization was applied, followed by a $\log_2$ transformation. The LIMMA package was used to fit a linear model to each probe and (moderated) *t* tests or *F* tests were performed on the groups being compared. To control the expected proportions of false positives, the FDR for each unadjusted *p* value was calculated using the Benjamini and Hockberg method implemented in LIMMA. The microarray data are available through the National Center for Biotechnology Information Gene Expression Omnibus (GSE87801). Pathway analysis was performed using Gene Set Enrichment Analysis (GSEA), a non-parametric annotation-driven statistical analysis method [36], to assess which biological processes are associated with the different LTBI and BCG scar groups. We tested gene sets from the Molecular signature Database (MsigDB, http://www.broad.mit.edu/gsea/msigdb Hallmark collection (h.all.v5.0.symbols.gmt) which summarize and represent specific well-defined biological states or processes displaying coherent expression. Statistical significance was set for *p* value below 0.05.

3 Results

3.1 Participant characteristics

The flow of the participants through the study and recruitment details have been described elsewhere [7]. Of the twenty-nine mothers considered for the WBA/Luminex analysis, 12 had a LTBI and 16 had a BCG scar. Three mothers had missing information on BCG scar and were not included in the analysis. Mothers with and without a BCG scar were comparable in terms of age (25 years versus 26 years, p = 0.78), LTBI (31% versus 50%, p = 0.42) and gravidity status (37% versus 50% primigravida, p = 0.70). Their infants were comparable in terms of birth weight (3.09 versus 3.22, p = 0.47) and gender (19% versus 40% male, p = 0.38). Ninety-three mothers were considered for the gene expression microarray, and of these, 21 had a LTBI and 38 had a BCG scar. Mothers with and without a BCG scar were comparable in terms of age (24 years versus 25 years, p = 0.34), LTBI (26% versus 41%, p = 0.26), gravidity status (39% versus 45% primigravida, p = 0.78). Their infants were comparable in terms of birth weight (3.24 versus 3.21, p = 0.77) and gender (40% versus 47% male, p = 0.77) (Table 1).

3.2 The innate immune responses to the different stimuli

The median cytokine and chemokine responses to the different stimuli were analysed. Supplementary Tables 1A and 1B illustrate these for mothers and infants, respectively. There

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were overall low to moderate concentrations of cytokines, chemokines and growth factors in both maternal and cord blood samples, except for IL-6, IL-8, MCP-1, MIP-1α, MIP-1β and IP-10 (to TLR 7/8 agonist) where concentrations were high across the different stimuli.

3.3 The association between maternal LTBI, maternal BCG scar and innate immune responses in mothers and their offspring

Cytokine and chemokine responses were analysed for associations with maternal LTBI and maternal BCG scar.

For the combined results, maternal responses were not associated with their own BCG scar, except for VEGF where mothers without a BCG scar, compared to those with, had higher concentrations (p = 0.031, Fig. 1A). For IL-4, mothers with a BCG scar, compared to those without, had higher responses (p = 0.012, Supplementary Table 1). Maternal LTBI was positively associated with cord blood IP-10 responses, with an aGMR [95% CI] of 5.10 [1.21, 21.48], p = 0.026 (data not shown).

Cord blood samples obtained from infants of mothers with a BCG scar, compared to those without BCG scar, had overall higher responses to innate stimuli for the following analytes: IFN-γ (aGMR 2.69 [1.15, 6.17]), IL-12p70 (1.95 [1.10, 3.55]), IL-10 (1.82 [1.07, 3.09]), VEGF (3.55 [1.07, 11.48]) and IP-10 (6.76 [1.17, 38.02] There was a similar, but weaker, trend for the proinflammatory cytokines TNF-α (aGMR 1.99 [0.69, 5.89]) and IL-1β (1.55 [0.37, 6.61]). (Fig. 1B, and Supplementary Tables 2 and 3).

The associations between infant responses to the different stimuli and maternal LTBI (Supplementary Figs. 1A and 1B) and maternal BCG scar (Supplementary Figs. 2A and 2B) were analysed. The following CpG-specific cytokine and chemokines were positively associated with maternal LTBI: IL-12p70 (p = 0.014), MCP-1 (p = 0.011) and MIP-1β (p = 0.007) (Supplementary Fig. 1B). Cytokines and chemokines that were positively associated with maternal BCG scar included: IL-10 (p = 0.017) and GM-CSF (p = 0.042) to PAM3Cys-Ser; TNF-α (p = 0.044), IL-2 (p = 0.019), IL-1β (0.005), IL-6 (p = 0.017), IL-10 (p = 0.001), GM-CSF (p = 0.014) and VEGF (p = 0.048) to FSL-1; TNF-α (0.017) to LPS; IFN-γ (p = 0.018), IL-12p70 (p = 0.023), GM-CSF (p = 0.047) to CL097; IL-2 (p = 0.048), IL-1β (0.017), IL-10 (p = 0.040), IL-8 (p = 0.011), GM-CSF (p = 0.027) to Mannan; TNF-α (p = 0.027), IL-12p70 (P = 0.012) and VEGF (P = 0.003) to Curdlan (Supplementary Figs. 2A and 2B).

3.4 Principle component analysis of infant innate immune responses

We observed correlations among the cytokines and chemokines measured and this was summarized using PCA. For the mothers, two principle components (PCs) were identified, which together, accounted for 43% of the variance in the dataset. The first PC explained 25% of the total variance and was characterized by IFN-γ, TNF-α, IL-12p70, IL-1β, IL-6, IL-4, IL-10, IL-13 and the second PC explained a further 18% of the total variance and was characterized by MCP-1, MIP-1α, MIP-1β, IL-8, and IL-17A based on factor loadings > 0.1 (Fig. 2A). Neither Maternal LTBI (data not shown) nor maternal BCG scar (Fig. 2B) was associated with the mothers' own PC scores.

For the infants, two PCs identified accounted for 53% of the variance in the dataset. The first PC explained 39% of the total variance and was characterized by most of the cytokines and growth factors measured (IFN-γ, TNF-α, IL2, IL-12p70, IL-4, IL-13, IL-10, IL-1β, IL-6, IL-8, VEGF and GM-CSF) (Fig. 2C). The second PC explained a further 14% of the total variance and was characterized by MCP-1 and MIP-1β. Infants with a high response in PC1 were born to mothers with a BCG scar (Fig. 2D).

These results are illustrated in Fig. 3. There were no associations between maternal LTBI and levels of PCs in the infants (Fig. 3A and B), and no associations between maternal BCG scar and levels of PCs in the mothers (Fig. 3C and D). Maternal BCG scar was associated with high levels of PC1 in the infants (median level of scores: 1.44 in scar-positive versus −0.94 in scar-negative, p = 0.020, Fig. 3E). There was no association between maternal BCG scar and levels of PC2 in the infants (median level of scores: −0.002 in scar-positive versus 0.754 in scar-negative, p = 0.065, Fig. 3F).

The correlations among the cytokines and chemokines measured are shown in Supplementary Table 4.

3.5 Analyses of clusters of innate cytokines and chemokines

In addition to the PCA, we performed a hierarchical bicluster analysis of the innate responses to further identify sets of cytokines and chemokines that might be coordinately expressed in infants of mothers with and without a BCG scar using R programming. Three clusters (C) of cytokines were identified (illustrated in Fig. 4): MCP-1, MIP-1α, MIP-1β, IL-17A (C1), VEGF, GM-CSF, IL-12p70 (C2) and IL-1β, IL-8, TNFα, IFN-γ, IL-2, IL-4, IL-10 (C3). Eleven cytokines formed an additional cluster (C4) that contained high concentrations of the proinflammatory cytokines produced by infants of mothers with a BCG scar.

3.6 Gene expression profiles in infants of mothers with and without LTBI, and in the infants of mothers with and without a BCG scar

In order to further examine the associations we found with the innate responses using the Luminex® assay, gene expression microarray analysis was performed using blood obtained from 42 and 51 infants at one and six weeks post-BCG, respectively, using RNA extracted from unstimulated whole blood. Gene expression from infants of mothers with and without LTBI and those with and without a BCG scar were compared. Infants of mothers with LTBI, compared to those of mothers without LTBI, had downregulated interferon and inflammation pathways one week after BCG immunisation (Fig. 5A), but up-regulated interferon and inflammation pathways at six weeks post immunisation (Fig. 5B). In contrast, the interferon and inflammation pathways were both up regulated in infants of mothers with a BCG scar at one (Fig. 6A and Supplementary Fig. 3A) and six (Fig. 6B and Supplementary Fig. 3B) weeks after BCG immunisation.

4 Discussion

This study reports an unexpected finding about the association between maternal BCG scar and infant responses in a birth cohort. We have shown that infants of mothers with a BCG

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scar have enhanced proinflammatory responses. The concentrations of proinflammatory cytokines measured in cord blood in response to stimulation with innate stimuli using the Luminex® assay were increased in infants of mothers with a BCG scar. The expression of genes in the interferon and inflammation responses pathways measured using gene transcription microarray was also increased in infants of mothers with LTBI at six week post BCG immunisation, and in infants of mothers with a BCG scar at one and six weeks after BCG immunisation.

Innate immune responses may determine the effectiveness of adaptive responses [37] and lead to either biased [38] or regulatory immune profiles [39–41]. The increased responses reported here may therefore impact on immune responses to vaccines administered at birth and on the course of infections and disease in childhood. Further studies of human innate immune profiles in response to immunisation, and during infections and disease, are needed.

There were no associations between maternal BCG scar and the mothers' own innate immune responses: associations were manifested only in the infants. The presence of a maternal BCG scar was taken to indicate BCG immunisation of a mother during infancy. There are suggestions of positive associations between IFN-γ responses and reactions at the site of BCG immunisation [42,43], and presence of a scar has been associated (in other studies) with protection against LTBI [27,28]. Scar might therefore be a good measure of protective immune responses. However, it is difficult to reconcile how a response to a vaccine administered to mothers in their infancy would exert its effects several years later in the offspring. It is possible that there may be common genetic factors between the mothers and their infants that determine scar formation and subsequent responses in the infants, or that the factors associated with scar formation in the mothers are transmitted to the infants. The lack of association between maternal BCG scar and the mother's own responses could be attributed to cumulative life-time exposures that alter the initial maternal innate immune responses after BCG immunisation. We did not collect data on scarring in these infants, but an ongoing larger study with a longer follow up will provide the opportunity to assess relationships between scarring and immune responses in mothers and their infants.

The development of a scar is also dependent upon the strain, dose and method of administration of the BCG vaccine [44]. The Danish strain of BCG vaccine, compared to BCG Russia, has been shown to elicit stronger responses in infants one year later and to cause more scarring [23–25,45,46], and the intradermal route of administration is associated with the formation of distinctive scars [47,48]. We were unable to ascertain the strain, the dose and the method of administration of BCG vaccine in these women, although the most common strain and the method used in this setting are BCG Russia and the intradermal method, respectively. Since BCG immunisation is administered in the neonatal period, it is difficult to obtain information about BCG immunisation status of adults in a country where hospitals do not routinely record vaccine strain. There is therefore the possibility of misclassification of women based on the presence or absence of a scar. It is possible that the scar-negative women may have been BCG vaccinated without developing a scar, or that scars were lost with time. Our observed differences in infant response may therefore relate either to the mother's BCG immunisation status or to the quality of the mother's response to BCG immunisation.

Previous studies have reported the presence [49,50] or absence [51] of maternal cells in cord blood samples. It is therefore possible that the high proinflammatory response observed in cord blood could be due to responses from maternal cells in cord blood, but the method we used for collecting cord blood (by needle and syringe, with no "milking" of the cord, coupled with the use of trained midwives) minimized contamination. Previous tests carried out on maternal and cord blood samples in our studies (comparing levels of β-human chorionic gonadotropin) showed that contamination of cord blood by maternal blood was rare (unpublished data).

Interferon and inflammatory pathways were down-regulated in infants of mothers with LTBI at one week, but up-regulated at six weeks after BCG immunisation; this offers some support to the hypothesis that prenatal exposure to maternal LTBI modifies the infant response to BCG, but the change in direction of effect as the immune response matured was unexpected, and these findings would need to be confirmed in a larger study.

Limitations of the study were its observational and explorative nature, its small sample size relative to the many outcomes assessed. Maternal and infant factors such as maternal age, gravidity status, infant birth weight and gender were not adjusted for since these were not crudely associated with infant responses, and the numbers involved were generally small.

In summary, maternal BCG scar had a stronger association with infant responses than maternal LTBI, with an increased proinflammatory profile of immune responses. The mechanisms that underlie this association need to be further examined in a larger study.

Appendix A. Supplementary material

Refer to Web version on PubMed Central for supplementary material.

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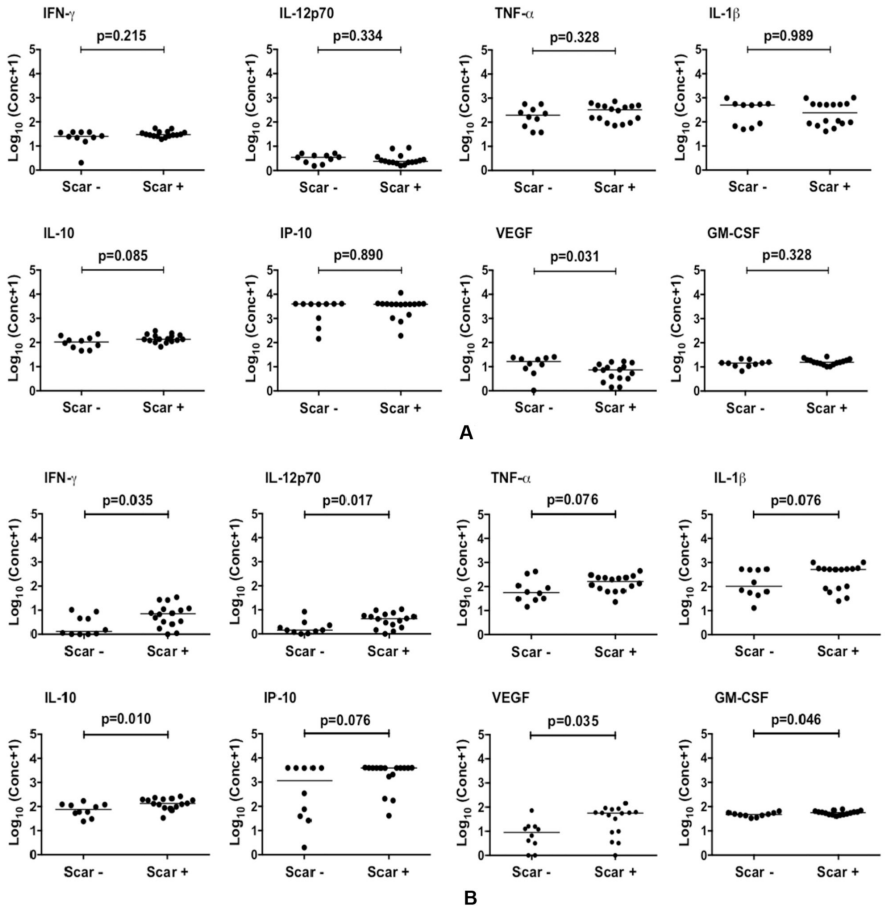

Fig. 1.

The association between maternal BCG scar and infant innate responses. Combined median cytokine or chemokine production following overnight stimulation with lipopolysaccharide (LPS) (toll-like receptor (TLR) 4 agonist), FSL-1 (TLR2/6 agonist), CpG-ODN2006 (TLR9 agonist), PAM3Cys-Ser (TLR1/2 agonist), CL097 (TLR7/8 agonist), Mannan (DC-SIGN agonist) and Curdlan (Dectin-1 agonist). Cytokines representing Th1/proinflammatory (IFN-γ, IL-12p70, TNF-α and IL-1β), immunoregulatory responses (IL-10) and chemokines/ growth factors (IP-10, VEGF and GM-CSF) measured by Luminex® assay are shown for the

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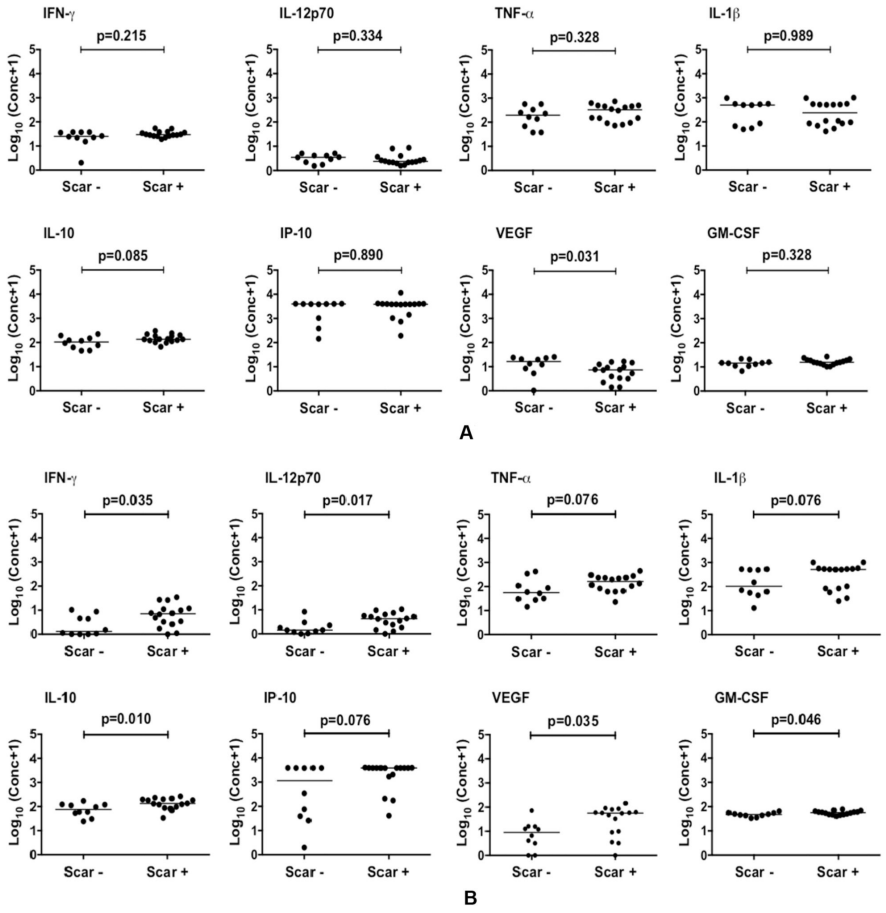

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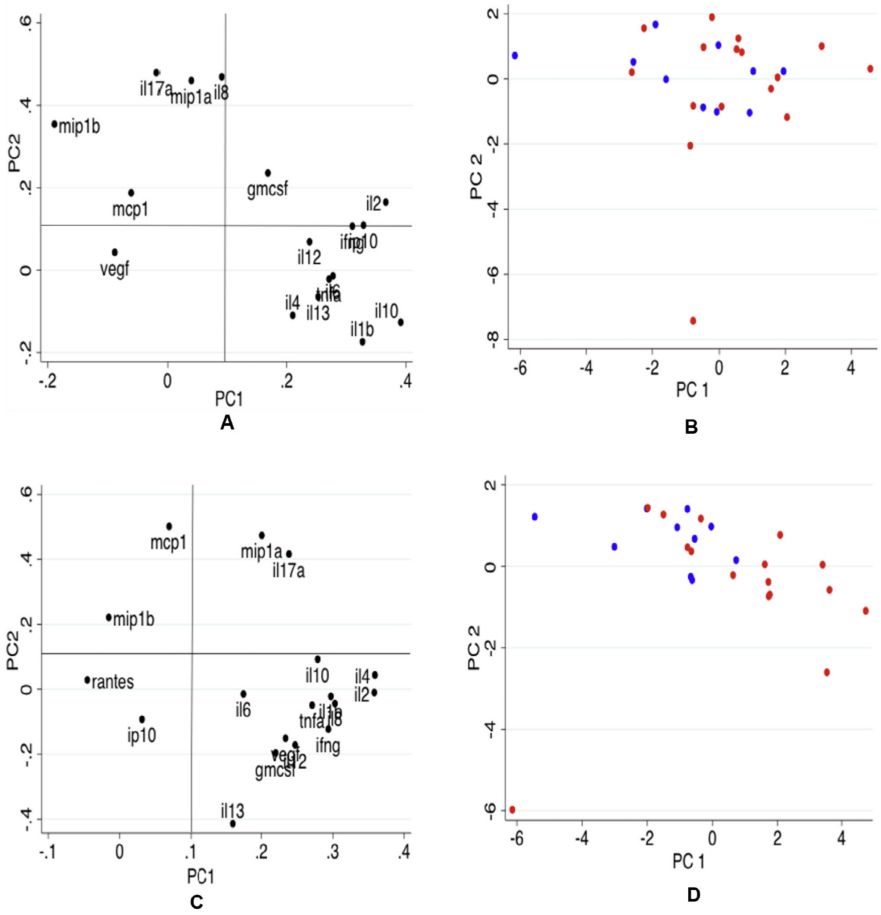

Fig. 2.

Scatterplots of first and second factor loadings for maternal and cord blood, derived from Principal Component Analysis of 17 analytes, showing cytokines and chemokines (A and C), individual mothers (B) and neonates (D). For mothers, the first principal component (PC) was characterized by a mixture of cytokines and the second PC consisted of chemokines. For neonates, the first PC was characterized by proinflammatory cytokines and the second PC consisted of chemokines, based on factor loadings >0.1. Red circles represent BCG scar-positive (Scar+) mothers and their infants. BCG scar-negative (Scar–) mothers

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and their infants are represented by blue triangles. One infant had overall high background responses (unstimulated samples) for most cytokines/chemokines measured. Subtracting the unstimulated values from antigen stimulated values gave overall low net values, thus the negative PC scores (−6.311 for PC1 and −6.228 for PC2). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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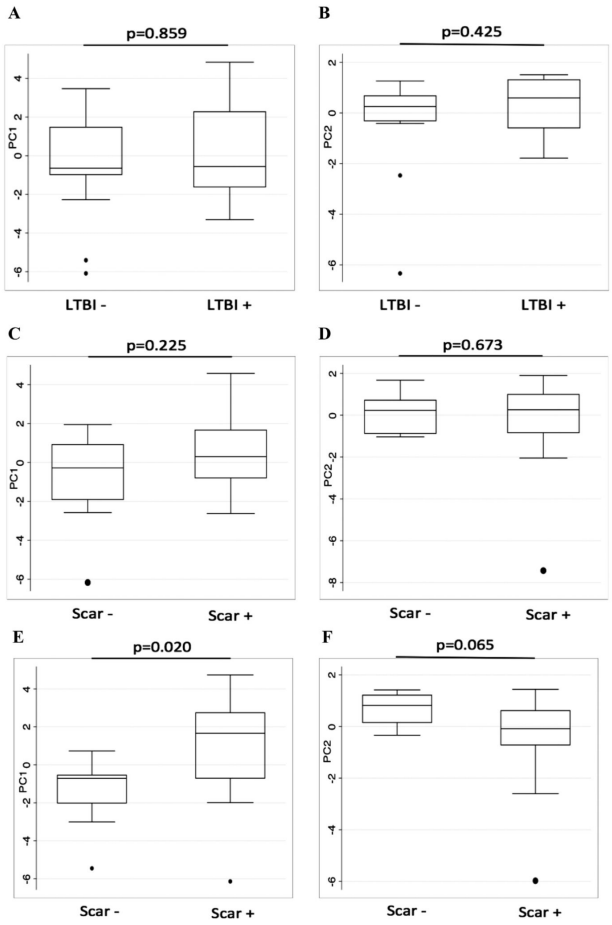

Fig. 3.

The association between maternal LTBI, maternal BCG scar and the innate immune responses in mothers and neonates. PCA was used to assess the association between maternal LTBI, maternal BCG scar and infant responses. The association between maternal LTBI and infant innate responses (A and B), and the association between maternal BCG scar and maternal (C and D) and infant (E and F) responses are shown. Two PCs that explained 43% and 53% of the variance in the dataset for mothers and neonates, respectively, were identified. The box plots represent the median and the interquartile range of the levels of the

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two PCs. The whiskers show the minimum and maximum values. P values are from Wilcoxon rank sum test.

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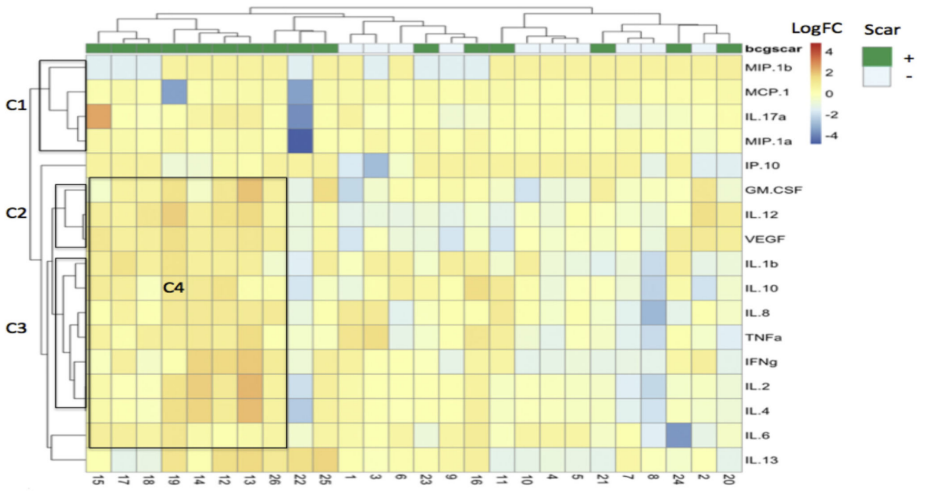

Fig. 4.

Cluster analysis of the innate cytokines and chemokines using the average linkage distance between clusters using R. Clusters go from root to leaf node for each cytokine and for the individual infants. Clusters in between are based on their agglomerative value. The branch shows the similarity, the shorter the branch, the more similar. Expression levels of individual cytokines ($\log_{10}$ [pg/ml]) are represented by shades of blue to red based on their correlations according to the dendrogram on the left, with highest values in dark red and the lowest in dark blue. Three distinct sets of correlated cytokines "clusters" are indicated as C1, C2 and C3 on the left. In addition, eleven cytokines (C4) form a cluster that has mainly inflammatory cytokines. Most infants of mothers with a BCG scar (top, green) clustered together in one discrete group, distinct from infants of mothers without a BCG scar (top, light blue). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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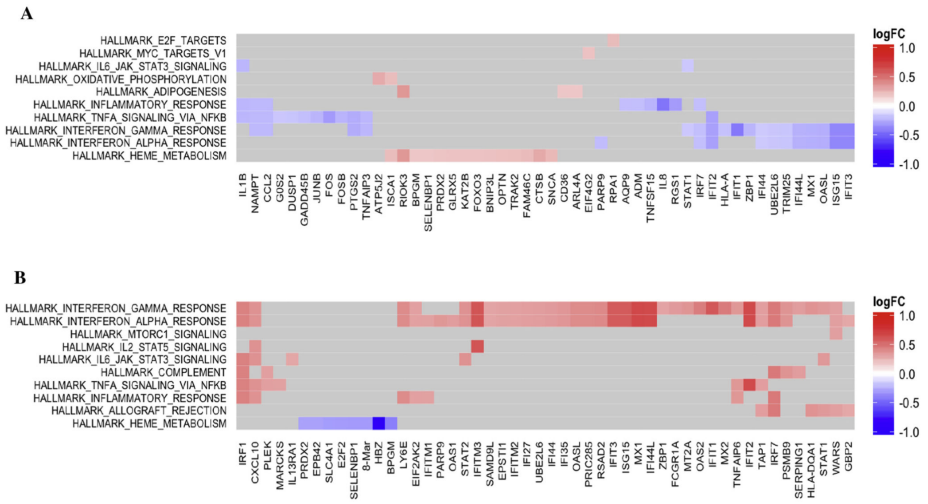

Fig. 5.

Gene Set Enrichment Analysis for the comparison of infants of mothers with and without LTBI. A checkerboard map showing top enriched pathways on y-axis and top leading edge genes (gene members contributing most to the enrichment score) on the x-axis. Scale at the right represents the gene expression fold change (log2 (exposed/unexposed)). Red (blue) indicates genes that are up-regulated (down-regulated) among infants of mothers with LTBI mothers. Interferon and inflammation response pathways were up regulated in infants of mothers with LTBI at six weeks. FDR adjusted *p*-value cut off of <0.25 was applied for pathways significance. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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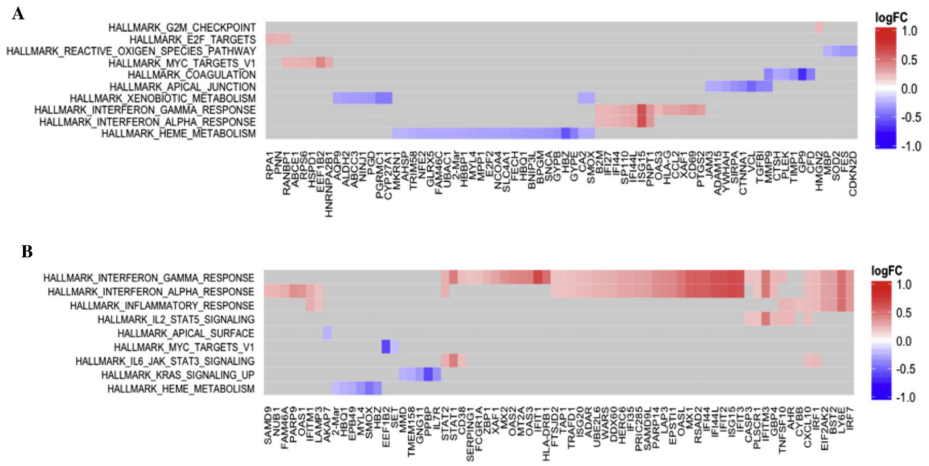

Fig. 6.

Gene Set Enrichment Analysis for the comparison of infants of mothers with and without a BCG scar. A checkerboard map is presented showing top enriched pathways on y-axis and top leading edge genes (gene members contributing most to the enrichment score) on the x-axis. Scale at the right represents the gene expression fold change (log2 (scar+/scar–). Red (blue) indicates genes that are up-regulated (down-regulated) among infants of scar-positive mothers. Interferon and inflammation response pathways are up regulated in infants of mothers with a BCG scar at one and six weeks after BCG immunisation. FDR adjusted p-value cut off of <0.25 was applied for pathways significance. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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Table 1

Characteristics of participants by maternal BCG scar status. The figures are given as numbers with percentage (%) in brackets, or as mean values. *P* value is based on unmatched *t* test for differences in maternal age and infant birth weight, and a two-sided Fisher's exact test for differences in maternal LTBI, parity and infant gender between scar-positive and scar-negative groups.

Characteristics	Participants for Luminex assay			Participants for microarray		
	Maternal BCG Scar present (n = 16)	Maternal BCG Scar absent (n = 10)	*P* value	Maternal BCG Scar present (n = 38)	MaternalBCG Scar absent (n = 22)	*P* value
Mothers						
Age, mean (years)	25	26	0.78	24	25	0.39
Latent TBI status, Present, no (%)	5 (31)	5 (50)	0.42	10 (26)	9 (41)	0.26
Gravidity, Primigravida, no (%)	6 (37)	5 (50)	0.70	14 (39)	10 (45)	0.78
Infants						
Sex, Male, no (%)	3 (19)	4 (40)	0.38	14 (40)	8 (47)	0.77
Mean birth weight (kg)	3.09	3.22	0.47	3.24	3.21	0.77

London School of Hygiene & Tropical Medicine
Keppel Street, London WC1E 7HT
www.lshtm.ac.uk

Registry
T: +44(0)20 7299 4646
F: +44(0)20 7299 4656
E: registry@lshtm.ac.uk

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Student	Sarah Prentice
Principal Supervisor	Stephen Cose
Thesis Title	Investigating the Non-Specific Effects of BCG in Neonates

If the Research Paper has previously been published please complete Section B, if not please move to Section C

SECTION B – Paper already published

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frontiers in Immunology

REVIEW
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They Are What You Eat: Can Nutritional Factors during Gestation and Early Infancy Modulate the Neonatal Immune Response?

*Sarah Prentice**

Clinical Research Department, London School of Hygiene and Tropical Medicine, London, United Kingdom

The ontogeny of the human immune system is sensitive to nutrition even in the very early embryo, with both deficiency and excess of macro- and micronutrients being potentially detrimental. Neonates are particularly vulnerable to infectious disease due to the immaturity of the immune system and modulation of nutritional immunity may play a role in this sensitivity. This review examines whether nutrition around the time of conception, throughout pregnancy, and in early neonatal life may impact on the developing infant immune system.

Keywords: nutrients, immunity, ontogeny, neonatal, pregnancy, infection, supplements

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Kirsty Le Doare,
Imperial College London,
United Kingdom

Reviewed by:
Daniel Munblit,
Imperial College London,
United Kingdom
Stephen M. Todryk,
Northumbria University,
United Kingdom

***Correspondence:**
Sarah Prentice
sarah.prentice@lshtm.ac.uk

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INTRODUCTION

Nearly 3 million deaths occur annually in children less than 30 days old, principally in low and middle-income countries (1). Improvements in neonatal mortality rate have proved difficult to achieve. Low-cost, easily implementable interventions are urgently needed.

Infections directly account for approximately one-third of neonatal deaths and are likely to contribute to deaths from other causes such as prematurity and in cases where babies are stillborn (1). Neonates show heightened susceptibility to infectious diseases due to a functionally immature immune system (2). Innate immune components are compromised by impaired mucosal surface integrity (3), lower levels of complement proteins (4), and reduced phagocytic capacities (5). Adaptive immune responses to pathogens are attenuated compared to adult responses, with children under 2 months old tending toward more regulatory responses with strong Th-2 and Th-17 cell polarization and weak Th-1 polarization (2, 6, 7). This is partly necessary to produce a tolerogenic environment, stopping rejection at the maternofetal interface and reducing reactions to self-antigens, and partly due to lack of primary exposure to antigens necessary to build up the adaptive immune responses. This functional immaturity of responses leaves the neonate particularly vulnerable to infectious pathogens. Decades worth of research has been directed at identifying interventions to improve neonatal immune responses to infections.

Various organs are sensitive to nutrition during embryonic and fetal development. Nutritional status can have short-term impacts on both fetal and childhood growth and development and longer term influences on adult health. Infants born following periods of nutritional deprivation, such as the Dutch Hunger Winter and identified in The Hertfordshire cohort, show increased risks of coronary heart disease, stroke, type-2 diabetes and metabolic syndrome when subsequently exposed to periods of nutrient sufficiency (8, 9). The concept that undernutrition during gestation may contribute to adult disease by having permanent effects on the structure, function and metabolism of

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the developing fetus, is known as the Developmental Origins of Health and Disease (DOHaD) theory. It has subsequently been shown to extend to a range of other diseases including psychiatric illnesses and cancers (10). Excess macronutrient consumption in mothers has also been associated with long-term sequelae in their offspring (11). Micronutrient deficiencies have long been known to have impacts on organogenesis, with iodine deficiency leading to congenital hypothyroidism (12) and folate deficiency increasing the risk of neural tube defects (13). Therefore, it has been hypothesized that the developing immune system is likely to be similarly sensitive to nutrition and that optimizing a mother's nutritional state during pregnancy will have long-term benefits for the immune responses during the neonatal period and beyond.

Early human evidence that nutritional factors during gestation might specifically influence adult immune responses came from longitudinal studies carried out in The Gambia in the 1990s (14). The Gambia has a strong bimodal seasonality that has major effects on the nutritional status of the population. The dry season, running from November to June, is a time of relative nutrient security. With the previous seasons crops being harvested, macronutrients are in greater supply and manual labor levels tend to be lower. In contrast, the rainy season, running from July to October, is characterized by declining levels of food availability and higher manual labor demands as the next season's crops are planted but the previous seasons supply is running short. This leads to deficits of both energy and micronutrient intakes that are particularly pronounced for women, who bare the brunt of much of the agricultural work (15). Analysis of demographic surveillance data available for the population from the 1940s revealed that people born during the "hungry" rainy season had a threefold higher risk of mortality from infectious diseases as adults than those born during the dry season (14). These findings were independent of subsequent nutritional status, as demonstrated by anthropometric and hematological status at 18 months of age, suggesting that the effector of these changes occurred earlier on in development. These data suggested that environmental factors, most likely nutrition, during conception, gestation and early postnatal life can have marked effects on the immune system that are stable, durable and not susceptible to modification by later improvements in nutritional status.

Nutrient intake of the mother and neonate is theoretically amenable to modification *via* supplements, which represent low-cost, easily implementable public health interventions. As such, there has been huge interest in the provision of nutritional supplements during gestation and early infancy to improve neonatal outcomes. This review summarizes the evidence regarding the impact of early life nutrition on biochemical immune markers and clinical infectious diseases outcomes in neonates.

POTENTIAL MECHANISMS FOR NUTRITIONAL INFLUENCES ON THE DEVELOPING NEONATAL IMMUNE SYSTEM

Studies in older children and adults have demonstrated the important influence that different nutrients have on the immune system. These effects, and the impacts of deficiencies on susceptibility to infectious diseases, are summarized in **Table 1**. Although the influence of nutrients on the developing immune system *in utero* and in early neonatal life may be similar to that of older children and adults, the impact of the nutritional state of the mother on the neonatal immune system is less well described.

Mother's nutritional status may hypothetically affect the neonatal immune system by influencing:

- *The mother's own immune system:* Optimizing maternal nutrition could directly enhance the neonatal immune system by increasing the quality and quantity of antibody and other immune factors available for passive transfer across the placenta and in breast milk. It could also indirectly improve neonatal immunity, by reducing the likelihood of maternal infections that may lead to preterm birth, a known cause of IgG deficiency in neonates due to reduced third-trimester antibody transfer (57). Increased maternal infections may also influence neonatal immune development *via* effects on the hypothalamic–pituitary–adrenal (HPA) axis (see below).
- *Placentation:* Maternal nutrient availability has been shown in animal and human studies to affect placentation, with affects on size, morphology, nutrient transfer receptors and vascular flow (58–63). This may theoretically affect passive transfer of antibodies and other immune factors to the fetus as well as altering the efficiency of nutrient transfer for fetal immune system development.
- *The maternal HPA axis:* The HPA axis is activated in times of low nutrient availability [particularly protein-energy malnutrition (64) and zinc deficiency (65, 66)] leading to increased circulating glucocorticoids. Increased cortisol levels can lead to both immunosuppression and altered placental function in the mother, with downstream effects for the fetus as described above, as well as directly impacting on the fetal immune system *via* actions on its own HPA axis.
- *The maternal gut microbiome:* The human intestinal tract contains more than 10^{14} bacteria and other organisms (67). These commensal microflora have evolved a complex symbiotic relationship with humans, and are increasingly recognized as essential for many aspects of human health (68). Nutrient intake influences the composition of the gut microbiota, which in turn can influence the availability of nutrients for absorption from food (69–71). The gut microbiome is crucial for the development and functioning of the mucosal immune system (72). Healthy gut flora help to promote mucosal tolerance to non-pathogenic antigens, reduce the overgrowth of pathogenic microorganisms and enhance absorption of nutrients that are potentially important for systemic immune system development (68). Dysbiosis (altered microbiome) has been associated with increased risk of immune-mediated diseases such as allergy, asthma, and inflammatory bowel diseases, as well as increased risk of infections (73). Animal models suggest that the immune development of the offspring may be influenced by the maternal microbiota in the following ways [reviewed in detail in Ref (74)]: (1) alteration of nutrient uptake having direct effects on maternal immunity and hence the availability of antibodies and immune factors for transfer to the offspring,

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TABLE 1 | Nutrients and their effects on immunity.

Nutrient	Effect on immunity	Effect of deficiency on clinical immune outcomes	Reference
Protein energy	*Innate* Epithelial integrity Complement levels NK-cell activity *Adaptive* T-lymphocyte number and function, particularly Th1-type cytokines Delayed type hypersensitivity Effect on B-lymphocytes less clear	Increased bacterial, viral, and fungal infections	(16, 17)
n-3 PUFAs	Activity is largely immunosuppressant with reductions in: *Innate* Leukocyte chemotaxis and adhesion NK-cell function Innate cytokine production *Adaptive* T-lymphocyte signaling	Theoretical increases in inflammatory-mediated diseases and allergy. Trials suggest that supplementation reduces the risks of inflammatory-mediated diseases such as rheumatoid arthritis and improves responses to infectious disease	(18–25)
Vitamin A	*Innate* Epithelial integrity Neutrophil, monocyte, macrophage, and NK-cell number and function *Adaptive* T-lymphocyte differentiation and migration T-lymphocyte numbers, especially CD4 B-lymphocyte numbers Antibody production and may affect the balance of production of different IgG subclasses	Increased susceptibility to infections, particularly diarrhea, respiratory infections and measles. Supplementation of children from 6 months to 5 years in areas at risk of deficiency reduces all cause mortality, diarrhea incidence and mortality and measles incidence and morbidity on meta-analysis	(26–28)
B vitamins	*Vitamin B2 (riboflavin)* Phagocyte activation *Vitamin B6* Dendritic cell function Lymphocyte maturation and growth T-lymphocyte activity and delayed type hypersensitivity B-lymphocyte activity and antibody production *Vitamin B9 (folate)* Epithelial integrity NK-cell activity T-lymphocyte proliferation and response to mitogenic activation Cytotoxic T-lymphocyte activity *Vitamin B12* NK-cell activity CD8+ T-cell activity B-lymphocyte activity and antibody production		(29–39)
Vitamin C	*Innate* Epithelial integrity Phagocyte production Antioxidative functions *Adaptive* T-lymphocyte maturation Interferon production	Association with increased incidence and severity of pneumonia. Supplementation in the elderly shows possible reductions in pneumonia incidence and duration	(40)
Vitamin D	*Innate* Macrophage activity (cathelecidin antimicrobial peptide expression, induction of reactive oxygen intermediaries, activation of autophagy) *Adaptive* T-lymphocyte number and function Th1/Th2 balance skewed to Th2 Unclear effect on B-lymphocytes (in humans)	Increased susceptibility to infections, particularly of the respiratory tract. Meta-analysis shows reduced acute respiratory tract infections when routine supplementation is given in the context of deficiency	(41–43)
Vitamin E	*Innate* Epithelial barrier integrity NK-cell activity *Adaptive* T-lymphocyte proliferation and function Delayed type hypersensitivity reactions Vaccine-mediated antibody responses	Supplementation is suggested to lead to reduced respiratory tract infections in the elderly	(37, 44, 45)

(Continued)

Appendix 2

TABLE 1 | Continued

Nutrient	Effect on immunity	Effect of deficiency on clinical immune outcomes	Reference
Zinc	*Innate* Epithelial barrier integrity Proinflammatory cytokine production Neutrophil oxidative burst NK-cell function *Adaptive* T-cell maturation and proliferation Th1/Th2 balance skewed to Th1	Increased bacterial, viral and fungal infections: particularly diarrhea and pneumonia. Routine supplementation of children in at-risk areas leads to reductions in duration of diarrhea and incidence of pneumonia, in children >6 months on meta-analysis, but not in children 2–6 months old	(46–50)
Selenium	*Adaptive* CD4+ T-lymphocyte proliferation and function	Increased viral virulence	(51–54)
Iron	*Innate* Neutrophil, NK-cell, and macrophage activity Innate cytokine production *Adaptive* T-lymphocyte numbers No apparent effect on B-lymphocyte number and function	May enhance or protect from infections with bacteria, viruses, fungi and protozoa depending on the level of iron. Although supplementation may theoretically enhance immunity to infectious diseases, untargeted supplementation may increase availability of iron for pathogen growth and virulence and increase susceptibility to, particularly, malaria and bacterial sepsis	(55, 56)

(2) alteration of the repertoire of antibodies passively transferred to the neonate, which may alter the degree of mucosal tolerance in the neonate, and hence its own microbiome composition (75, 76), (3) bacterial metabolites derived from the microbiota may be transferred to offspring across the placenta and in breastmilk and may impact on the offspring's developing immune system (77), and (4) organisms from the maternal microbiota can be found in placental tissue (78) and this exposure may impact directly on the developing infant immune system and indirectly by altering gestational length.

The mother's nutritional status may also affect the neonatal immune system by directly altering the nutrients available to the developing embryo/fetus. This may theoretically have long-term effects on offspring immunity *via*:

- *Epigenetic modification:* Epigenetic modification is the process by which stable alterations to gene expression, and thus the phenotype of cells, are induced without changes to the primary DNA sequence (79, 80). These modifications may be altered in response to environmental factors, persist following cell division, and, in some cases, are heritable—providing a means by which the environment may have permanent and multigenerational impacts on phenotype (81). The three main types of epigenetic modification are (1) DNA methylation; where the degree of methylation at, primarily, CpG dinucleotide rich sites in gene-specific promoters affects the degree of expression of that gene, (2) histone modification; where the accessibility of promoter regions of genes to transcription machinery is altered by additions to protein tails, affecting the degree to which DNA transcription occurs, and (3) non-coding RNAs, where small lengths of RNA bind to target mRNA, altering its subsequent translation (81). Of these, DNA methylation has emerged as a strong candidate effector mechanism to explain the DOHaD theory as it largely occurs during embryogenesis or early postnatal life, and produces durable effects (82). Alterations in DNA methylation of key metabolic genes induced by famine exposure in early life persist for at least six decades (83, 84). Epigenetic modification could theoretically have similar long-term impacts on the expression of genes important for the immune system.
- *Organogenesis and lymphopoiesis:* The process by which organs develop during embryonic and fetal life is highly sensitive to environmental influences. It has long been known that exposure to adverse factors at critical windows of organogenesis can lead to permanent changes in organ growth and function. Development of the infant immune system is likely to be similarly susceptible to environmental influences, including nutrient levels. In older children, both the thymus and hematopoietic branches of immunity are acutely sensitive to undernutrition, with reductions in thymus size and blood cell functioning shown to occur in both acute and chronic starvation conditions (85). As both immune compartments undergo massive expansion during the gestational period, with the thymus being at its largest as a proportion of body size at birth, it is highly plausible that nutritional conditions *in utero* would impact on the neonatal immune system. Studies in animals support a link between maternal macro/micronutrient deficiency and reduced thymic size and function (86–88), which may not be fully reversible by later improvements in nutrition (89).
- *Immunoregulatory mechanisms, e.g., the neonatal HPA axis:* Maternal cortisol levels (which may be altered by nutrient availability, see above), can influence the development of the fetal HPA axis, with long-term consequences for neuroendocrine-immune interactions (90, 91). Although the developing fetus is generally protected from maternal cortisol fluctuations by the function of 11 B-hydroxysteroid dehydrogenase in the placenta, levels of this enzyme are decreased by undernutrition (92). Evidence from animal studies suggests that stimulation of the fetal HPA axis can lead to lower lymphocyte proliferation, reduced NK-cell activity, and reduced antibody responsiveness in offspring (93), as well as increasing the responsiveness of the HPA axis to stressors later in life. These effects are hypothesized to be mediated through epigenetic programming of glucocorticoid receptors (91).

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- *The neonatal gut-microbiome:* The neonatal gut microbiome is strongly influenced by the maternal microbiome. Colonization of the gastrointestinal tract occurs around the time of birth (and possibly even earlier) with organisms acquired from the mother's gastrointestinal tract, vagina, skin, and breast milk, and is influenced by delivery type, gestational age, and feeding method among other factors (94). Modification of the maternal microbiome may thus be hypothesized to influence the developing neonatal immune system both directly, by altering the neonatal microbiome composition, and indirectly, by altering the nutrient status of the mother and hence the availability of nutrients for immune system development during fetal life.

A conceptual framework for the potential influences of early life nutrition on the developing infant immune system is shown in **Figure 1**. Evidence for such effects occurring in humans is discussed below.

EVIDENCE FOR THE INFLUENCE OF PRE- AND PERICONCEPTIONAL NUTRITION ON THE INFANT IMMUNE SYSTEM

Epigenetic Modification of the Early Embryo

Specific evidence for the impact of periconceptional nutrition on later immune functioning through epigenetic modifications has been suggested from the previously described Gambian cohort. The plasma levels of 1-carbon metabolites crucial for DNA methylation undergo seasonal variations in pregnant women. Higher levels of folate, methionine, and riboflavin, and reduced homocysteine levels occur in the nutritionally challenged rainy season (95–97). Although counterintuitive, this may be due to increased consumption of green leafy vegetables during this period, due to the need to food diversify (98). The increased level of these methyl-donor intermediaries correlates with increases in DNA methylation seen at metastable epialleles (MEs) (see **Box 1**) in children conceived in the rainy season (and thus born in the dry season, correlating with reduced later infectious disease mortality) (96, 99). A metastable epiallele VTRNA2-1, involved in tumor suppression and viral immunity, has been identified that is differentially methylated according to season of conception (and hence nutritional status), and is stable for at least 10 years (100). This provides the first in-human evidence that periconceptional nutrition could directly influence subsequent immune functioning. Although the clinical relevance of the variability in methylation of this ME in susceptibility to infections has yet to be proven, it provides a tantalizing suggestion that the seasonal variation in adult infectious disease mortality is mediated, at least in part, through nutritionally sensitive epigenetic modifications.

A number of epidemiological studies have now linked DNA methylation status at the promoter region of inflammatory mediators to nutritional status in pre- and early postnatal life (107–109), although the timing of nutritional influences causing these epigenetic modifications is difficult to prove. Methylation status of these genes has been correlated with later markers of biochemical inflammation, though effects on clinical outcomes have yet to be shown (107). Intriguingly, animal models have shown that alterations to paternal diet can alter DNA methylation in offspring, with resultant phenotypic changes increasing the risk of obesity and metabolic syndromes (110–113). The

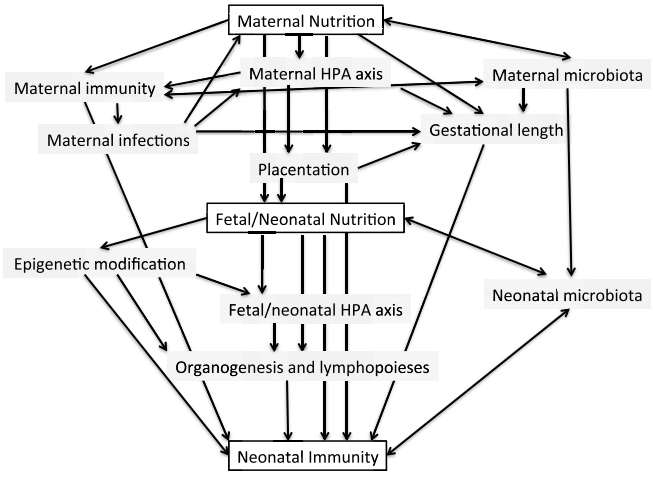

FIGURE 1 | Conceptual framework for the potential interactions between maternal and early neonatal nutrition and the developing infant immune system.

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BOX 1 | Metastable epialleles. A tool for investigating the influence of the periconceptional environment on offspring epigenomes.

The inherent tissue specificity of many epigenetic changes creates challenges for the study of the influence of epigenetic modifications on adult phenotypes (99). While epidemiological association studies between gene variants and risk of disease may use easily obtainable peripheral blood draws, studies investigating epigenetic influences on disease etiology may require tissue-specific samples that are often not as accessible. Metastable epialleles (MEs) are regions of DNA where methylation is established stochastically in the early embryo and is subsequently maintained throughout all three germ-layer lineages (101). Thus, methylation of MEs occurring in the early embryonic period (pregastrulation) may be determined from peripheral blood samples.

Differential methylation of MEs in mice has been shown to have dramatic phenotypic consequences including alterations in fur color (102), tail-kinking (4, 103), and propensity to obesity (104). Methylation of murine MEs is strongly influenced by maternal nutrition and other environmental factors in the periconceptional period (105, 106). MEs in humans may have effects on adult disease and provide an easily accessible method of investigating the epigenetic pathways that may be involved in the DOHaD theory.

potential transgenerational influence of paternal diet on the health outcomes of offspring has also been suggested in humans from epidemiological studies carried out in Sweden. These showed a correlation between reduced food availability during the father's, and even grandfather's, preadolescence and increased life expectancy, with reduced risk of cardiovascular and diabetes-related mortality (114). Other studies have linked early onset of paternal obesity with increased liver enzymes and long-term changes in percentage body fat in offspring. These effects are likely to be mediated by epigenetic modification of spermatozoa, and may be sex specific (115). Thus, it may be that paternal diet is also ultimately shown to produce lasting effects on the immune system of offspring.

Although most human studies have focused on DNA methylation as a mediator of long-term effects of periconceptional environment on the health of off-spring, animal studies suggest that histone modification (116) and microRNAs (117, 118) may also play a role in the developmental origins of disease, though their importance in immune system development has yet to be investigated. Thus, it appears likely that immune system functioning is influenced by interacting and overlapping epigenetic modifications induced by nutritional status, and other environmental factors, occurring around the time of conception, during gestation and in early postnatal life.

Placentation

Although evidence for the importance of several micronutrients including vitamin D, zinc, folate, calcium, and iron on placental growth and function exists (58, 59), studies directly investigating the effects of periconceptional maternal nutrition on placentation and subsequent fetal immunity are limited. One study that randomized non-pregnant women of child-bearing age to a multiple-micronutrient (MMN) supplementation or placebo and followed up subsequent pregnancies, showed minimal improvements in placental vascular function with MMN supplementation, but no improvements in other markers of placental function (plasminogen activation inhibitor 1 and 2 ratio) and transfer of maternal measles antibody at birth (119).

EVIDENCE FOR THE INFLUENCE OF GESTATIONAL NUTRITION ON THE INFANT IMMUNE SYSTEM

Macronutrients

Protein Energy

The relationship between maternal nutrition and fetal growth is complex, involving maternal metabolic and endocrine, as well as placental, functioning (2, 120). However, the neonatal presentation of protein-energy malnutrition is assumed to be infants who are born small-for-gestational age (SGA). Infants born SGA or low-birth weight (LBW) have an increased risk of infectious mortality in the neonatal period and beyond (121–124). SGA/LBW infants show altered immunology, with lower complement and IgG (125), lower plasmacytoid dendritic cells, higher NK-cells and higher IgM (126), and higher inflammatory activation and T-cell turnover (127), compared to those delivered at an appropriate weight. Gambian infants born in the nutritionally deprived rainy season (a presumptive marker of reduced macronutrient supply in late gestation) show smaller neonatal thymus size (128), and have some changes to thymic function (129). These immune changes do not appear to be long lasting, however, and a seasonal effect of infectious disease incidence may contribute to these findings (130, 131). Intrauterine growth restriction has been associated with reduced vaccine responses in childhood, though inconsistently (132–135).

Given the suggested link between macronutrient deficiencies and neonatal morbidity, a number of maternal protein supplementation strategies have been evaluated (136). Balanced protein energy supplementation (containing up to 20% of energy as protein) leads to modest increases in birth weight (up to 324 g) (137), and reduces the number of SGA infants born by around a third (136). Reductions in neonatal deaths as a result of supplementation have not been clearly shown, however, with meta-analysis of the three published studies reporting neonatal mortality showing only non-significant improvements in neonatal outcomes (136, 138–140). Even if these non-significant reductions in mortality are true findings, the causal mechanisms underlying such effects are unknown, with reductions in prematurity likely to play a significant role. No clear link between maternal protein energy supplementation and improvement in neonatal immunity has been demonstrated. Maternal protein supplementation has no proven impact on later vaccine responses, mucosal immunity and delayed-type hypersensitivity reactions (130) or thymus size (141), although impacts on thymic function at the cellular level were not assessed. The lack of substantial demonstrable neonatal benefits from maternal protein energy supplementation may reflect the heterogeneous etiologies of SGA and LBW, with factors such as poor placentation and environmental toxin exposure not addressed by supplementation. It may also be due to challenges with targeting the intervention to the most at-risk subjects within populations. Subgroup analysis of supplementation studies suggest that the intervention is only beneficial when provided to malnourished individuals, and that high protein supplements may even impair fetal growth when given in the context of adequate diets (136).

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Lipids

Maternal PUFA supplementation during gestation is associated with reductions in preterm births and small increases in birth weight (142) on systematic review. However, impacts on the immune system are less clear. Most research has been directed on the effect of fish-oil supplementation on reduction in atopy risk in offspring. Systematic reviews have suggested reductions in offspring IgE-mediated allergy and eczema following gestational/lactational n-3 PUFA supplementation, though the duration of these effects is not clear and the relative importance of the timing of supplementation during gestation or lactation is difficult to determine (143, 144). Murine studies suggest that n-3 PUFA supplementation of mothers can improve offspring responses to infections, with enhanced vaccination responses shown in mice fed high n-3 PUFA diets during gestation and lactation (145). In humans, docosahexaenoic acid (DHA) supplementation during gestation and lactation was associated with reductions in CD8+ T-cells, increases in naive CD4CD45RA+ helper cells and reductions in lymphocyte IFNγ production (146). However, this trial did not show changes to immunoglobulin levels, vaccination responses or clinical outcomes and may have been confounded by the high baseline dietary DHA levels of all participants. One trial of prenatal DHA supplementation has shown reduction in incidence and duration of cold symptoms during infancy (147). No significant evidence of reductions in neonatal outcomes such as sepsis, morbidity or mortality have been shown in systematic review of human studies, though adequately powered trials to assess these outcomes are lacking (148).

Micronutrients

Micronutrient deficiencies are estimated to affect approximately 2 billion people worldwide. They are often particularly severe in women of reproductive age due to the high demands of pregnancy and lactation (149). Optimization of micronutrient levels in pregnant women has therefore been proposed as a strategy to enhance neonatal immunity.

Specific Micronutrient Supplementation during Gestation

Zinc

Overt zinc deficiency is now rare but moderate deficiency is common worldwide (150). Zinc supplementation of mothers leads to biochemical improvements in their zinc status and that of their offspring (151, 152). Thymus size in infants correlates with cord-blood zinc levels (153), although a recent study showed no impact of maternal zinc supplementation on infant thymic size (154). Improved hepatitis B vaccine antibody responses and delayed type hypersensitivity reactions to BCG vaccination have been shown following maternal zinc supplementation (154), but no effect on haemophilus influenza B conjugate vaccine responses has been found (155). Theses studies suggest some influence of maternal zinc supplementation on infant immune development, but the clinical impact of this is uncertain. A recent systematic review of 21 trials (>17,000 mother–infant dyads) suggests no benefit of maternal zinc supplementation for IUGR, LBW, stillbirth, and neonatal death, though small reductions in preterm birth were shown (156). No significant reduction in neonatal infective outcomes, including neonatal sepsis, umbilical infections, fever, and necrotizing enterocolitis (NEC), was seen but the number of studies reporting these outcomes was small. One study from Bangladesh showed reduced acute diarrheal and impetigo episodes in the first 6 months of life following maternal zinc supplementation, though no difference in persistent diarrhea, cough, and LRTI (157, 158). A study from Indonesia similarly reported reduced diarrheal incidence in infants <6 months old following maternal supplementation with zinc, but this was at the expense of increased episodes of cough (159). Conversely, a study in Peru did not report any benefit for diarrheal prevalence (160).

Vitamin D

Vitamin D deficiency is common worldwide due to lack of UV exposure in northern latitudes, darker skin pigmentation in southern latitudes, covering the skin with clothes, and vegetarian diets. There are strong correlations between maternal and umbilical cord vitamin D with deficiency or insufficiency in the mother likely to cause deficiency in offspring (161). Systematic reviews of supplementation in pregnancy suggest reduced risk of vitamin D deficiency in offspring and slight increases in birth weight (162, 163). However, no evidence for improvement in any other neonatal outcomes including neonatal mortality has been shown (162). Impacts of vitamin D deficiency on the developing immune system have been shown with reduced thymus size in offspring (164) and an association with increased CRP [although this trend is reversed with vitamin D sufficiency (>50 nmol/L) (165, 166)]. Maternal vitamin D supplementation during gestation results in increased Th1 and Th2 cytokine gene expression and reduced pattern recognition receptor expression in cord blood, following stimulation with PHA (167). Clinically, vitamin D deficiency in cord blood has been associated with increased risk of lower respiratory tract infections, wheeze, and eczema in a number of observational studies, suggesting long-term impacts on immune ontogeny, although causation is difficult to prove (168, 169). Of four studies assessing the impact of maternal vitamin D supplementation on infant risk of respiratory infections and wheeze (170–173), only one showed significant reductions in incidence of acute respiratory tract infections in offspring (170). In this study the intervention was combined with postnatal vitamin D supplements so the contribution of maternal supplementation *per se* is difficult to assess. A recent systematic review of vitamin D supplementation in pregnancy and early life did not show any reduction in the risk of persistent wheeze, eczema, or asthma, though the quality of available evidence was low (174).

Vitamin A

Vitamin A deficiency is associated with increased susceptibility particularly to diarrhea, respiratory infections, and measles (27). Infants born to mothers with low serum retinol had increased all-cause neonatal mortality in a study in Malawi (175). Nepali infants born to mothers with xeropthalmia (the clinical manifestation of severe vitamin A deficiency) had a 63% increased mortality within the first 6 months of life, which was reduced following maternal supplementation (176). However, large randomized controlled trials of vitamin A supplementation

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including more than 310,000 mother–infant pairs have failed to show benefits for perinatal and all-cause neonatal mortality on systematic review, despite reductions in maternal night-blindness and possible reductions in maternal infections (177). There is some evidence, though, that vitamin A supplementation of women may lead to long-term enhancement of natural antibody levels in offspring, perhaps acting through impacts on early lymphopoiesis (178). This suggests that long-term alterations to the neonatal immune system may occur following vitamin A supplementation, but that more sensitive outcome measures are required to identify these changes than all-cause neonatal mortality.

Iron

Fetal iron acquisition occurs actively across the placenta, mainly in the last trimester of pregnancy, and is highly regulated (179, 180). Direct correlations between maternal and fetal iron status are not consistently seen, as neonatal iron levels are likely to be preserved at the expense of maternal stores, but severe maternal anemia is associated with reductions in neonatal iron (181). Iron deficiency is thought to be the most prevalent micronutrient deficiency worldwide (182). It occurs particularly in low-income countries where diets tend to be low in absorbable iron and parasitic burden can be high. Systematic reviews support the use of daily or intermittent iron supplementation during pregnancy for improvement of maternal iron status and reduction in anemia (182, 183). However, no evidence for improvements in other maternal or neonatal outcomes has been found. There is a current paucity of evidence regarding specific impacts, whether beneficial or detrimental, of maternal oral iron supplementation on neonatal infection risks (184). Similarly, studies investigating a direct impact of fetal iron status on immune system ontogeny are lacking.

B-Vitamins, Including Folic Acid

Folate (vitamin B9) has been widely studied as a pregnancy supplement, due to its role in the reduction of neural-tube defects. A systematic review of 31 studies, mainly carried out in Europe in the 1960s and 1970s, showed a modest increase in birth weight (136 g) following maternal folate supplementation, but no reduction in preterm birth, still-birth, or neonatal death (all cause) (185). The impact of folate supplementation in pregnancy on neonatal immune parameters and infective outcomes has not been investigated. More recently, concerns have been raised that folate supplementation given beyond the first trimester, or in excessive doses during pregnancy, may be linked to an increased risk of allergy/asthma, but the evidence is largely from observational studies and is not yet conclusive (186).

Vitamin B12 deficiency is associated with an increased risk of preterm birth (187), but its supplementation in pregnancy has been little studied. One study in Bangladesh confirmed that maternal oral vitamin B12 supplementation during pregnancy and lactation led to significant increases in infant B12 levels, but this was not associated with improvements in passive transfer of influenza antibodies or levels of acute inflammation markers (188). A significant reduction in number of infants with raised CRP was shown, but the number of infants with the outcome was small and the influence of timing of supplementation during pregnancy or lactation could not be distinguished.

A systematic review of three randomized controlled trials of maternal supplementation with vitamin B6 has been shown to result in a significant reduction in mean birth weight (217 g) (189). The impact of supplementation on neonatal mortality or infections has not been studied (190).

One study of vitamin B2 supplementation during pregnancy and lactation exists, which showed modest increases in infant riboflavin levels, but did not report neonatal outcomes (191). Sole supplementation with other B-vitamins has not been studied in the context of pregnancy and their impacts on the developing neonatal immune system are unknown.

Other Vitamins and Trace Elements

A number of other micronutrients with known immunomodulatory effects in adults have been little studied in neonates. Longitudinal studies of the influence of maternal diet on infant respiratory outcomes have suggested inverse associations between maternal vitamin E intake and infant asthma/wheeze (192–194), however, this has not been borne out in randomized controlled trials of maternal supplementation (195). Maternal selenium deficiency leads to low selenium status of neonates and is associated with reduced circulating adaptive immune cells and *in vitro* thymocyte activation (196). Observational studies have associated maternal selenium deficiency with enhanced risk of infant infections in the first 6 weeks of life, but these studies are at high risk of confounding (197). One supplementation study of selenium in HIV positive mothers showed a possible reduced risk of all-cause child mortality after 6 weeks of life, but a non-significant increase in fetal deaths (198). No studies have yet investigated maternal vitamin C, vitamin E, or selenium supplementation for neonatal immune outcomes specifically. There is also no current evidence for reductions in the more gross markers that may be associated with neonatal immune function (IUGR, LBW, preterm birth, perinatal, or neonatal death) from supplementation in pregnancy of vitamin C (199), vitamin E (200), copper (201), or selenium (198).

Multiple Micronutrient Supplementation during Gestation

When micronutrient deficiencies exist they are often multiple, due to poor quantity and diversity of available foodstuffs (149). Identification and targeted treatment of specific deficiencies in pregnant women is expensive and programmatically challenging. Therefore many studies aiming to enhance micronutrient levels in pregnancy use multiple micronutrient (MMN) supplements that provide the recommended daily allowance of all vitamins and minerals in one tablet (202). However, the evidence supporting the use of MMNs for neonatal outcomes in general, and neonatal immunity specifically is not clear. Meta-analysis of studies involving more than 135,000 women showed modest increase in birth weight (22–54 g), with corresponding reduction in babies born SGA or LBW, following MMN supplementation compared to standard iron and folic acid supplementation (203). These improved birth outcomes did not translate into improvements in neonatal and infant morbidity/mortality including from

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infectious disease (204). No MMN supplementation studies to date have investigated neonatal immune parameters specifically, although one randomized controlled trial from The Gambia is due to report shortly (205).

Probiotics, Prebiotics, and Synbiotics

Studies of maternal supplementation with probiotics (live microorganisms that contribute to a "healthy" gut microbiota), prebiotics [nutrients that promote growth of healthy bacteria, such as non-digestible oligosaccharides (206)], and synbiotics (a combination or pro- and prebiotics), for modulation of the neonatal immune system have been conducted in humans, but are relatively limited. A number of randomized controlled trials have shown that maternal consumption of probiotics or synbiotics can lead to measurable changes in the composition of their offspring's microbiome (207–210) and to changes in immune markers in the mother (211). However, alterations in infant immune markers following maternal supplementation, such as vaccine responses and cytokine levels, have been harder to show (212). Reduced incidence of eczema, though not asthma and wheeze, in infants has been suggested from systematic reviews of trials of prenatal supplementation but the effects may not be durable (72, 213–216). One small trial has shown reduced gastrointestinal infections in infants born to mothers supplemented with probiotics (211), and another a reduction in respiratory infections (217), but these findings need to be confirmed in larger studies.

EVIDENCE FOR THE INFLUENCE OF EARLY POSTNATAL NUTRITION ON THE INFANT IMMUNE SYSTEM

The major nutritional influence on neonatal immunity is breast milk, which contains immunological components such as antibodies, anti-inflammatory cytokines and other antimicrobial factors, as well as the macro and micronutrients to support neonatal immune system development (218). Its benefits over formula milk for protection against various infections, atopy, and allergy are well reviewed elsewhere (219, 220). Here, we focus on the potential impact of supplementary nutritional interventions for the breastfeeding mother and neonate on the developing neonatal immune system.

Lactational Supplementation

The composition of breast milk is highly regulated according to the neonate's needs with the concentrations of many components maintained independently of maternal nutritional status and diet (221). Some immunomodulatory micronutrients, such as iron, folate and zinc (222, 223) and macronutrients such as arachadonic acid (224, 225) are not altered in the breast milk according to maternal diet. Therefore, maternal supplementation of these nutrients would likely have little or no impact on neonatal immune outcomes and they are not discussed further in this section. However, some immunoactive nutrients in breast milk are impacted by diet and their concentrations in milk vary worldwide. These include vitamin A, vitamin D, B vitamins, selenium, and PUFAs, particularly DHA (34, 221).

Micronutrient Supplementation of Lactating Mothers

Vitamin A

Vitamin A is not only necessary for the developing neonatal immune system, its presence in breast milk is also important for the regulation of a number of breast milk proteins important for host defense (226). Infants are born with low vitamin A stores in the liver, and breast milk is the main source of vitamin A for infants during the first 6 months of life (227). Numerous reports have shown decreased breast milk vitamin A concentration with maternal deficiency, and increased concentrations with high exogenous vitamin A levels (228, 229). However, the results of postnatal maternal vitamin A supplementation studies for neonatal outcomes have been inconclusive. Systematic reviews of both lower dose (200,000 IU) and higher dose (400,000 IU) postpartum maternal vitamin A supplementation have shown only small increases in breast milk retinol concentrations (230) and a lack of supporting evidence for reduced infant morbidity (including from infections) to 6 months of age (230, 231). As a result, WHO no longer recommends routine postpartum vitamin A supplementation for women in low- and middle-income countries (WHO 2017). Studies on the effects of postpartum vitamin A supplementation on immunological outcomes specifically are limited and inconclusive. Studies variously report increases and no change to sIgA following postpartum vitamin A supplementation (226, 232). Further studies looking at a wider array of immunological parameters, and altering the timing of vitamin A supplementation are ongoing (226).

Vitamin D

Vitamin D deficiency is relatively common in breastfed infants, with low concentrations in milk even from vitamin D sufficient mothers (233). Studies investigating maternal postpartum supplementation have shown variable results, though on balance suggest supplementation may enhance infant vitamin D status (234–238). At present, however, direct neonatal supplementation of with vitamin D is the preferred method of enhancing neonatal vitamin D status (see below). Studies investigating the impact of vitamin D supplementation in breast-feeding women for neonatal immunological outcomes are lacking.

B-Vitamins

B-vitamins levels in the breast milk are largely amenable to improvements with supplementation of the mother (with the exception of folate) (34, 239), but there are no studies looking at the impact of lactational B-vitamin supplementation on neonatal immune outcomes.

Selenium

Selenium levels in breast milk are sensitive to dietary intake (240) and can be increased by supplementation (240, 241) [although these effects have not been consistently shown (197, 242)] and alter infant selenium status (243). Although selenium deficiency in infants has been associated with increased risk of respiratory infections in the first 6 weeks of life (197), large studies investigating maternal postpartum selenium supplementation for infant infectious morbidity have not been conducted.

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Multiple Micronutrients

Given the high prevalence of coexisting micronutrient deficiencies world-wide, there is a surprising lack of studies investigating the impact of multiple micronutrient supplements in breastfeeding mothers for infant outcomes (34). Only two small trials (52 women total) have compared MMN supplementation with nothing/placebo in breast feeding mothers, and reported on neither infant morbidity nor immunological outcomes (34, 232).

Lipid Supplementation of Lactating Mothers

The concentration of PUFAs, particularly DHA, in breast milk is highly affected by maternal diet (244), and PUFA supplementation increases levels in breast milk (245). Breast milk n3:n6 ratios have been associated with risk of allergy and atopy in infants in observational studies (246–248) although not consistently (249). Fish oil supplements provided during lactation alter cytokine production in the infant for at least 2.5 years, favoring faster immune maturation and Th1 polarization (250). Given the increasing existence of imbalanced n3:n6 ratios in westernized diets, there has been interest in providing PUFA supplements to lactating women for allergy prevention in infants, although concerns exist about potential negative impacts on infectious disease susceptibility (251, 252). However, at present only two studies (667 participants) have investigated postnatal maternal PUFA supplementation specifically, and although persisting alterations in cytokines have been shown, the studies were underpowered to detect differences in infant atopic disease or infectious morbidity (143, 250).

Probiotic, Prebiotic, and Synbiotic Supplementation of Lactating Mothers

Supplementation of lactating mothers with probiotics has been associated with alterations to breast milk cytokines and infant fecal IgA (253), and changes to the breast milk and infant microbiomes (254). Studies supplementing mothers with probiotics during lactation suggest a reduced risk of dermatitis, but interventions tended to combine pre- and postnatal supplementation, so the specific impact of lactational supplementation is difficult to determine (255). As with prenatal maternal supplementation, effects on infant immune outcomes following lactational supplementation require further evaluation (72, 256).

Neonatal Supplementation

Direct supplementation with crucial nutrients in the neonatal period has also been assessed as a strategy to protect infants from deficiency. However, in the majority of cases, despite improvements in the nutrient status of infants, no clear evidence for improvements in clinical or biochemical immune outcomes has been shown.

Micronutrient Supplementation of the Neonate

Zinc

Zinc use in older infants has been associated with reductions in diarrhea duration (48) and lower respiratory tract infections incidence (47), but results following supplementation in the neonatal period have been more equivocal (257–261). One small study of zinc supplementation as an adjunct to antibiotics in neonates with sepsis showed a reduction in treatment failures and a non-significant 43% reduction in mortality (262). A larger study to investigate this is currently ongoing (263). Studies directly investigating the impact of neonatal zinc supplementation on immunological markers are limited. Routine zinc supplementation has not been associated with improvements in OPV seroconversion rates (264), although its use as an adjunct to antibiotics in neonatal sepsis has been associated with significantly reduced serum calprotectin, IL-6, and TNFα and a non-significant reduction in mortality (265).

Vitamin D

Vitamin D supplementation is recommended routinely in many countries for its impact on calcium and bone metabolism, but large-scale evidence for postnatal supplementation on any immunological disease outcomes (infection or allergy) is lacking (266). A recent systematic review of supplementation in children below 5 years of age did not show reductions in diarrhea and pneumonia incidence despite raised vitamin D levels in supplemented children, though supplementation in the neonatal period was not looked at specifically (42). One trial of maternal and infant vitamin D supplementation has suggested lower numbers of respiratory infection primary care visits following high dose maternal and infant supplementation, compared to low dose (170). A large trial to investigate immunological outcomes following neonatal vitamin D supplementation in breastfed infants is currently underway (266).

Vitamin A

Vitamin A supplementation in children from low- and middle-income countries aged 6 months to 5 years is associated with reductions in all-cause mortality of around one-third on systematic review (28). In contrast, a large systematic review of trials including more than 168,000 infants from low- and middle-income countries did not show any benefit of vitamin A supplementation when given in the neonatal period (267). Effects of supplementation may differ by underlying vitamin A status of the population, as reductions in all-cause mortality were suggested in the South Asian studies but not in the African studies. The African studies also showed concerning side-effects with increased transient bulging of the fontanelle and interactions of vitamin A with routine immunizations, particularly in female infants (268, 269). Studies investigating the effects of neonatal vitamin A on immunological parameters are limited. One study conducted in Guinea Bissau showed no effect of neonatal vitamin A supplementation on BCG vaccination responses at 6 months of age (270), although some evidence of reduced TNFα and IL-10 production in girls who have not received DTP vaccination (271). Two RCTs are currently ongoing to specifically investigate the effects of neonatal vitamin A supplementation on the immune system, but these have yet to report (226, 272). Routine vitamin A supplementation in children below 6 months of age is not currently recommended.

Iron

The provision of iron supplements to neonates deserves special mention due to its potential for increasing susceptibility to

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infections by enhancing iron availability for pathogens (55). Studies conducted in the 1970s showed that injecting neonates with iron dextran at birth significantly increased the risk of *Escherichia coli* meningitis and sepsis (273) and enhanced *in vitro* bacterial growth (274, 275). This may have been partly due to the mode of delivery, as parenteral iron administration is not subject to regulated uptake in the gut and therefore may overwhelm iron homeostatic mechanisms in iron replete children, but similar concerns exist with the untargeted provision of oral iron supplements. Older children given iron supplements from 4 months of age have increased risk of gastrointestinal infections (276), adult studies show increased *in vitro* bacterial growth in serum after oral iron supplementation (277) and there are suggestions that malaria risk is increased when oral iron is provided to iron replete children in endemic countries (55, 278). Human breast milk contains low levels of iron and has specific iron chelating agents such as lactoferrin. Our group and others have also shown that serum iron drops rapidly and profoundly in the first 12 h of life that and persists at low levels for at least 4 days. This low serum iron is associated with reduced *ex vivo* bacterial growth (279, 280). Taken together, this evidence suggests that humans may have evolved to mitigate against the enhanced pathogen susceptibility and oxidative stress that results from high iron loads. Therefore provision of exogenous iron to the neonate, except in specific situations where severe iron deficiency anemia has been diagnosed, may do more harm than good. In fact, there is increasing interest in novel therapeutics, such as lactoferrin and hepcidin agonists, that reduce serum iron in the context of neonatal infections (281–283). However, as preterm and growth-restricted infants have lower iron stores from birth, routine iron supplementation is often given, starting from 4 weeks of age, in high-income countries (284). In these settings, where infectious disease burden is low, no adverse infective outcomes have been shown on systematic review (285).

Other Vitamins and Trace Elements

Parenteral selenium supplementation of very LBW infants in NICU has been shown to increase selenium levels and reduce the incidence of neonatal sepsis, but systematic review of available evidence does not show improvements in survival (286, 287). No similar studies of oral supplementation in normal weight, term, breastfed infants in areas of selenium deficiency have been conducted. Studies looking at the effects of neonatal selenium, B-complex vitamins, vitamins C and E, or combined micronutrient supplements on immunological parameters specifically are lacking.

Probiotic, Prebiotic, and Synbiotic Supplementation in the Neonate

Interest in the provision of probiotics, prebiotics, or synbiotics directly to neonates that are at risk of dysbiosis of the gut microbiome has exploded in recent years (255). Preterm infants are at particular risk of dysbiosis, not only due to gut immaturity, but because they often have reduced or delayed enteral feeds and increased exposure to antibiotics. Failure to establish normal gut flora is linked to higher risk of NEC and nosocomial sepsis (288). Systematic review of studies providing probiotics to low-birth weight infants in neonatal units, suggest a reduction in grade II or III NEC and all-cause mortality, though no significant reductions in sepsis (289, 290). Not all studies have shown clear benefits for NEC, however, and multistrain probiotics appear more beneficial than single strain organisms (291). Prebiotic supplements have not been shown to result in significant reduction in NEC, all-cause mortality or sepsis when given to preterm infants (292). The long-term health implications of use of pre- and probiotic supplements in preterm infants are not currently known. Provision of probiotics and prebiotics to formula fed infants, in attempts to produce a gut microbiome profile similar to breastfed infants, has also been extensively studied. Although beyond the scope of this review, these studies suggest reductions in atopic disease (though few studies have follow-up of sufficient duration to assess long-term effects) (293) and some limited evidence on systematic review for reductions in gastrointestinal and respiratory infections (294, 295). More excitingly, a recent randomized controlled trial in breastfed infants in rural India showed that synbiotic administration during the first 7 days of life led to a 40% reduction in sepsis and all-cause mortality in the first 60 days of life (296). This suggests that in certain situations even the breastfed microbiome may be altered for immunological benefits in the early neonatal period. However, further studies to examine the effect of different strains, dosages and durations, as well as the long-term consequences of synbiotic administration, will be needed before synbiotics could be considered as a public health intervention for neonatal sepsis.

SUMMARY

Despite multiple animal and human studies associating nutrient deficiencies with adverse immunological outcomes, there is strikingly little evidence to suggest nutritional supplementation during gestation and early infancy has benefits for neonatal responses to infection or allergic disease prevention.

There are a number of plausible explanations for the lack of significant and consistent impacts of individual or combined nutrient supplements on neonatal outcomes. First, it may reflect the heterogeneity of the studied populations in-terms of their underlying nutritional status. Improvements in clinical outcomes are likely to be most where deficiencies are highest. The transfer of many nutrients across the placenta, such as vitamin A (177) and iron (179), occurs actively and is regulated by the fetus, meaning that even in the context of maternal insufficiency the fetus remains relatively protected. As a result, maternal supplementation might only benefit infants born to mothers with critical deficiencies. Large population studies including non-deficient participants will have reduced power to detect clinical benefit. Maternal vitamin A supplementation, for instance, had larger effects on maternal and neonatal outcomes in Nepal (297), where severe deficiency is common, compared to Ghana (298) and Bangladesh (299) where levels of deficiency are more moderate (177). Second, in many studies iron and folate were provided to mothers in the non-intervention arm. As these can also impact on neonatal infective outcomes, this may have confounded the results (156). Third, the optimal level of supplementation of micro- and macronutrients for neonatal outcomes is not known and dosages often differ between studies (300). Micronutrients

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have nutrient–nutrient interactions that may alter the availability of other immunity modulating nutrients and have a rate-limiting effect on immune development (301). High levels of iron, zinc, and protein, for instance, can have counterintuitively negative effects on the immune system, and may have detrimental outcomes when given to sufficient women (302). If this is the case, then population-based treatment as a public health intervention becomes challenging and less measurably effective. Fourth, it may be that the onset of maternal supplementation in the studies was too late in gestation to have lasting effects on immune system development. Supplementation was commenced after 12 weeks of age in many studies, which would miss an early programming effect of nutrients if one exists. As a number of supplementation studies reported improvements in mothers nutrient status following supplementation, but no improvements in clinical outcome for the offspring, it would be interesting to know whether this enhanced nutritional status had positive impacts on future pregnancies, by improving nutrient status during the periconceptional period. Lastly, despite the large number of studies investigating maternal nutrient supplementation, those designed specifically to look at the effects on neonatal immune development and infectious/allergic disease outcomes are limited and further research with more sensitive outcome markers is warranted.

Although the evidence for the benefits of nutritional supplements in pregnancy and early infancy has so far been disappointing, some exciting possibilities remain. The persisting epigenetic changes induced by nutritional factors around the time of conception, which may impact on immune functioning in later life, warrants further study to assess their impact on neonatal infections, allergy and the amenability to supplementation. The potential benefit of probiotics and synbiotics for infectious disease and allergic outcomes in infancy is also extremely exciting. The World Allergy Organisation has recently recommended probiotic use during gestation, lactation and early life for infants at high risk of atopic disease (303), but further work to determine the most effective strains, dosage and duration, and whether these vary by geographical region, will be needed before their widespread use as a public health intervention against neonatal infections can be recommended.

AUTHOR CONTRIBUTIONS

SP was responsible for all parts of this article.

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Conflict of Interest Statement: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The reviewer DM and handling editor declared their shared affiliation.

Enrolment and Eligibility Form DBS 10/3/2014 Participant ID Sticker:

DBS
First Eligibility Checklist – Mother, Pre-Delivery

Maternal Screening Number DBS7|___|___|___| **SCN** Maternal Initials |___|___|___| **MIN**

Infant ID Number DBS6|___|___|___| **ID** **(FILL IN ONCE INFANT RANDOMISED)**

Date (dd/mm/yy) |___|___|/|___|___|/|___|___| **DATE**

Please tick:	**YES (=1)**	**NO (=2)**	
1. Does the mother reside in the Entebbe or Katabi subcounty, and does she expect to be there for the 10 weeks of the study?	☐	☒	**EBBRES**
2. Is the mother known to be HIV positive?	☒	☐	**HIV**
3. Is the mother or anyone living in the same house as the infant currently being treated for TB?	☒	☐	**TBHO**
4. Has mother, or anyone living in the same house as the infant, had any of the following symptoms?			
• Cough for more than 2 weeks	☒	☐	**COUGH**
• Recent blood stained sputum on coughing	☒	☐	**BSPUT**
• Weight loss more than 3kg in past month	☒	☐	**WTLOSS**
• Fever/chills or night sweats for past week or more	☒	☐	**FEVER**

If ANY tick in a GREY column the mother is NOT ELIGIBLE for inclusion in the study.
Please file this form in the enrolment folder and take no further action

If ALL ticks are in the WHITE column then the infant is ELIGIBLE for inclusion in the study
Please provide mother with information about the study and consent the mother

Staff Name…….…….. Staff Initials |___|___|___| **SIN**

Jan 2014-01-30. V2

Enrolment and Eligibility Form DBS 10/3/2014 Participant ID Sticker:

DBS
Second Eligibility Checklist: Infant, Post-delivery

Maternal Screening Number DBS7|___|___|___| **SCN** Maternal Initials |___|___|___| **MIN**

Infant ID Number DBS6|___|___|___| **ID** **(FILL IN ONCE INFANT RANDOMISED)**

Date (dd/mm/yy) |___|___|/|___|___|/|___|___| **DATE**

Please tick:	**YES (=1)**	**NO (=2)**	
1. Has mother consented to be in the study and signed the consent form?	☐	☐	**CON**
2. Was cord blood successfully collected?	☐	☐	**CORD**
3. What time was cord blood collected?	|___|___|:|___|___|		**CBT**
4. Does the baby have any major congenital malformations?	☐	☐	**MALF**
5. Is the baby unwell as judged by a doctor or midwife?	☐	☐	**UNWELL**

If ANY tick in a GREY column the mother is NOT ELIGIBLE for enrolment in the study. Kindly inform the mother that unfortunately the infant can't be included in the study. File this form in the enrolment folder and take no further action.

If ticks are all in the WHITE boxes then the infant is ELIGIBLE for inclusion in the study.
Please proceed to the demographic and birth details form.

Staff Name…….…….. Staff Initials |___|___|___| **SIN**

Jan 2014-01-30. V2

Delayed BCG Study – Demographic form 10/3/2014 Participant ID Sticker:

DBS
Maternal Demographics and Infant Birth Details Form

Maternal Screening number: DBS7|___|___|___|**SCN** **Mother Initials** |___|___|___| **MIN**

Participant ID Number DBS6|___|___|___| **ID**

Date (dd/mm/yy) |___|___|/|___|___|/|___|___| **DATE**

Infant Details

1. Date of Birth	|___|___|/|___|___|/|___|___|	**DOB**
2. Time of Birth	|___|___|**:**|___|___|	**TOB**
3. Gender	|___| *M=1 F=2*	**SEX**
5. Maturity of the child	|___| **MAT**	
	Premature=1 Term=2 Postmature=3	
6. Birth Weight	|___|___|___|___|**g**	**BW**
7. Head Circumference	|___|___|**cm**	**OFC**

Maternal Details

8. Maternal Age	|___|___|	**MA**
9. Maternal Parity	|___|___|	**PAR**
10. Is mother on iron supplements?	|___| *(1=Yes 2=No)*	**IRON**

Socio-Economic Indices

11. Current Marital Status: |___| **MSTAT**
(1=Single, 2=Married / living as married, 3=Widow, 4=Divorced/separated 5=prefers not to say)

12. Ethnic group/Tribe (choose from list)
a) What tribe is the babies Father? |___| **FTRIBE**
b) What is your tribe (morther)? |___| **MTRIBE**

1 = Ganda	**12 = Nyole**	**22 = Ruli**
2 = Ankole	**13 = Gwere**	**23 = Maadi**
3 = Nyoro	**14 = Samya**	**24 = Nubian**
4 = Tooro	**15 = Jopadhola**	**25 = Sudan**
5 = Lugbara	**16 = Teso**	**26 = Rwanda**
6 = Kiga	**17 = Kumam**	**27 = Congo**
7 = Fumbira	**18 = Lango**	**28 = Burundi**
8 = Konio	**19 = Acholi**	**29 = Kenya**
10 = Gisu	**20 – Alur**	**30 = Tanzania**
11 = Soga	**21 = Lugbara**	**99 = Other, Specifiy ……**

Appendix 3c)

Delayed BCG Study – Demographic form 10/3/2014 Participant ID Sticker:

13. Highest level of education attained: |___| **MEDUC**

(1=None 2=Primary 3=Secondary 4=Tertiary 5= prefers not to say)

14. Do you smoke? |___| **SMOKE** *(1=Yes, 2=No)*

15. Do you drink alcohol? |___| **ALC** *(1=Yes, 2=No)*

16. What material is your house roof predominantly made of? |___| **ROOF**
(1=Dry banana leaves/fibre, 2=Grass, 3=Tins, 4=Iron sheets, 5=Tiles 6 = Absetos 9 = Other, specifiy……..)

17. What material are your house walls predominantly made of? |___| **WALL**
(1=mud/wattle 2=metal 3 = bricks 4=Wood 5= Iron sheets 6 = Prefers not to say)

18. How many rooms are in your house? |___| **ROOM**

(Include bedroom & sitting room but not bathroom and kitchen)

19. How many people live in your house (including yourself)? |___|___| **PEOPLE**

20. Which of the following do you or (you and your husband) own? (choose from list) |___| Assests

1 = bed
2 = mobile phone
3 = radio
4 = television
5 = bicycle/motorbike
6 = car
7 = all of the above
8 = none of the above

21. What fuel is primarily used for cooking in your home? |___| **FUEL**

(1=firewood 2=charcoal, 3=paraffin 4=gas/electricity)

Name of staff filing out form …… …**Initials |__|__|__|** SIN

WHEN COMPLETE PLEASE CONTINUE TO FOLLOWTHE RANDOMISATION INSTRUCTIONS

DBS Routine Clinical Review Form V3 April 2014 Participant ID Sticker:

DBS
ROUTINE CLINICAL REVIEW FORM

Date (dd/mm/yy) |___|___|/|___|___|/|___|___| **DATE** **Participant IDNO** DBS6|___|___|___| **ID**

Mother's Initials |___|___|___| **PIN**

Age |___| y |___|___| m |___|___| w |___|___| d **AGE**

Weight |___|___| . |___|___|kg **WEIGHT** Length |___|___|cm **LENGTH**

Temperature (Axilla) |___|___| . |___|^{0}C **TEMP** Respiratory Rate |___|___|___| br/m **RESP**

Heart Rate |___|___|___| bpm **HR** Capillary Refill Time |___| secs **CRT**

Feeding status |___| **FS** *(B = Breastfeeding exclusively, M = Mixed Feeding, F = Formula exclusively)*

(1) Parent Recall of Clinical Episodes

Since your last clinic visit has your child been unwell? |___| *Yes = 1 No =2* **ILL**

	Episode 1 Duration (d)		**Episode 2** Duration (d)		**Episode 3** Duration (d)	
Symptoms *(Yes = 1 No = 2)*						
Fever	|___| |___|___|	**1A**	|___| |___|___|	**2A**	|___| |___|___|	**3A**
Convulsions	|___| |___|___|	**1B**	|___| |___|___|	**2B**	|___| |___|___|	**3B**
Diarrhoea (<3 motions/day)	|___| |___|___|	**1C**	|___| |___|___|	**2C**	|___| |___|___|	**3C**
Diarrhoea (>3 motions/day)	|___| |___|___|	**1D**	|___| |___|___|	**2D**	|___| |___|___|	**3C**
Dysentry	|___| |___|___|	**1E**	|___| |___|___|	**2E**	|___| |___|___|	**3E**
Vomiting	|___| |___|___|	**1F**	|___| |___|___|	**2F**	|___| |___|___|	**3F**
Thirsty and drinks eagerly	|___| |___|___|	**1G**	|___| |___|___|	**2G**	|___| |___|___|	**3G**
Not able to drink/feed well	|___| |___|___|	**1H**	|___| |___|___|	**2H**	|___| |___|___|	**3H**
Cough	|___| |___|___|	**1I**	|___| |___|___|	**2I**	|___| |___|___|	**3I**
Difficulty in breathing	|___| |___|___|	**1J**	|___| |___|___|	**2J**	|___| |___|___|	**3J**
Sore mouth	|___| |___|___|	**1K**	|___| |___|___|	**2K**	|___| |___|___|	**3K**
Skin rash	|___| |___|___|	**1L**	|___| |___|___|	**2L**	|___| |___|___|	**3L**
Eye/Ear discharge	|___| |___|___|	**1M**	|___| |___|___|	**2M**	|___| |___|___|	**3M**
Poor growth/weight loss	|___| |___|___|	**1N**	|___| |___|___|	**2N**	|___| |___|___|	**3N**
Headache	|___| |___|___|	**1O**	|___| |___|___|	**2O**	|___| |___|___|	**3O**
Dry cough, mainly at night	|___| |___|___|	**1P**	|___| |___|___|	**2P**	|___| |___|___|	**3P**
TB contact	|___| |___|___|	**1Q**	|___| |___|___|	**2Q**	|___| |___|___|	**3Q**
Measles contact	|___| |___|___|	**1R**	|___| |___|___|	**2R**	|___| |___|___|	**3R**
Other (specifiy)	|___| |___|___|	**1S**	|___| |___|___|	**2S**	|___| |___|___|	**3S**

Appendix 3d)

DBS Routine Clinical Review Form V2 10/3/2014 **Participant IDNO** DBS6|___|___|___| **ID**

Participant Initials |___|___|___| **PIN**

Management of each episode	**Episode 1**	**Episode 2**	**Episode 3**
Medical Review	|___| **MR1**	|___| **MR2**	|___| **MR3**

Medical Review: *0=None, 1=Local Health Clinic, 2=Research Clinic, 3=Government Hospital, 4=Traditional Healer 5=private hospital*

Treatment	|___| **TR1**	|___| **TR2**	|___| **TR3**

Treatment: *0=None, 1=Antibiotics, 2=Anti-pyretics, 3=Analgesics, 3=Ointment, 4=Other, specify*

Outcome	|___| **O1**	|___| **O2**	|___| **O3**

Outcome*: 1=Improved 2=unchanged 3=deteriorated 4=died*

Comments:

(2) Current Clinical Episodes

Are you concerned your child is currently unwell? |___| *Yes (1) No (2)* **CCE**

If YES move to Illness Episode Form

(3) Current Examination Findings

Are there any new abnormal clinical findings on examination? |___| *Yes (1) No (2)* **CCF**

If YES move to Illness Episode Form

(4) Blinding

Was blinding broken during this clinic visit? |___| *Yes (1) No (2)* **BB**

If Yes why was blinding broken |___| **BBR**

Maternal concern about BCG site = 1 TB contact = 2 Illness consistent with neonatal TB = 3 Accidental = 4

Other = 5 (please specify)……

Clinician Name…….. Clinician Signature……..

Clinician Initials |___|___|___| **SIN**

Tick and initial the appropriate area on the 'Routine Clinic Visit Checklist' to confirm that review has occurred, and send the participant to the phlebotomist

Phlebotomy Form – Routine Clinic Visit. DBS V2 10/3/2014 Participant ID Sticker:

DBS
Phlebotomy and Vaccinations Paper Form - Routine Clinic Visit

1. **Participant IDNO** DBS6|___|___|___| **ID** **1b. Mother's Initials** |___|___|___| **PIN**
2. **Date** (dd/mm/yy) |___|___|/|___|___|/|___|___| **DATE**
3. **Time** (hr:min)|___|___|:|___|___| **TIME**
4. **Clinic Visit Number** |___| **CVN**

Please open the brown envelope attached to the visit checklist. This details what blood samples and vaccines are required

Blood Samples

4. Are blood tests required during this clinic visit? |___| *(Yes= 1 No = 2)* **BTR**
If yes continue to fill out this section
If no move on to BCG vaccine section

5. Was Lithium Heparin (green) successfully collected? |___| *(Yes= 1 No = 2)* **LHC**

If yes what was the approx. volume |___|.|___|ml **LHV**

If no why was the draw not successful? |___| **LHU**
3 unsuccessful attempts (1) Mother refused (2) Other (please specify) (3)

6. Was EDTA (purple) successfully collected? |___| *(Yes= 1 No = 2)* **EDC**

If yes what was the approx. volume |___|.|___|ml **EDV**

If no why was the draw not successful? |___| **EDU**
3 unsuccessful attempts (1) Mother refused (2) Other (please specify) (3)

Make sure all samples are labelled with Participant ID, Todays Date and the Blood Sample Number
Place all samples in the transport container for transfer to UVRI

BCG Vaccination

7. Is BCG vaccination required at this clinic visit? |___| *(Yes= 1 No = 2)* **BCGV**
If yes vaccinate using study BCG, fill out this section and the infant's personal participant plans
If no move on to BCG scar measurement section

8. BCG batch number |___|___|___|___|___|___|/|___| **BCGB**

9. BCG expiry date (dd/mm/yy) |___|___|/|___|___|/|___|___| **BCGE**

2 **Participant IDNO** DBS6|___|___|___| **ID**
Participant Initials |___|___|___| **PIN**

BCG Scar Measurement

10. Is the child **9 weeks** old or more? |___| *(Yes= 1 No = 2)* **NW**

If YES (i.e. the child is 9 weeks of age or older) then remove the BCG plaster and measure the BCG scar diameter

If no move onto next section

11. BCG Scar diameter |___|___|**mm** **SCAR**

Routine Immunisations

12. Are primary immunisations required at this clinic visit? |___| *(Yes= 1 No = 2)* **PIR**
If yes either provide them yourself (or send to MCHC) and document below.

If primary immunisations are required please ask mother if she has brought a stool sample with her. If so, label and give to the laboratory technicians for processing

13. Has a stool sample been received? |___| *(Yes= 1 No = 2)* **SSR**

Once procedures are complete double check the both copies of the personal participant plans have been completed

Tick and initial the box on 'Routine clinic visit checklist' to show which procedures were completed

Send the participant back to reception

Staff Member Name……Staff Member Initials |___|___|___| **SIN**

Appendix 3f)

Delayed BCG Study 2014

Participant Sticker:

DBS
Routine Clinic Visit Checklist

To be completed before transport reimbursement given

Participant IDNO: DBS|_6__|___|___|___| **ID** Mother's Initials: |___|___|___| **PIN**

Date: |___|___|/|___|___|/|___|___| **DATE** Clinic Visit Number |_**1**_| **CVN**

Check that the following have been done:

	Response (Initial and tick when done)	**Comment, if any:**
Receptionist		
Participant checked-in (ticked in diary and entered into attendance register)	☐ _____	
Plaster placed on R deltoid	☐ _____	
Both copies of Participant Study Card put in the brown envelope	☐ _____	
Nurse		
Anthropometry and vital signs measured and recorded	☐ _____	
Physician		
Clinical review completed	☐ _____	
Phlebotomist	Required and taken ☐ _____	
Blood samples taken if required	Not Required ☐ _____	
Vaccinations given if required (or sent to MCHC)	Required and given ☐ _____ Not Required ☐ _____	
Both copies of Participant Study Card updated	☐ _____	
Receptionist		
Participant copy of Study Card updated and given back to mother (coloured card)	☐ _____	
White paper copy of Study Card filed at the front of the file. Other paper forms filed in the appropriate section of the file	☐ _____	
Mother reminded of date of next clinic visit	☐ _____	
Transport re-imbursement given	☐ _____	

If any boxes un-ticked, please complete that activity or explain why it could not be done

Name of receptionist …….Receptionist Initials |___|___|___|

V2 April 2014

DBS Illness Event Record V3 7/4/2014 Participant ID Sticker:

DBS - ILLNESS EVENT RECORD

Please complete after writing full paper notes in the participants record

Date of visit (dd/mm/yy) |___|___|/|___|___|/|___|___| **DATE** **Mother's IDNO** DBS6|___|___|___| **ID**

Participant Initials |___|___|___| **PIN**

1. Measurements

Age |___| y |___| m |___|___| w|___|___| d **AGE**	Temperature (Axilla) |___|___| . |___| ^{0}C **TEMP**
Weight |___|___|___|___| g **WEIGHT**	Respiratory Rate |___|___|___| br/m **RESP**
Length |___|___| cm **LENGTH**	Heart Rate |___|___|___| bpm **HR**
	Capillary Refill Time |___| secs **CRT**

2.Symptoms

Please Indicate All Symptoms That Apply *(Yes = 1 No=2)*

Fever	|___|	**FEV**	Sore Mouth	|___|	**SOM**
Convulsions	|___|	**CON**	Skin Rash	|___|	**SKR**
Diarrhoea (<3 motions/day)	|___|	**DIA**	Eye/Ear discharge	|___|	**EED**
Diarrhoea (>3 mothions/day)	|___|	**DIAT**	Poor growth/weight loss	|___|	**WTL**
Dysentry	|___|	**DYS**	Headache	|___|	**HEA**
Vomiting	|___|	**VOM**	Dry cough, mainly at night	|___|	**DCO**
Thirsty and drinks eagerly	|___|	**THI**	TB contact	|___|	**TBC**
Not able to drink/feed well	|___|	**NDR**	Measles contact	|___|	**MEC**
Cough	|___|	**COU**	Injury	|___|	**INJ**
Difficulty in breathing	|___|	**DIB**	Other (specify)……		

3. Signs

Please Indicate All Signs That Apply *(Yes = 1 No=2)*

General

Restless / Irritable	|___| **RES**	Pallor	|___| **PAL**
Lethargic / unconscious	|___| **LTH**	Jaundice	|___| **JAU**
Sunken eyes	|___| **SKE**	Cyanosis	|___| **CYN**
Slow / very slow skin pinch	|___| **SKP**	Oedema	|___| **OED**
Brown thin hair	|___| **BTH**	Conjuctivitis	|___| **CJS**
Oral sores	|___| **ORS**	Corneal clouding	|___| **CCD**
Koplik's spots	|___| **KPS**	Lymph node enlargement	|___| **LNE**
Finger clubbing	|___| **FCB**		

Cardiovascular

Abnormal heart rate	|___| **AHR**	Abnormal Heart sounds	|___| **AHS**
Other cardiovascular abnormality present	|___| **OCA (**Specify……)		

Respiratory

Difficulty in breathing	|___| **DFB**	Fast breathing	|___| **FBG**
Decreased breath sounds	|___| **DBS**	Wheeze / rhonchi	|___| **WHZ**
Bronchial breathing	|___| **BBG**	Crepitations	|___| **CRP**
Stridor	|___| **STR**	Croup	|___| **CRO**
Other respiratory abnormality	|___| **ORA** (Specify……)		

Appendix 3g)

DBS Illness Event Record

Participant IDNO DBS6|___|___|___| **ID**
Mother's Initials |___|___|___|**PIN**

Abdominal System			
Abdominal swelling	|___| **ABS**	Ascites	|___| **ASC**
Hepatomegaly	|___| **HPM**	Splenomegaly	|___| **SPM**
Other Abdominal mass	|___| **ABM**	Other abdominal abnormality (Specify……)	|___| **OAA**
Skin			
Maculopapular rash	|___|**MPR**	Pustules	|___|**PUR**
Vesicular rash	|___|**VSR**	Abscess	|___|**ABR**
Petechiae	|___|**PTR**	Ulcer	|___| **ULR**
Other skin abnormality	|___|**OSA**	(Specify……)	
ENT			
Otitis media	|___| **OTM**	Otitis externa	|___| **OTE**
Tonsillitis	|___| **TNS**		
Central Nervous System			
Impaired consciousness	|___| **CNS**	Bulging fontanelle	|___| **BFT**
Seizures	|___| **SEZ**	Sunken fontanelle	|___| **SFT**
Stiff neck	|___| **STN**	Positive kerning's sign	|___| **PKS**
Focal Neurological Deficit	|___| **FND**	Other neurological abnormality (Specify……)	|___| **ONA**
Musculoskeletal System			
Abnormality present	|___| **AMS** (Specify……)		

4. Where Investigations Ordered During This Clinic Visit? |___| **INV** *Yes = 1 No = 2*
(Paper Investigations Sheet)

5. What is Your Provisional Diagnosis/Diagnoses? (Please see attached coding numbers)
|_____| **PDI** |_____| **PD2** |_____| **PD3** |_____| **PD4**

Other …… **PD0**

6. Was The Child Hospitalised For This Illness? |___| **HSP** *Yes = 1 No = 2*

7. Has a Follow-up Appointment Been Made? |___| **FUA** *Yes = 1 No = 2*
Date (dd/mm/yy) |___|___|/|___|___|/|___|___| **FUD**

8. Was Blinding Broken During this Clinic Visit? |___|**BB** *Yes = 1 No = 2*

8b) If Yes why was blinding broken? |___| **BBR**

Maternal concern about BCG site = 1 *TB contact = 2* *Illness consistent with neonatal TB = 3* *Accidental = 4*
Other = 5 (please specify)……

Clinician's name: …… Clinician Signature……

Clinician Initials |___|___|___| **SIN**

Appendix 3h)

Extra follow-up sheet for illness events DBS V3 7/4/2014 Participant ID Sticker:

DBS- Follow-up Appointment Sheets
Fill in after writing normal paper notes in the patient's file

1. Participant ID DBS6|___|___|___| **IDNO** 1b Mother's Initials |___|___|___| **PIN**
2. Follow-up Appointment Number |___| **FUA**
3. Date (dd/mm/yy) |___|___|/|___|___|/|___|___| **DATE**

4. Age |___| y |___|___| m |___|___| d **AGE**
5. Weight |___|___| . |___| kg **WEIGHT**
6. Length |___|___| cm **LENGTH**

7. Temperature (Axilla) |___|___| . |___|___| ^{0}C **TEMP**
8. Respiratory Rate |___|___|___| br/m **RESP**
9. Heart Rate |___|___|___| bpm **HR**
10. Capillary Refill Time |___| secs **CRT**

11. Outcome |___| *1 = improved, 2 = unchanged, 3 = deterioration, 4 = dead* **FUAO**

12. New investigations? |___| *Yes = 1 No = 2* **NI**

13. Was the child hospitalised for this illness? |___| *Yes = 1 No = 2* **HSP**

14. What is the current diagnosis? Please see coding chart

|___|___|___|**CD1** |___|___|___|**CD2** |___|___|___|**CD3** |___|___|___|**CD4** Other (specify……)

15. Is this the final diagnosis ? |___| *Yes = 1 No = 2* **FD**

15. Has another follow-up appointment been made? |___| *Yes = 1 No = 2* **FUA**

Date |___|___|/|___|___|/|___|___| **FUD**

16. Was blinding broken during this clinic visit? |___| *Yes = 1 No = 2* **BB**

16 b) If Yes why was blinding broken |___| **BBR**

Maternal concern about BCG site = 1 TB contact = 2 Illness consistent with neonatal TB = 3 Accidental = 4
Other = 5 (please specify)……

Clinician's name: …….. **Clinician's signature:** ……..

Clinician's initials |___|___|___| SIN

Appendix 3h)

Participant ID Sticker:

Delayed BCG Study 2014

DBS
Illness Clinic Visit or Follow-up Checklist

To be completed for all illness visits

Participant IDNO: DBS|_6__|___|___|___| **ID** Mothers Initials |___|___|___| MIN

Date: |___|___|/|___|___|/|___|___| **DATE**

Check that the following have been done:

	Response (tick and initial when done)	**Comment, if any:**
Receptionist Participant checked in (logbook and/or diary)	☐ _____	
Plaster placed on R deltoid	☐ _____	
Both copies of Personal Participant Plan sealed in the brown envelope	☐ _____	
Nurse Anthropometry and vital signs measured	☐ _____	
Clinician and phlebotomist Clinical review completed	☐ _____	
Treatment and follow-up organised if required	Required and given ☐ _____ Not Required ☐ _____	
Blood samples taken if required	Required and taken ☐ _____ Not Required ☐ _____	
Diagnostic tests carried out if required	Required and done ☐ _____ Not Required ☐ _____	
Receptionist Participant study card given back to mother Paper study card version filed at front of file	☐ _____	
Forms filed in the 'Illness Episode' section	☐ _____	
Mother reminded of date of next clinic visit (follow-up or routine)	☐ _____	

If any boxes un-ticked, please complete that activity or explain why it could not be done

Name of receptionist……Initials of receptionist |___|___|___|

V2 April 2014

This child received BCG vaccination at birth as part of a research study. If you believe this child has contracted TB please inform the EMaBS clinic team in writing at:

The Delayed BCG Study
MRC/UVRI Uganda Virus Research Institute on AIDS
51-59 Nakiwogo Road
Entebbe
PO BOX 49

If this card is found please return it to:
EMaBS Clinic, Near Grade A Hospital, Entebbe

Affix Participant ID Label Here

DBS
Personal Participant Plan

Mother Name:_____

Participant Name:_____
Date of Birth: |__|__|/|__|__|/|__|__|__|

This infant is in the
BCG AT BIRTH GROUP

Randomisation Group: C1

I confirm the mother has consented for her child to be in the Delayed BCG Study:

Staff Name_____ Signature_____ Date___/___/___

Clinic Visit Number	Expected Attendance Date	Procedures	Actual Date of Procedures
Birth	**Date of Birth** |__|__|/|__|__|/|__|__|__|	BCG	|__|__|/|__|__|/|__|__|__| *Batch* |__|__|__|__|__|__|/|__| *Expiry* |__|__|/|__|__|__|__|
		OPV	|__|__|/|__|__|/|__|__|__|
1st Routine Visit	**5 days of age** |__|__|/|__|__|/|__|__|	Blood sample 1	|__|__|/|__|__|/|__|__|__|
2nd Routine Visit	**6 weeks of age** |__|__|/|__|__|/|__|__|	Blood sample 2	|__|__|/|__|__|/|__|__|__|
		First dose primary immunisations (DTP/Hib/HepB PCV and OPV)	|__|__|/|__|__|/|__|__|__|
		Stool Sample	|__|__|/|__|__|/|__|__|__|
3rd Routine Visit	**10 weeks of age** |__|__|/|__|__|/|__|__|	Second dose primary immunisations (DTP/Hib/HepB PCV and OPV)	|__|__|/|__|__|/|__|__|__|
		Stool Sample	|__|__|/|__|__|/|__|__|__|

Appendix 3j)

INFANT BCG STUDY, IMMUNOLOGY SAMPLE COLLECTION FORM

Baby's Initials |__|__|__|PIN
DBS STUDY |_6_|__|__|__|ID

Participant ID sticker

Date of Sample: |__|__|/|__|__|/|__|__|__|__|**SDATE**

Sample: 70=Cord blood; 72=Baby 1week; 73=Baby 6 weeks 75=Baby 10weeks; 77=Baby sick: 79=other, specify..... |__|__| **SAMP**

Blood sample time (24hr): |__|__|:|__|__|**STIME**

Please tick|__√__| the specimen boxes below if sample taken off.

Heparin tube (Green) |__| **LHC** approx. vol. |__|. |__|ml **LHV**

EDTA tube (Purple) |__| **EDC** approx. vol. |__|. |__|ml **EDV**

If blood sample was not successful, why not? |__| **EDU**
3 unsuccessful attempts (1), Mother refused (2) Other please specify (3) ……..

Name of person taking off specimen: ……Initials |__|__|__|**INS1**

To be completed by staff at Rabbit House Immunology Laboratory

Date blood sample received: |__|__|/|__|__|/|__|__|__|__|**RDATE**

Lab Number: |__|__|__|__|__|__| **LABNO**

Time sample received (24 hr): |__|__|: |__|__|**RTIME**

Comments e.g. Sample received in good condition, satisfactory e.t.c.....**COM**

Name of person receiving specimen: ……....Initials |__|__|__|**INS2**

Appendix 3j)

DBS Haematology Form V0.2 April 2014 Participant ID Sticker:

DBS HAEMATOLOGY LAB FORM

Participant initials: |___|___|___| **PIN** **Participant ID DBS** |_6_|___|___|___| **ID**

Date sample received in Lab |____|____|/|____|____|/|____|____|____|____| **LDATE**

Time sample received in Lab |____|____|/|____|____|/|____|____|____|____| **LTIME**

Sample: **Illness Episode?** 1=yes, 2=no |____| **IES**
OR Routine Sample 1=yes, 2=no |____| **RS** **Sample Number** |____| **RSN**

Lab NO: |___|___|___|___|___|___| **SLAB**

IS SAMPLE SUITABLE FOR ANALYSIS? 1=yes, 2=no |____|

If **no**, comment…… **COM1**

FBC **(FILL IN BELOW OR STAPLE AN AUTOMATIC PRINT OUT TO THE FORM)**

WBC |____|____|.|____| **x10^3/µl WCC**

RBC	|____|.|____|____|	x10^6/µl	**RBC**
HGB	|____|____|.|____|	g/dL	**HB**
HCT	|____|____|.|____|	%	**HCT**
MCV	|____|____|____|	fL	**MCV**
MCH	|____|____|.|____|	pg	**MCH**
MCHC	|____|____|.|____|	g/dL	**MCHC**

DIFFERENTIAL (ABSOLUTE CELL COUNTS)

NE#	|____|____|.|____|____|	x10^3/µl **NEU**
LY#	|____|____|.|____|____|	x10^3/µl **LYM**
MO#	|____|____|.|____|____|	x10^3/µl **MONO**
EO#	|____|____|.|____|____|	x10^3/µl **EOS**
BA#	|____|____|.|____|____|	x10^3/µl **BABS**

PLT |____|____|____|____| 10^3/µl **PLT** **MPV** |____|____|.|____| fL **MPV**

Lab comments: …… **COM2**

Name and signature of person reporting the result.....

Date of reporting result |____|____|/|____|____|/|____|____|____|____|**RDATE**

Appendix 3k)

Delayed BCG STUDY, PARTICIPANT FINAL STATUS FORM

Mother's Initials |__|__|__| MINS **|_D_|_B_|_S_|STUDY |__|__|__|__|II**
Participant Date of Birth |__|__|/|__|__|/|__|__|

Date of Form: |__|__|/|__|__|/|__|__|__|__| **DATEFIN**

Final status of this participant: **|__|**
1=Enrolled but discontinued follow up **DISCON**
2=Enrolled but lost to follow up **LOST**
3=Enrolled and completed study **COMPLETE**

A. If follow up discontinued, give reason: **|__|** **REASON2**
1=Mother opted out of the study **OPTOUT**
2=Mother travelling and couldn't attend routine visits **TRAV**
3=Death **DIE**
4=Baby received none study BCG at community clinic **OBCG**
5=Other reasons, specify…….. **OTR**

B. If lost to follow up, give reason: **|__|** **REASON3**
1=Mothers address and contact details not taken **ADDNIL**
2= Mother changed address and couldn't be reached **ADDCHANGE**
3=Mother moved out of study area **RELOCATE**
4=Other, specify…….. **OTR**

BCGC
C. If follow-up was discontinued or infant lost to follow up, did we confirm that the baby had received BCG? **|__|** **BCGB**
BCG6
1= Yes, received at birth during the study prior to discontinuation (BCG at birth group)
2= Yes, received at 6 weeks during the study prior to discontinuation (BCG at 6 weeks group) **MCH6**
COM6
3= Yes, given by MCHC upon discontinuation of the study (BCG at 6 weeks group)
4= Yes, verbal confirmation from mother/father that the infant was taken to a community health center and received BCG (BCG at 6 weeks group) **NOS**
5=No, specify why not……

Name of study staff…… Initials|__|__|__| **INS**

Appendix 4a)

Information Sheet – Delayed BCG Study

MRC/UVRI/Entebbe Hospital/Kisubi Hospital

Does neonatal BCG vaccination provide short and longer-term protection against heterologous invasive infectious disease by enhancing the innate immune system?

Dear Mothers,

We would like to invite your child to participate in a research study.

What is the purpose of this study?

All babies in Uganda should receive BCG vaccination soon after birth. This is designed to protect them against TB. Some scientists think that BCG vaccination might have some other beneficial effects that might protect babies from other infections, but no one knows whether this is really true. We would like to know whether giving BCG at birth has such beneficial effects, and this is the reason for this study. We will be measuring whether BCG vaccination improves your baby's defenses against other infections and whether this happens for a short amount of time after vaccination or lasts for a longer time.

In this study, some children will receive BCG vaccination on the day that they are born and others will have it when they are six weeks old. We will ask to take two blood samples from your child to see whether there is a difference in the immune responses to infection depending on when the infants are vaccinated. We will also regularly review all children at the clinic for illness until they are ten weeks old.

We hope that this study will provide important information that will help us design better vaccination programmes to protect babies from a range of infectious diseases.

Measurements and samples collected from your child

If you agree for your child to take part in this study this is what will happen:

- At delivery, a sample of cord blood (2 teaspoons) will be taken after your baby has been delivered.
- The rest of the following procedures will only take place if the delivery goes well, the cord blood is obtained successfully, and your baby is healthy.
- After delivery you will be asked questions about your health and home environment.
- You will be asked to pick between a number of envelopes which will determine when your baby receives BCG vaccination (immediately or delayed to 6 weeks of age). The midwife will administer the BCG vaccination if your baby is to receive it immediately. All babies will have Oral Polio Vaccine as usual.
- We will ask you to bring your baby to clinic for their normal vaccinations, 2 small blood test (less than ½ a teaspoon each), up to 3 swabs of the nose and 2 samples of faeces, before the age of 10 weeks. This is likely to be four visits to the clinic.
- If your baby did not receive BCG vaccination at birth, we will ask you to bring them to the clinic at 6 weeks of age to have the vaccination.

Information Sheet – Delayed BCG Study

- Whenever your baby is brought to clinic they will be reviewed by a Doctor and his or her findings will be documented. If your baby is unwell they will receive investigations and treatment free of charge.
- Transport will be provided to take you home from the hospital and field workers may visit you at home to remind you about your follow up visits. A transport refund will be given for each visit when you are asked to come to the clinic.
- We will also encourage you to bring your child for review by a Doctor in the research clinic if you are concerned that they are unwell at any time during the study. Investigations and treatment will be free of charge.
- Your child's participation in the study is complete when they reach 10 weeks old.

Are there any risks in taking part?
Some people think that delaying BCG might pose a risk to your child from other infections. We do not know whether this is actually the case though, and is the reason for our study. To protect your child as much as possible, all children in the study will be regularly reviewed by a Doctor to check that they are well and will be treated as a priority if they are not. We will also contact you by telephone in between clinic visits to check that your child remains well. You are free to bring your child to the clinic at any point during the study if you are concerned that they require medical attention.

If your child has BCG vaccination delayed to 6 weeks there may be a slight increased risk of them getting TB during this time. We believe the risk is very low as it is unusual for babies to get TB so young. Many children in Uganda do not have BCG vaccination by 6 weeks of age and do not develop TB. Some scientists also think that giving BCG vaccination when your child is slightly older may improve the way your child fights off TB in the long-term, so may actually be good for your child. To make sure your child remains healthy during the study we will ask you to bring the child to clinic if either you or someone else living in the same house as the child is diagnosed with TB. We will also tell you what signs of illness to look out for that would mean that we would like to see your child at the clinic more quickly. All children in the study will be reviewed regularly by a Doctor, free of charge, to check that they are well and they will be treated as a priority if they are not.

There is likely to be a small amount of discomfort caused from having blood samples taken, but our staff are very experienced and so this is minimal. Each blood sample is very small (less than ½ a teaspoon) which will not cause harm to your child.

Data and Sample Storage
Data collected in this study will be anonymous i.e. someone looking at the data would not be able to identify you or your child from it. Any data collected will also be completely confidential and accessible only by members of the research team. If you agree to it, other researchers in the future might also use the anonymous data collected to continue to improve our understanding of the protective effects of BCG vaccination. Part of each blood sample may also be stored for other tests in the future. Some of these stored samples may be used for genetic studies. These studies look at whether differences in your family history explain why some people respond better to vaccinations and diseases than others. All the information collected, and the results of the tests, will be completely confidential. We will not contact you with the results of future studies as the work is for research purposes

only and not for identifying illnesses in your child. If you allow your child's blood to be used we may be able to find out information that will help to provide better services for people in Uganda and elsewhere in the future.

Your right to refuse or withdraw from the research study

Your participation in this study is voluntary. You are free to withdraw your child from the study at any time. Dropping out of the study will not affect your entitlement to routine government health care.

If you have any questions about your participation in this study, please feel free to ask the responsible midwife, doctor or field worker.

If you prefer, you may speak to one of the principal investigators for this study:

Dr Sarah Prentice (041 7704180)

Dr Stephen Cose (041 7704180)

Dr Alison Elliott (041 7704180)

If you have any questions about your rights as a research subject, you may also speak with the Ethics Committee Chairman from Uganda Virus Research Institute: (0414 321962)

Thank you very much.

Appendix 4b)

Consent Form – Delayed BCG Study

Participant ID Sticker

MRC/UVRI/Entebbe Hospital/Kisubi Hospital

Consent for procedures to investigate the impact of neonatal BCG vaccination on the innate immune response to heterologous pathogens

Mother's names ……..

Mother's IDNO |___|___|___|___|

I have read and/or been fully explained the information sheet concerning my child's participation in this study and I understand what will be required if I agree for them to take part in the study.

Our participation is voluntary.

My questions concerning this study have been answered by …….

I understand that at any time I may withdraw my child from this study without giving a reason and without affecting their entitlement to routine government health care and management.

Name of mother …… Date……

"My signature / thumb print below indicates that I agree for my child to take part in this study, for BCG vaccination to be randomly assigned and for blood to be drawn from them."

……
Signature

Or right thumb print

Witness*:

Name …… Signature …….

Date ……
**for those using a thumb print, this witness must not be a member of the research staff or a study participant*

Person Taking Consent:

Name …… Signature …….

Date ……
Note: form to be completed and signed in duplicate. One copy to be given to the mother; one copy to be retained at the clinic.

Appendix 4b)

Consent Form – Delayed BCG Study

Participant ID Sticker

MRC/UVRI/Entebbe Hospital/Kisubi Hospital

Consent to use samples and records for future studies

Mother's names ……..

Mother's IDNO |___|___|___|___|

I have been asked for permission to use the samples and records of my child for future studies. I have read the foregoing information or it has been fully explained to me. I had the opportunity to ask questions about it and any questions I have asked have been answered to my satisfaction.

Name of mother …… Date……

"My signature / thumb-print below indicates that I agree for part of my child's blood sample to be stored for future studies".

……
Signature

Or right thumb print

"My signature / thumb print below indicates that I do not agree for part of my child's blood sample to be stored for future studies".

……
Signature

Or right thumb print

Witness*:
**for those using a thumb print, this witness must not be a member of the research staff or a study participant*

Name …… Signature ……

Date ……

Person taking consent:

Name …… Signature ……

Date ……
Note: form to be completed and signed in duplicate. One copy to be given to the mother; one copy to be placed in infants study file

Appendix 4c)

Advice Leaflet - Delayed BCG Study
MRC/UVRI/Entebbe Hospital/Kisubi Hospital

Your child has received BCG vaccination at birth

We hope your child will be fine and healthy during this study, but we would like you to look out in case they become unwell and bring them to the clinic if you have any worries. This could be for any kind of illness, but particularly if they have:

Fever
Cough with rapid or noisy breathing
Diarrhoea especially if lots or with blood
Vomiting and unable to keep any milk down
Not feeding well
Having very few wet nappies
Skin rash
Bulging soft spot in head
Unusually sleepy or unable to wake-up
Crying unusually without settling

We are happy to see your child in clinic, free of charge, if you have any other worries about them, even if the concerns are not on the list above.

Whenever you come to the clinic please bring your vaccination card and plan with you

Contacts to call if you have any concerns

Dr Sarah Prentice (English Language): 0778-013944
Dr Dorothy Aibo (Luganda): 0771-021104
MAB clinic: 0414-320448

Summary of Product Characteristics

1 NAME OF THE MEDICINAL PRODUCT

BCG VACCINE SSI

Powder and solvent for suspension for injection.

2 QUALITATIVE AND QUANTITATIVE COMPOSITION

After reconstitution, 1 dose (0.1 ml) for adults and children aged 12 months and over contains:
Mycobacterium bovis BCG, Danish strain 1331, live attenuated, 2-8 x 10^5 cfu.

After reconstitution, 1 dose (0.05 ml) for infants under 12 months of age contains:
Mycobacterium bovis BCG, Danish strain 1331, live attenuated, 1-4 x 10^5 cfu.

This is a multidose container. See section 6.5 for the number of doses per vial.

For a full list of excipients, see section 6.1

3 PHARMACEUTICAL FORM

Powder and solvent for suspension for injection.

White crystalline powder (hardly visible due to the small amount of powder in the vial). The solvent is a colourless solution without any visible particles.

4 CLINICAL PARTICULARS

4.1 Therapeutic Indications

Active immunization against tuberculosis.

4.2 Posology and method of administration

Posology:
Children at least 12 months of age and adults:
0.1ml of the reconstituted vaccine strictly by intradermal injection. National recommendations should be consulted regarding the need for tuberculin testing prior to administration of BCG vaccine SSI.

Infants under 12 months of age:
0.05ml of the reconstituted vaccine strictly by intradermal injection.

Method of Administration
When drawn up into the syringe the vaccine suspension should appear homogeneous, slightly opaque and colourless.

BCG Vaccine SSI should be administered with a syringe fitted with a short bevel needle (25 G/ 0.50 mm or 26G/0.45mm).
BCG Vaccine should be administered by personnel trained in the intradermal technique.

Jet injectors or multiple puncture devices should not be used to administer the vaccine.
The injection site should be clean and dry;
Antiseptics should not be used prior to administration.
If alcohol is used to swab the skin, it must be allowed to evaporate before the vaccine is injected.
The vaccine should be injected strictly intradermally in the arm, over the distal insertion of the deltoid muscle onto the humerus (approx. one third down upper 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 is best left uncovered to facilitate healing.

4.3 Contraindications

BCG vaccine SSI should not be administered to persons known to be hypersensitive to any component of the vaccine. Normally the vaccination should be postponed in persons with pyrexia or generalised infected skin conditions. Eczema is not a contraindication, but the vaccine site should be lesion free. BCG Vaccine SSI should not be given to persons receiving systemic corticosteroids or immunosuppressive treatment including radiotherapy, to those suffering from malignant conditions (e.g. lymphoma, leukaemia, Hodgkin's disease or other tumours of the reticuloendothelial system), those with primary or secondary immunodeficiencies, those with HIV infection, including infants born to HIV positive mothers. The effect of BCG vaccination may be exaggerated in these patients, and a generalised BCG infection is possible. In areas where the risk of contracting tuberculosis and HIV is high, it may be appropriate to vaccinate asymptomatic HIV-positives with BCG according to WHO recommendations.

BCG Vaccine SSI should not be given to patients who are receiving anti-tuberculous drugs.

4.4 Special warnings and precautions for use

Although anaphylaxis is very rare, facilities for its management should always be available during vaccination.

Tuberculin positive persons (consult national recommendations for the definition of a positive tuberculin reaction) do not require the vaccine. Administration of the vaccine to such persons may result in a severe accelerated local reaction.

Injections made too deeply increase the risk of lymphadenitis and abscess formation.

4.5 Interaction with other medicinal products and other forms of interaction

BCG may be given simultaneously, at a separate site, with all other vaccines and immunoglobulins.
Intradermal BCG vaccination may be given concurrently with inactivated killed or live vaccines, including combined the measles, -mumps and –rubella vaccines.
Other vaccines to be given at the same time as BCG Vaccine SSI should not be given into the same arm. If not given at the same time an interval of not less than four weeks should normally be allowed to lapse between the administration of any two live vaccines.
No further vaccination should be given for at least three months in the arm used for BCG vaccination, because of the risk of regional lymphadenitis

4.6 Fertility, pregnancy and lactation

Although no harmful effects to the foetus have been associated with BCG vaccine, vaccination is not recommended during pregnancy or lactation. However, in areas with high risk or tuberculosis infection, BCG may be give during pregnancy or lactation if the benefit of vaccination outweights the risk.

4.7 Effects on ability to drive and use machines

No effect on ability to drive and use machines has been observed.

4.8 Undesirable effects

The expected reaction to successful vaccination with BCG Vaccine SSI includes induration at the injection site followed by a local lesion that may ulcerate some weeks later and heal over some months leaving a small, flat scar. It also may include enlargement of a regional lymph node to < 1cm.

Undesirable effects of the vaccine include the following:

Uncommon	Systemic: Headache, fever. Local: Enlargement of regional lymph node > 1cm. Ulceration with a discharging ulcer at the site of injection.
Rare (<1/1000)	Systemic: Disseminated BCG complications such as osteitis or osteomyelitis. Allergic reactions, including Anaphylactic reactions. Local: Suppurative lymphadenitis, abscess formation.

During post-marketing safety surveillance syncope among patients receiving injections have been reported. Also seizures and convulsions have been reported infrequently.

An excessive response to the BCG Vaccine SSI may result in a discharging ulcer. This may be attributable to inadvertent subcutaneous injection or to excessive dosage. The ulcer should be encouraged to dry and abrasion (by tight clothes, for example) avoided.

BCG Danish strain 1331 is susceptible to most commonly used anti-tuberculous drugs. However the MIC of isoniazid for the BCG Danish strain 1331 is 0.4 mg/ml [Bactec 460]. There is no consensus as to whether M. bovis should be classified as susceptible, intermediately resistant or resistant to isoniazid when MIC is 0.4mg/ml. However, based on criteria set for Mycobacterium tuberculosis, the strain could be considered to be of intermediate susceptibility. Expert advice should be sought regarding the appropriate treatment regimes for systemic infections or persistent local infections following vaccination with BCG Vaccine SSI.

Though anaphylactoid reactions are extremely rare, facilities for their management should always be available.

4.9 Overdose

Overdosage increases the risk of supperative lymphadenitis and may lead to excessive scar formation.
Gross overdosage increases the risk of undesirable BCG complications.
For treatment of generalised infections with BCG, refer *to section 4.8*.

5 PHARMACOLOGICAL PROPERTIES

5.1 Pharmacodynamic properties

Pharmacotherapeutic group (ATC code): J 07 AN 01.

The vaccine contains Mycobacterium bovis BCG (Bacillus Calmette-Guerin) of the Danish strain 1331. BCG is an attenuated strain of Mycobacterium bovis. Vaccination with BCG Vaccine SSI elicits a cell-mediated immune response that confers a variable degree of protection to infection with M. tuberculosis.

Vaccinated persons normally become tuberculin positive after 6 weeks. A positive tuberculin skin test does indicate a response of the immune system to the BCG vaccination or to a mycobacterial infection, however the relationship between post vaccination tuberculin skin test reaction and the degree of protection afforded by BCG remains unclear.

The duration of immunity after BCG vaccination is not known, but there are some indications of a waning immunity after 10 years.

5.2 Pharmacokinetic properties

Not relevant for vaccines.

5.3 Preclinical safety data

Not available.

6 PHARMACEUTICAL PARTICULARS

6.1 List of excipients

BCG Vaccine SSI:
Sodium glutamate

Diluted Sauton SSI:
Magnesium sulphate heptahydrate
Dipotassium phosphate
Citric acid monohydrate
L-asparagine monohydrate
Ferric ammonium citrate
Glycerol 85%
Water for injections.

6.2 Incompatibilities

Only Diluted Sauton SSI may be used for reconstitution of BCG Vaccine SSI.
In the absence of compatibility studies BCG VACCINE SSI must not be mixed with other medicinal products.

6.3 Shelf life

12 months.

Use immediately after reconstitution.

6.4 Special precautions for storage

BCG Vaccine SSI:
Store in a refrigerator (2^0 C – 8^0 C).
Keep the vials in the outer carton in order to protect from light.

Diluted Sauton SSI:
Do not freeze.

6.5 Nature and contents of container

Nature and content:
BCG Vaccine SSI, amber Type I glass (Ph. Eur.).
Diluted Sauton SSI, colourless Type I glass (Ph. Eur.).

Presentations:
5 vials BCG Vaccine SSI (0.75 mg BCG) + 5 vials Diluted Sauton SSI (1 ml) packed in the same box.

One vial of reconstituted vaccine contains 1 ml, corresponding to 10 doses for adults and children aged 12 months and over (0.1 ml) or 20 doses for infants under 12 months of age.

6.6 Special precautions for disposal of a used medicinal product or waste materials derived from such medicinal product and other handling of the product

Reconstitution:
Only the solvent provided with the BCG VACCINE should be used for reconstitution.

The rubber stopper must not be wiped with any antiseptic or detergent. If alcohol is used to swab the rubber stopper of the vial, it must be allowed to evaporate before the stopper is penetrated with the syringe needle.

The vaccine should be visually inspected both before and after reconstitution for any foreign particulate matter prior to the administration.

Using a syringe fitted with a long needle, transfer to the vial the volume of solvent given on the label. Carefully invert the vial a few times to resuspend the lyophilised BCG completely. DO NOT SHAKE. Gently swirl the vial of resuspended vaccine before drawing up each subsequent dose. When drawn up into the syringe the vaccine suspension should appear homogeneous, slightly opaque and colourless.

The reconstituted vaccine should be used immediately.

Any unused vaccine or waste material should be disposed of safely in accordance with local requirements.

7 MARKETING AUTHORISATION HOLDER

Statens Serum Institut
5, Artillerivej
DK-2300 Copenhagen S
Denmark

8 MARKETING AUTHORISATION NUMBER

PA 0798/002/001

9 DATE OF FIRST AUTHORISATION/RENEWAL OF THE AUTHORISATION

Date of first authorisation: 14 December 2001

Date of last renewal: 14 December 2006

10 DATE OF REVISION OF THE TEXT

February 2007

Delayed BCG Study
Serious Adverse Event Reporting Form

Please give to Sarah Prentice or Steve Cose within 24 hours of notification of event

Patient Initials: ..... Patient Study No: ☐☐☐☐☐☐☐☐

Date of Birth: ☐☐ ☐☐ ☐☐☐☐ (d d m m m y y y y)

Treating Clinician: ..... Hospital: .....

Type of Report
☐
1=First
2=Interim
3=Final

Sex
☐
1= Male
2= Female

Height
☐☐☐ cm

Weight
☐☐☐.☐ kg

Did Unblinding Occur?
☐
0= No,
1=yes

Trial Arm if Unblinding Occured
☐
1= BCG at Birth
2= BCG at 6 weeks of age

Why was the event serious? *(choose most serious)*
☐
1= Resulted in death
2= Life-threatening
3= Required inpatient hospitalisation or prolongation of existing hospitalisation
4= Resulted in persistent or significant disability/incapacity
5= Resulted in congenital anomaly/birth defect
6= Other medically important event

Where did the SAE take place?
☐
1= Hospital
2= Out-patient clinic
3= Home
4= Nursing home
5= Hospice
6= Other, specify……..

Briefly describe SAE (*include relevant symptoms, body site, and relevant lab tests, treatments received)*
continue on a separate sheet if necessary

Details of SAE

Serious Adverse Event Name:	**Duration of SAE** (dd mmm yy)	**SAE Status** 1= Resolved 2= Resolved with sequelae 3= Persisting 4= Worsened 5= Fatal 6= Not assessable	**Expectedness** 1= Expected* 2= Unexpected
Name	Date of Onset ☐☐ ☐☐☐ ☐☐ Date Resolved ☐☐ ☐☐☐ ☐☐ or tick box if ongoing ☐	☐	☐

** Was the event one of the recognised undesirable effects of the trial medication? See BCG product insert*

Trial Treatment

Causal relationship to event 1=Definitely 2= Probably 3= Possibly 4= Unlikely 5= Not related 6=Not assessable	**Action Taken** 0=None 1=Dose reduction 2=Treatment delayed 3=Treatment delayed and reduced 4=Treatment permanently stopped
☐	☐

Appendix 6

DBS

Patient's Study Number ☐☐☐☐☐☐☐☐

Other treatments at time of event (include concomitant medication, radiotherapy, surgery, palliative care, continue on a separate sheet if necessary) Exclude any therapy given for management of SAE

Treatment Give generic name of drugs/treatment given in the last 30 days.	**Total Daily Dose**	**Route of Administration** 1=Oral 2=Intravenous 3=Subcutaneous 4=Other, specify	**Start Date** (dd mmm yy)	**Currently Ongoing?** 0= no 1=Yes	**End Date** (dd mmm yy)	**Action Taken** 0=None 1=Dose reduction 2=Treatment delayed 3=Treatment delayed and reduced 4=Treatment permanently stopped
			☐☐ ☐☐☐ ☐☐	☐	☐☐ ☐☐☐ ☐☐	☐
			☐☐ ☐☐☐ ☐☐	☐	☐☐ ☐☐☐ ☐☐	☐
			☐☐ ☐☐☐ ☐☐	☐	☐☐ ☐☐☐ ☐☐	☐
			☐☐ ☐☐☐ ☐☐	☐	☐☐ ☐☐☐ ☐☐	☐

Other relevant information to facilitate assessment
(Include medical history, drug or alcohol abuse, family history, findings from special investigations)

Was this event expected in view of the patient's clinical history?
☐ 0= No
1= Yes

Additional Information:

Signature
Authorised Health Professional .....

Print name.....

Date of report ☐☐ ☐☐☐ ☐☐
d d m m m y y

Contact telephone no.....

OFFICE USE ONLY

Was SAE drug related? Yes☐ No☐

Was event unexpected? Yes☐ No☐

Was the event a SUSAR? Yes☐ No☐

Event No ☐☐☐☐☐☐☐

Comments:

Date entered on database ☐☐ ☐☐☐ ☐☐
d d m m m y y

MEDRA code ☐☐☐☐☐☐☐☐

Form checked by staff (signature) .....

Date ☐☐ ☐☐☐ ☐☐
d d m m m y y

Checked by clinical reviewer (signature) .....

Date ☐☐ ☐☐☐ ☐☐
d d m m m y y

Final Version 1.0; 04/03/2014

Appendix 7a)

London School of Hygiene & Tropical Medicine
Keppel Street, London WC1E 7HT
United Kingdom
Switchboard: +44 (0)20 7636 8636

www.lshtm.ac.uk

Observational / Interventions Research Ethics Committee

Sarah Prentice
Research Fellow
CR / ITD
LSHTM

9 January 2014

Dear Dr. Prentice,

Study Title: **Does neonatal BCG vaccination provide protection against heterologous invasive infectious disease by enhancing the innate immune system?**
LSHTM ethics ref: **6545**

Thank you for your letter of 8 January 2014, responding to the Interventions Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Chair.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised, subject to the conditions specified below.

Conditions of the favourable opinion

Approval is dependent on local ethical approval having been received, where relevant.

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document	Version	Date
LSHTM ethics application	n/a	
Protocol	2.2	02/01/2014
Information Sheet	V2	02/01/2014
Consent form	V1	August 2013
Appendix 1a) Enrolment and Eligibility Form	V1	August 2013
Appendix 1 b) Maternal Demographics and Infant Birth Details Form		August 2013
Appendix 1 c) Personal Participant Study Plan (Example)		August 2013
Appendix 1 d) Routine Clinical Review Form		July 2013
Appendix 1 e) Illness event record		August 2013
BCG at birth - Advice Leaflet	V1	02/01/2014
BCG at 6weeks - Advice Leaflet	V1	02/01/2014

After ethical review

Any subsequent changes to the application must be submitted to the Committee via an Amendment form on the online application website. All studies are also required to notify the ethics committee of any serious adverse events which occur during the project via an Adverse Event form on the online application website. An annual report form is required on the anniversary of the approval of the study and should be submitted during the lifetime of the study on the online application website. At the end of the study, please notify the committee via an End of Study form on the online application website.

Professor John DH Porter
Chair
ethics@lshtm.ac.uk
http://www.lshtm.ac.uk/ethics/

Appendix 7b)

UNHRO
Uganda National Health Research Organisation

Uganda Virus Research Institute
Plot 51-59, Nakiwogo Road, Entebbe
P.O. Box 49, Entebbe-Uganda
Tel: +256 414 320 385 / 6
Fax: +256 414 320 483
Email: directoruvri@uvri.go.ug

REPUBLIC OF UGANDA

Our Ref: GC/127/13/11/432
Your Ref: ……

26th November 2013

Drs. Steven Cose, Sarah Prentice.

RE: UVRI SEC review of protocol titled **"Does Neonatal BCG Immunisation Provide Protection against Heterologous Invasive Infectious Disease by Stimulating the Innate System."**

Thank you for submitting your responses to the queries addressed to you by UVRI SEC during the SEC meeting of 14th November 2013.

This is to inform you that your responses dated 20th November 2013 were reviewed and met the requirements of the UVRI Science and Ethics Committee.

UVRI SEC annual approval has been given for you to conduct your research up to 26th November 2014. Annual progress report and request for extension should be submitted to UVRI SEC prior to the expiry date, to allow timely review.

The reviewed and approved documents included;

1. UVRI-SEC application form
2. Project Protocol Version 2.1 20/11/2013
3. Information sheet and consent forms.
4. Applicants' CVs

You can now continue with your study after registration with the Uganda National Council for Science and Technology (UNCST).

Note: UVRI SEC requires you to submit a copy of the UNCST approval letter for the above study before commencement.

Yours sincerely,

[redacted]

Mr. Tom Lutalo
Chair, UVRI SEC
C.C Secretary, UVRI SEC

Appendix 7c)

Uganda National Council for Science and Technology

(Established by Act of Parliament of the Republic of Uganda)

17/02/2014

Our Ref: HS 1524

Dr. Sarah Prentice
MRC/Uganda Virus Research Institute on AIDS
Uganda Virus Research Institute
Entebbe

Re: Research Approval: **Does Neonatal BCG Vaccination provide protection against heterologous invasive infectious disease by stimulating the innate immune system?**

I am pleased to inform you that **05/12/2013**, the Uganda National Council for Science and Technology (UNCST) approved the above referenced research project. The Approval of the research project is for the period of **05/12/2013** to **05/06/2016**.

Your research registration number with the UNCST is **HS 1524**. Please, cite this number in all your future correspondences with UNCST in respect of the above research project.

As Principal Investigator of the research project, you are responsible for fulfilling the following requirements of approval:

1. All co-investigators must be kept informed of the status of the research.
2. Changes, amendments, and addenda to the research protocol or the consent form (where applicable) must be submitted to the designated local Institutional Review Committee (IRC) or Lead Agency for re-review and approval **prior** to the activation of the changes. UNCST must be notified of the approved changes within five working days.
3. For clinical trials, all serious adverse events must be reported promptly to the designated local IRC for review with copies to the National Drug Authority.
4. Unanticipated problems involving risks to research subjects/participants or other must be reported promptly to the UNCST. New information that becomes available which could change the risk/benefit ratio must be submitted promptly for UNCST review.
5. Only approved study procedures are to be implemented. The UNCST may conduct impromptu audits of all study records.
6. A progress report must be submitted electronically to UNCST within four weeks after every 12 months. Failure to do so may result in termination of the research project.

Below is a list of documents approved with this application:

	Document Title	Language	Version	Version Date
1	Research proposal	English	2.1	20 Nov 2013
2	Information sheet	English, Luganda	1.0	05 Aug 2013
3	Consent form	English, Luganda	1.0	05 Aug 2013

Yours sincerely,

[redacted]

Leah Nawegulo Omongo
for: Executive Secretary
UGANDA NATIONAL COUNCIL FOR SCIENCE AND TECHNOLOGY

cc Chair, Uganda Virus Research Institute SEC, Entebbe

LOCATION/CORRESPONDENCE

Plot 6 Kimera Road, Ntinda
P. O. Box 6884
KAMPALA, UGANDA

COMMUNICATION

TEL: (256) 414 705500
FAX: (256) 414-234579
EMAIL: info@uncst.go.ug
WEBSITE: http://www.uncst.go.ug

Appendix 7d)

DRUG NATIONAL AUTHORITY

Safe Drugs Save Lives

23/10/2014

382/ESR/NDA/DID-10/2014

Principal Investigator
MRC/UVRI Uganda Research Unit on AIDS
Plot 51 – 59 Nakiwogo Road
PO Box 49, Entebbe, Uganda
Tel. 0792925686

Dear Dr. Stephen Cose,

RE: NO OBJECTION LETTER

Reference is made to your application dated the 17th September 2013 seeking approval of the study entitled *"A randomized controlled trial of BCG vaccination at birth compared to at 6 weeks of age to investigate whether BCG provides protection against heterologous invasive infectious disease by stimulating the innate immune system"*.

Following a favorable hearing of your appeal in meeting held on the 17th September 2014 between Medical Research Council (MRC), National Drug Authority (NDA) Experts, the National Drug Authority here by issues a "No Objection Letter" (NOL) to you in accordance with the National Drug Policy and Authority (Conduct of Clinical Trials) Regulations, 2014.

This approval however is subject to the following provisions:

(a) NDA shall be informed immediately of any toxic effects or death, which may occur during the Clinical Trial and of any data received which, might cast doubt on the validity of the continuation of the Clinical Trial.

(b) NDA shall be notified of any decision to discontinue the Clinical Trial. The reason for such cancellation shall be stated.

(c) The Clinical Trial shall be conducted in accordance with the NDA approved protocol. Any amendment(s) to the protocol shall first be submitted to the NDA for approval. All clinical trials shall be conducted in accordance with International Conference on Harmonization (ICH) and Good Clinical Practice (GCP) Guidelines.

(d) The medicine shall be administered by or under the direction of the authorized Principal Investigator. In the case where the Principal Investigator permits another medical practitioner to administer a medicine, which is exempted from the registration for the purpose of the Trial, the Principal Investigator shall remain responsible for [redacted] arising from such usage.

HEAD OFFICE
Plot 46-48 Lumumba Avenue
P.O.Box 23096, Kampala, Uganda
Tel: (+256) 414 255665/347391/347392
Fax: +256 414 255758
Hotline: (+256) 414 344052, 776 110 008, 712 001 199
Website: www.nda.or.ug, Email: ndaug@nda.or.ug
Facebook: Uganda National Drug Authority
Twitter: @UNDAuthority

OUR MISSION
To ensure access to quality, safe and efficacious human and veterinary medicines and other healthcare products through the regulation and control of their production, importation, distribution and use.

REGIONAL OFFICES
Central Region, Nakawa -Tel: +256 312 261 548,
Western Nile Region, Arua -Tel: +256 372 260 085/7,
South Western Region, Mbarara -Tel: +256 485 421 088,
South Eastern Region, Jinja -Tel/Fax +256 434 122 176,
Eastern Region, Tororo -Tel: +256 454 445 195,
Western Region, Hoima - Tel:/Fax +256 465 440 688,
Northern Region, Lira -Tel/Fax +256 473 420 652,

Appendix 7d)

NUATION SHEET

(e) In the event that the authorized Principal Investigator ceases to participate in the clinical trial, NDA shall be informed and the reason for such cessation shall be given.

(f) Procedures for importation of the study product as stipulated by NDA shall be followed and the consignment verified accordingly.

This approval is valid up to 23/09/2015. If you have to continue with the study, a request for continuation should be made in writing to the Executive Secretary, National Drug Authority.

[redacted] 23/10/14

Gordon K. Sematiko
EXECUTIVE SECRETARY/REGISTRAR

Copy to: Head Inspectorate Services
Drug Information Department

HEAD OFFICE
Plot 46-48 Lumumba Avenue
P.O.Box 23096, Kampala, Uganda
Tel: (+256) 414 255665/347391/347392
Fax: +256 414 255758
Hotline: (+256) 414 344052, 776 110 008, 712 001 199
Website: www.nda.or.ug, Email: ndaug@nda.or.ug
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OUR MISSION
To ensure access to quality, safe and efficacious human and veterinary medicines and other healthcare products through the regulation and control of their production, importation, distribution and use.

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Eastern Region,Tororo -Tel: +256 454 445 195,
Western Region, Hoima - Tel:/Fax +256 465 440 688,
Northern Region, Lira -Tel/Fax +256 473 420 652,

Reagent	Supplier	Catalogue number
16% methanol free formaldehyde	Perbio Science	28908
Buffer PB	Qiagen	19066
ELISA set reagents (B)	BD Bioscience	550534
Ficoll-paque	Thermo FIsher Scientific	10379484
Glucose	Thermo FIsher Scientific	15023021
Glutamine	Thermo FIsher Scientific	10214683
Glycerol	Thermo FIsher Scientific	17904
Glycerol pure	SLS	CHE2066
Glycine	Invitrogen	15527013
H3K4me3 antibody	Diagenode	Pab-003-050
Hepcidin-25 ELISA	Bachem	H-5926
HEPES	Thermo FIsher Scientific	10041703
Histopaque	Sigma	H8889
IFNγ Human ELISA set	BD Bioscience	555142
IL-10 Human ELISA set	BD Bioscience	555157
IL-1b Human ELISA set	BD Bioscience	557953
IL-6 Human ELISA set	BD Bioscience	555220
H3K9me3 antibody	Diagenode	C15410193 (pAb-193-050)
MinElute PCR Purification Kit	Qiagen	28006
Phosphate Buffered Saline	Sigma	P4417-100TAB
Penicillin streptomycin	Thermo FIsher Scientific	10101043
Polyinosinic acid sodium salt	Sigma	P1530-25mg
Protein A/G beads	Santa Cruz	Sc-2003
Protease inhibitor complex	Roche	11836153001
Proteinase K	Qiagen	19131
RNA later	Thermo FIsher Scientific	AM7021
RPMI 1640 w/o phenol red	Thermo FIsher Scientific	32404-014

Sodium dodecyl sulfate	Thermo FIsher Scientific	28364
Sterile water	Scientific Laboratory Supplies	dd69801
TNFα Human ELISA set	BD Bioscience	555212
Tryptone Soy Broth	Scientific Laboratory Supplies	CM129B
Tryptophan blue	Sigma-Aldrich	T8154-20ML

Appendix 9. Results Tables

1. Epigenetic sub-study

1.1 Study numbers

Total participants: 31.

BCG vaccinated: 16. 6 male, 10 female. 11 high BCG responders (scar≥5mm), 5 low BCG responders(scar≤4mm)

BCG unvaccinated: 15. 7 male, 8 female. 8 high BCG responders (scar≥4mm), 6 low BCG responders (scar≤3mm).

1.2 Cross-sectional comparisons of median epigenetic modification by BCG status

Table 1.2.1

	H3K4me3						H3K9me3					
	Cord			Six weeks			Cord			Six weeks		
	BCG+ve	**BCG-ve**	p-value	**BCG+ve**	**BCG-ve**	p-value	**BCG+ve**	**BCG-ve**	p-value	**BCG+ve**	**BCG-ve**	p-value
IL-6	0.05	0.02	0.06	0.14	0.17	0.61	0.05	0.04	0.84	0.08	0.21	0.41
TNFα	0.31	0.13	**0.04**	1.29	1.73	0.58	0.01	0.005	0.35	0.05	0.09	**0.03**
IL-1β	0.06	0.02	0.15	0.14	0.12	0.74	0.10	0.02	**0.03**	0.13	0.22	0.60
Combined	0.42	0.19	**0.04**	1.57	2.08	0.58	0.22	0.09	0.15	0.25	0.48	0.38

1.3 Median percentage recovery of epigenetically modified chromatin from the promoter region of pro-inflammatory cytokines.

Table 1.3.1 All Infants

	H3K4me3			H3K9me3		
	Cord	**6 weeks**	p-value	**Cord**	**6 weeks**	p-value
IL-6	0.02	0.14	**0.0001**	0.006	0.13	**0.004**
TNFα	0.19	1.51	**<0.0001**	0.012	0.05	**0.01**
IL-1β	0.04	0.12	**0.0006**	0.04	0.14	0.13
Combined	0.31	1.71	**<0.0001**	0.10	0.39	**0.05**

Table 1.3.2 By BCG status

	H3K4me3						H3K9me3					
	BCG +ve			**BCG -ve**			**BCG +ve**			**BCG -ve**		
	Cord	**6 weeks**	p-value	Cord	6 weeks	p-value	**Cord**	**6 weeks**	p-value	**Cord**	**6 weeks**	p-value
IL-6	0.05	0.14	**0.03**	0.02	0.17	**0.0007**	0.05	0.08	0.36	0.04	0.21	**0.002**
TNFα	0.31	1.29	**0.003**	0.13	1.7	**0.0007**	0.01	0.05	0.86	0.005	0.09	**0.001**
IL-1β	0.06	0.14	**0.08**	0.02	0.12	**0.002**	0.10	0.13	0.86	0.02	0.22	**0.03**
Combined	0.42	1.57	**0.007**	0.19	2.08	**0.0007**	0.22	0.25	0.86	0.09	0.48	**0.008**

1.4 Median within-infant changes overtime in epigenetic modification at the promoter region of pro-inflammatory cytokines, between birth and 6 weeks of age

Table 1.4.1 By BCG status

	H3K4me3			H3K9me3		
	BCG +ve	**BCG-ve**	p-value	**BCG +ve**	**BCG-ve**	p-value
IL-6	0.07	0.16	0.32	0.03	0.11	0.15
TNFα	0.80	1.69	0.27	0.005	0.09	**0.007**
IL-1β	0.05	0.09	0.33	0.02	0.12	0.21
Combined	1.05	1.89	0.27	0.04	0.31	0.12

1.5 Effects of sex on within-infant changes to epigenetic modification at pro-inflammatory promoters between birth and 6 weeks of age

Table 1.5.1 All infants

	H3K4me3			H3K9me3		
	Male	**Female**	p-value	**Male**	**Female**	p-value
IL-6	0.04	0.13	0.19	0.08	0.04	0.64
TNFα	0.59	1.44	0.30	0.04	0.02	0.80
IL-1β	0.09	0.05	0.78	0.07	0.02	0.67
Combined	0.71	1.60	0.30	0.2	0.07	0.87

Table 1.5.2 By BCG

	H3K4me3						H3K9me3					
	Male			**Female**			**Male**			**Female**		
	BCG +ve	**BCG -ve**	p-value	**BCG +ve**	**BCG -ve**	p-value	**BCG +ve**	**BCG -ve**	p-value	**BCG +ve**	**BCG -ve**	p-value
IL-6	0.01	0.16	0.15	0.13	0.15	0.93	0.08	0.07	0.68	0.02	0.14	**0.03**
TNFα	0.30	1.69	0.12	1.15	1.55	0.86	0.04	0.06	0.46	0.002	0.11	**0.006**
IL-1β	0.04	0.11	0.09	0.05	0.07	0.96	0.13	0.01	0.81	0.02	0.20	0.13
Combined	0.36	2.01	0.15	1.42	1.72	0.86	0.42	0.09	0.94	0.04	0.46	0.08

	H3K4me3						H3K9me3					
	BCG +ve			BCG –ve			BCG +ve			BCG -ve		
	Male	**Female**	p-value	**Male**	**Female**	p-value	**Male**	**Female**	p-value	**Male**	**Female**	p-value
IL-6	0.01	0.13	0.07	0.16	0.15	0.64	0.08	0.02	0.09	0.07	0.14	0.16
TNFα	0.30	1.15	0.10	1.69	1.55	1.0	0.04	0.002	0.36	0.06	0.11	0.07
IL-1β	0.04	0.05	0.28	0.11	0.07	0.56	0.13	0.02	0.71	0.01	0.20	0.30
Combined	0.36	1.42	0.08	2.01	1.72	1.0	0.42	0.04	0.46	0.09	0.46	0.35

1.6 Effects of BCG response, as measured by scar size at 10 weeks, on within-infant changes to epigenetic modification at pro-inflammatory promoters between birth and 6 weeks of age

Table 1.6.1 All infants

	H3K4me3			H3K9me3		
	High responders	**Low responders**	p-value	**High responders**	**Low responders**	p-value
IL-6	0.13	0.10	0.61	0.04	0.09	0.65
TNFα	1.5	0.60	0.56	0.02	0.06	0.24
IL-1β	0.06	0.10	0.65	0.02	0.16	0.38
Combined	1.64	1.02	0.58	0.09	0.27	0.71

Table 1.6.2 By response status (10 weeks)

	H3K4me3						H3K9me3					
	High responders			**Low responders**			**High responders**			**Low responders**		
	BCG +ve	**BCG -ve**	p-value	**BCG +ve**	**BCG -ve**	p-value	**BCG +ve**	**BCG -ve**	p-value	**BCG +ve**	**BCG -ve**	p-value
IL-6	0.13	0.14	0.84	0.05	0.17	**0.04**	0.03	0.09	0.41	0.03	0.11	0.09
TNFα	1.22	1.83	0.62	0.51	1.55	**0.03**	0.005	0.07	0.07	0.003	0.10	**0.03**
IL-1β	0.06	0.06	0.74	0.03	0.13	0.27	0.02	0.09	0.19	0.11	0.17	0.52
Combined	1.35	2.07	0.62	0.71	1.78	0.07	0.03	0.23	0.16	0.14	0.39	0.29

1.7 Effects of concomitant infectious illnesses on within-infant changes to epigenetic modification at pro-inflammatory promoters between birth and 6 weeks of age

Table 1.7.1 All infants

	H3K4me3			H3K9me3		
	Infection	**No infection**	p-value (rho)	**Infection**	**No infection**	p-value (rho)
IL-6	0.16	0.07	0.09 (0.31)	0.07	0.04	0.84 (-0.04)
TNFα	1.69	0.69	0.10 (0.30)	0.04	0.03	0.80 (0.05)
IL-1β	0.1	0.03	**0.04** (0.37)	0.01	0.03	0.62 (-0.09)
Combined	1.89	0.96	0.09 (0.31)	0.09	0.1	0.80 (-0.05)

Table 1.7.2 Effects of BCG vaccination and interim infections on epigenetic modification.

	H3K4me3						H3K9me3					
	BCG+ve			BCG-ve			BCG+ve			BCG-ve		
	Infection	**No infection**	p-value	**Infection**	**No infection**	p-value	**Infection**	**No infection**	p-value	**Infection**	**No infection**	p-value
IL-6	0.32	0.05	**0.05**	0.11	0.16	0.81	0.06	0.03	0.51	0.07	0.13	0.22
TNFα	2.11	0.64	0.18	1.69	1.15	0.46	0.02	-0.001	0.60	0.08	0.09	0.90
IL-1β	0.16	0.02	0.08	0.09	0.07	0.39	0.17	0.017	0.24	0	0.17	0.06
Combined	2.59	0.86	0.22	1.89	1.51	0.46	0.25	0.03	0.51	0.05	0.42	0.18

2. Cytokine sub-study

2.1 Study numbers, per protocol analysis (numbers of female infants in brackets)

2.1.1 Cross-sectional analysis

BCG at birth: n=112	Cord					S1 5 days of age					S2 6 weeks of age (pre-EPI1)					S3 6 weeks +5 days (post-EPI1 and BCG in delayed group)					S4 10 weeks of age (pre-EPI2)				
	TNFα	IL6	IL1β	IL10	IFNγ	TNFα	IL6	IL1β	IL10	IFNγ	TNFα	IL6	IL1β	IL10	IFNγ	TNFα	IL6	IL1β	IL10	IFNγ	TNFα	IL6	IL1β	IL10	IFNγ
Medium	112 (55)	102 (51)	112 (55)	112 (55)	110 (54)	51 (26)	46 (24)	51 (26)	51 (26)	51 (26)	43 (18)	41 (18)	43 (18)	43 (18)	42 (18)	40 (18)	36 (17)	40 (18)	40 (18)	40 (18)	42 (21)	35 (19)	42 (21)	42 (21)	41 (20)
PPD	112 (55)	109 (54)	112 (55)	112 (55)	110 (54)	51 (26)	49 (26)	51 (26)	51 (26)	50 (25)	43 (18)	43 (18)	43 (18)	43 (18)	42 (18)	40 (18)	39 (18)	40 (18)	40 (18)	40 (18)	42 (21)	41 (21)	42 (21)	42 (21)	41 (20)
Poly I:C	112 (55)	109 (54)	111 (55)	112 (55)	110 (54)	51 (26)	49 (26)	51 (26)	51 (26)	51 (26)	43 (18)	43 (18)	43 (18)	43 (18)	42 (18)	40 (18)	39 (18)	40 (18)	40 (18)	40 (18)	42 (21)	41 (21)	42 (21)	42 (21)	41 (20)
S.pneumoniae	112 (55)	109 (54)	112 (55)	112 (55)	110 (54)	51 (26)	49 (26)	51 (26)	51 (26)	51 (26)	43 (18)	43 (18)	43 (18)	43 (18)	42 (18)	40 (18)	39 (18)	40 (18)	40 (18)	40 (18)	42 (21)	41 (21)	42 (21)	42 (21)	41 (20)
S.aureus	112 (55)	109 (54)	112 (55)	112 (55)	110 (54)	51 (26)	47 (24)	51 (26)	49 (24)	51 (26)	43 (18)	43 (18)	43 (18)	43 (18)	42 (18)	40 (18)	39 (18)	40 (18)	40 (18)	40 (18)	42 (21)	41 (19)	42 (21)	41 (20)	41 (20)
E.coli	112 (55)	109 (54)	112 (55)	112 (55)	110 (54)	51 (26)	49 (26)	51 (26)	51 (26)	51 (26)	43 (18)	43 (18)	43 (18)	43 (18)	42 (18)	40 (18)	39 (18)	40 (18)	40 (18)	40 (18)	42 (21)	41 (21)	42 (21)	42 (21)	41 (20)
C.albicans	110 (54)	109 (54)	112 (55)	110 (54)	110 (54)	51 (26)	49 (26)	51 (26)	51 (26)	51 (26)	43 (18)	43 (18)	43 (18)	43 (18)	42 (18)	40 (18)	39 (18)	40 (18)	40 (18)	40 (18)	42 (21)	41 (21)	42 (21)	42 (21)	41 (20)

BCG at 6 weeks: n=112	Cord					S1 5 days of age					S2 6 weeks of age (pre-EPI1)					S3 6 weeks +5 days (post-EPI1 and BCG in delayed group)					S4 10 weeks of age (pre-EPI2)				
	TNFα	IL6	IL1β	IL10	IFNγ	TNFα	IL6	IL1β	IL10	IFNγ	TNFα	IL6	IL1β	IL10	IFNγ	TNFα	IL6	IL1β	IL10	IFNγ	TNFα	IL6	IL1β	IL10	IFNγ
Medium	111 (57)	103 (52)	112 (57)	112 (57)	110 (55)	53 (25)	52 (24)	52 (25)	53 (25)	53 (25)	46 (24)	44 (23)	46 (24)	46 (24)	44 (23)	50 (27)	49 (26)	50 (27)	50 (27)	49 (26)	50 (23)	46 (23)	50 (23)	50 (23)	49 (22)
PPD	112 (57)	109 (55)	111 (57)	112 (57)	110 (55)	53 (25)	53 (26)	52 (25)	53 (25)	52 (25)	46 (24)	46 (18)	46 (24)	46 (24)	44 (23)	50 (27)	49 (18)	50 (27)	50 (27)	49 (26)	50 (23)	50 (21)	50 (23)	50 (23)	49 (22)
Poly I:C	112 (57)	109 (55)	112 (57)	112 (57)	110 (55)	53 (25)	53 (26)	52 (25)	53 (25)	53 (25)	46 (24)	46 (18)	46 (24)	46 (24)	44 (23)	50 (27)	49 (18)	50 (27)	50 (27)	49 (26)	50 (23)	50 (21)	50 (23)	50 (23)	49 (22)
S.pneumoniae	112 (57)	109 (55)	112 (57)	112 (57)	110 (55)	53 (25)	53 (26)	52 (25)	53 (25)	53 (25)	46 (24)	46 (18)	46 (24)	46 (24)	44 (23)	50 (27)	49 (18)	50 (27)	50 (27)	49 (26)	50 (23)	50 (21)	50 (23)	50 (23)	49 (22)
S.aureus	112 (57)	109 (55)	112 (57)	112 (57)	110 (55)	53 (25)	53 (26)	52 (25)	50 (24)	53 (25)	46 (24)	45 (18)	45 (24)	45 (24)	44 (23)	50 (27)	49 (18)	50 (27)	50 (27)	49 (26)	50 (23)	47 (21)	50 (23)	47 (23)	49 (22)
E.coli	112 (57)	109 (55)	112 (57)	112 (57)	110 (55)	53 (25)	53 (26)	52 (25)	53 (25)	53 (25)	46 (24)	46 (18)	46 (24)	46 (24)	44 (23)	50 (27)	49 (18)	50 (27)	50 (27)	49 (26)	50 (23)	50 (21)	50 (23)	50 (23)	49 (22)
C.albicans	111 (57)	108 (55)	111 (57)	112 (57)	110 (55)	53 (25)	53 (26)	52 (25)	53 (25)	53 (25)	46 (24)	45 (18)	46 (24)	46 (24)	44 (23)	50 (27)	49 (18)	50 (27)	50 (27)	49 (26)	50 (23)	50 (21)	50 (23)	50 (23)	49 (22)

2.1.2 Within-Infant changes over time

BCG at birth	S1					S2					S3					S4				
	TNFα	IL6	IL1β	IL10	IFNγ	TNFα	IL6	IL1β	IL10	IFNγ	TNFα	IL6	IL1β	IL10	IFNγ	TNFα	IL6	IL1β	IL10	IFNγ
Cord	51 (26)	44 (24)	51 (26)	51 (26)	51 (26)	43 (18)	37 (17)	43 (18)	43 (18)	42 (18)	40 (18)	30 (13)	40 (18)	40 (18)	39 (17)	42 (21)	31 (17)	42 (21)	42 (21)	40 (19)
S1						18 (8)	15 (7)	18 (8)	18 (8)	17 (8)	16 (6)	13 (5)	16 (6)	16 (6)	16 (6)	12 (8)	11 (7)	12 (8)	12 (8)	12 (8)
S2											10 (4)	9 (4)	10 (4)	10 (4)	10 (4)	14 (5)	9 (5)	14 (5)	14 (5)	14 (5)
S3																12 (6)	7 (4)	12 (6)	12 (6)	11 (5)

BCG at 6 weeks	S1					S2					S3					S4				
	TNFα	IL6	IL1β	IL10	IFNγ	TNFα	IL6	IL1β	IL10	IFNγ	TNFα	IL6	IL1β	IL10	IFNγ	TNFα	IL6	IL1β	IL10	IFNγ
Cord	53 (25)	46 (21)	52 (25)	53 (25)	52 (24)	46 (24)	40 (20)	46 (24)	46 (24)	43 (22)	49 (27)	47 (25)	50 (27)	50 (27)	48 (25)	49 (23)	43 (22)	50 (23)	50 (23)	48 (21)
S1						16 (7)	15 (7)	15 (7)	16 (7)	15 (7)	17 (10)	17 (10)	17 (10)	17 (10)	16 (9)	17 (7)	13 (6)	17 (7)	17 (7)	17 (7)
S2											14 (8)	13 (7)	14 (8)	14 (8)	14 (8)	14 (7)	13 (7)	14 (7)	14 (7)	12 (5)
S3																19 (9)	18 (8)	19 (9)	19 (9)	19 (9)

2.2 Cross-sectional comparison of *in vitro* stimulated cytokine production

2.2.1 Geometric mean cytokine levels

		Cord Blood			5 days of age			6 weeks of age (pre-EPI1)			6 weeks of age (5d post-EPI1 +/-BCG)			10 weeks of age (pre-EPI2)		
		BCG at birth	**BCG at 6wks**	**p-value**	**BCG at birth**	**BCG at 6wks**	**p-value**	**BCG at birth**	**BCG at 6wks**	**p-value**	**BCG at birth**	**BCG at 6wks**	**p-value**	**BCG at birth**	**BCG at 6wks**	**p-value**
TNFα	**Medium**	4.49	4.49	0.54	4.49	4.49	0.89	4.49	4.49	0.32	4.49	4.49	0.52	7.16	6.03	0.92
	PPD	21.49	20.69	0.85	74.44	76.65	0.93	177.03	35.12	**<0.0001**	179.60	31.88	**<0.0001**	145.47	64.27	**0.005**
	Poly I:C	229.66	215.16	0.69	1279.91	1274.68	0.99	1060.56	786.00	0.18	999.86	924.08	0.60	1105.17	1067.40	0.86
	S.pneumoniae	687.98	655.19	0.78	1772.98	1860.62	0.73	1638.00	1654.52	0.96	1864.19	1403.68	**0.05**	1493.14	1753.77	0.31
	S.aureus	74.26	67.82	0.64	246.75	254.90	0.85	345.10	285.97	0.40	264.18	168.15	**0.05**	226.20	299.98	0.22
	E.coli	836.75	686.08	0.08	3069.85	2808.02	0.64	2172.65	2633.57	0.32	2806.61	2360.8	0.22	2112.47	2199.06	0.80
	C.albicans	77.87	98.13	0.40	87.30	125.67	0.39	131.37	98.44	0.42	109.09	113.04	0.99	138.77	148.38	0.85
IL-6	**Medium**	2.69	2.69	0.90	139.96	48.75	0.94	109.35	48.75	0.48	72.81	48.75	0.62	249.33	105.11	0.17
	PPD	4723.70	4363.90	0.62	8732.23	8938.70	0.91	9555.42	3255.28	**<0.0001**	10980.09	3609.23	**<0.0001**	8902.38	6376.13	0.07
	Poly I:C	15784.04	15406.88	0.81	39730.22	42387.86	0.65	27316.4	22898.76	0.21	31057.62	28146.06	0.47	31570.28	29763.1	0.65
	S.pneumoniae	67806.09	69227.55	0.82	83429.82	88370.33	0.58	54526.57	49612.31	0.43	65826.3	60303.27	0.35	51868.66	54826.57	0.63
	S.aureus	1773.32	1764.66	0.98	4588.19	5112.36	0.65	4258.28	3615.19	0.42	5088.71	3685.56	0.07	4168.86	4521.16	0.68
	E.coli	54140.54	51093.95	0.49	89928.44	88585.32	0.88	58856.48	54141.71	0.51	79023.18	72641.21	0.35	61301.12	66791.08	0.38

	C.albicans	22711.01	23376.33	0.89	9870.85	13352.39	0.45	9420.65	7242.43	0.49	9204.97	7946.49	0.71	15165.15	13144.9	0.67
IL-1β	**Medium**	2.24	2.24	0.28	30.58	11.91	0.34	19.13	2.24	**0.04**	2.24	4.63	0.36	32.90	13.92	0.11
	PPD	47.26	48.02	0.94	103.27	100.00	0.94	84.71	66.70	0.46	94.61	51.41	0.09	109.91	90.49	0.51
	Poly I:C	208.59	221.59	0.72	1733.38	1609.24	0.68	872.12	776.05	0.62	958.78	955.17	0.99	1597.64	1348.04	0.31
	S.pneumoniae	1409.41	1532.80	0.60	1576.76	1383.47	0.44	1557.48	1495.93	0.81	1479.20	1495.92	0.99	1619.04	1534.60	0.70
	S.aureus	350.60	305.04	0.35	718.49	690.78	0.80	637.68	710.40	0.50	647.64	601.53	0.61	765.99	690.13	0.40
	E.coli	1758.45	1623.08 (	0.54	4236.65	4016.13	0.75	3413.48	3943.37	0.38	5717.79	4534.11	0.06	4939.50	4475.66	0.48
	C.albicans	145.45	188.48	0.38	153.08	151.26	0.95	139.80	125.46	0.74	71.19	90.05	0.56	188.55	231.06	0.60
IL-10	**Medium**	4.49	4.49	0.71	4.49	6.58	0.59	6.79	4.49	0.97	7.56	4.49	0.13	7.95	7.15	0.47
	PPD	86.05	88.11	0.86	171.17	186.41	0.67	94.03	81.65	0.56	66.54	89.25	0.14	106.31	92.85	0.48
	Poly I:C	276.24	267.67	0.82	517.29	580.19	0.61	287.09	256.89	0.65	275.81	343.36	0.24	418.35	344.85	0.29
	S.pneumoniae	597.12	639.02	0.55	377.79	431.35	0.52	276.51	270.44	0.92	368.64	329.68	0.41	362.58	359.07	0.93
	S.aureus	39.20	36.85	0.68	68.62	67.62	0.97	51.95	60.24	0.52	55.67	53.38	0.85	68.75	56.89	0.37
	E.coli	1066.72	1044.96	0.78	1458.24	1470.81	0.95	863.65	986.56	0.48	1249.94	1226.81	0.85	1096.58	1127.00	0.87
	C.albicans	122.10	151.54	0.36	42.53	45.90	0.73	44.55	32.72	0.32	38.71	49.37	0.42	93.38	67.49	0.32
IFNγ	**Medium**	8.45	9.10	0.98	7.72	9.22	0.73	12.20	2.69	0.48	11.47	9.26	0.40	9.06	7.43	0.40
	PPD	13.83	13.13	0.82	24.25	20.88	0.63	1253.82	17.39	**<0.0001**	1138.62	23.91	**<0.0001**	922.86	487.73	**0.02**
	Poly I:C	20.51	17.39	0.53	100.00	115.43	0.70	172.96	168.91)	0.93	107.01	100.61	0.84	333.15	319.95	0.89
	S.pneumoniae	11.78	13.31	0.54	169.23	141.91	0.55	130.48	104.41	0.55	135.08	118.44	0.71	160.91	149.75	0.81

	S.aureus	9.43	12.73	0.17	89.26	67.93	0.41	159.42	98.85	0.21	85.77	90.02	0.91	175.02	154.44	0.74
	E.coli	22.74	26.47	0.49	225.08	132.48	0.17	233.24	226.93	0.93	366.82	245.96	0.32	345.23	274.82	0.51
	C.albicans	9.02	9.83	0.68	13.82	15.85	0.67	38.71	42.78	0.81	66.89	45.90	0.34	50.71	77.04	0.26

2.2.2 Medium subtracted geometric mean cytokine levels

		Cord Blood			5 days of age			6 weeks of age (pre-EPI1)			6 weeks of age (5d post-EPI1 +/-BCG)			10 weeks of age (pre-EPI2)		
		BCG at birth	**BCG at 6wks**	**p-value**	**BCG at birth**	**BCG at 6wks**	**p-value**	**BCG at birth**	**BCG at 6wks**	**p-value**	**BCG at birth**	**BCG at 6wks**	**p-value**	**BCG at birth**	**BCG at 6wks**	**p-value**
TNFα	**PPD**	9.70	8.98	0.78	45.00	39.13	0.70	136.07	16.99	**<0.0001**	142.03	15.79	**<0.0001**	112.88	38.84	0.004
	Poly I:C	207.61	179.70	0.48	1046.60	1040.81	0.99	892.09	731.82	0.50	980.64	907.30	0.61	1032.62	952.13	0.76
	S.pneumoniae	649.34	586.72	0.62	1725.58	1575.56	0.66	1570.59	1626.95	0.84	1844.00	1383.16	**0.05**	1450.49	1509.16	0.86
	S.aureus	51.82	47.33	0.72	197.76	152.72	0.43	261.31	227.81	0.68	244.67	132.24	**0.03**	168.22	229.61	0.34
	E.coli	823.61	641.91	0.06	3001.06	2671.38	0.55	2130.40	2587.06	0.33	2786.14	2343.41	0.22	2046.92	1942.27	0.82
	C.albicans	46.44	69.66	0.23	49.75	89.78	0.21	82.48	62.19	0.56	79.57	76.28	0.93	95.33	93.74	0.97
IL-6	**PPD**	4674.57	4176.08	0.50	7891.57	7372.40	0.81	7646.35	2658.00	**0.003**	11584.14	2872.35	**<0.0001**	6955.21	4232.48	0.18
	Poly I:C	15432.30	15051.24	0.82	38905.61	40141.73	0.83	26824.52	22965.36	0.28	30437.63	27796.45	0.52	29550.65	28549.15	0.81
	S.pneumoniae	68688.53	68817.06	0.98	83520.8	86346.68	0.74	53732.79	50254.59	0.59	66167.57	59811.2	0.30	51472.37	55113.5	0.59
	S.aureus	1564.38	1435.36	0.69	3928.24	4536.65	0.54	3351.02	2923.85	0.69	4989.96	3184.68	**0.03**	2524.86	3213.75	0.53
	E.coli	53780.34	51146.71	0.58	89179.45	85260.42	0.69	57863.96	55067.78	0.71	80592.52	72233.6	0.24	61216.92	68182.26	0.31
	C.albicans	19134.3	22436.07	0.56	8262.18	11493.8	0.48	8488.94	7107.81	0.66	8034.18	5900.32	0.53	12711.88	10394.01	0.65
IL-1β	**PPD**	29.77	28.17	0.84	56.00	65.53	0.71	38.02	43.64	0.76	56.02	26.60	0.09	49.37	48.99	0.99
	Poly I:C	173.11	168.56	0.91	1643.27	1523.31	0.68	758.60	746.78	0.95	827.52	922.43	0.66	1448.27	1303.86	0.56

	S.pneumoniae	1382.12	1468.30	0.69	1494.86	1317.67	0.45	1509.56	1464.40	0.86	1398.04	1452.80	0.87	1520.46	1484.67	0.87
	S.aureus	321.28	235.05	0.09	613.67	615.65	0.98	515.83	680.74	0.20	534.82	557.12	0.84	647.60	577.82	0.56
	E.coli	1739.34	1504.91	0.32	4071.97	3850.97	0.75	3185.24	3905.23	0.30	5671.20	4486.23	0.06	4818.33	4398.01	0.53
	C.albicans	110.46	140.71	0.48	93.58	100.65	0.86	63.85	88.77	0.46	43.83	51.72	0.75	100.41	155.87	0.34
IL-10	**PPD**	69.96	69.76	0.99	137.48	148.74	0.76	77.08	67.10	0.63	43.43	71.73	0.08	84.00	66.16	0.35
	Poly I:C	237.60	243.82	0.87	479.74	511.56	0.82	263.56	238.66)	0.72	241.65)	331.42	0.15	385.71	323.73	0.37
	S.pneumoniae	556.63	611.91	0.45	331.07	380.62	0.58	254.25	248.56	0.93	346.05	316.63	0.53	323.60	329.23)	0.91
	S.aureus	25.30	22.56	0.59	50.40	41.27	0.53	35.74	42.62	0.63	36.94	35.93	0.86	43.60	30.43	0.28
	E.coli	992.07	1006.97	0.89	1382.85	1342.85	0.89	817.34	967.67	0.43	1234.87	1212.67	0.85	1029.01	1105.24	0.68
	C.albicans	83.24	120.02	0.20	21.58	24.48	0.77	22.56	19.13	0.71	18.69	33.89	0.15	59.97	41.52	0.37
IFNγ	**PPD**	2.90	3.66	0.54	7.53	6.12	0.66	1183.17	5.42	**<0.0001**	985.97	8.43	**<0.0001**	760.66	448.64	0.11
	Poly I:C	6.01	4.92	0.58	59.67	61.92	0.93	129.66	118.84	0.84	51.04	59.96	0.74	247.73	249.66	0.98
	S.pneumoniae	2.77	3.49	0.48	97.07	92.33	0.91	77.13	46.35	0.34	74.38	64.29	0.77	95.99	105.76	0.82
	S.aureus	1.94	4.24	**0.03**	57.90	29.10	0.13	105.54	47.61	0.12	48.61	46.00	0.91	117.08	108.49	0.87
	E.coli	7.21	9.19	0.47	145.45	77.39	0.21	176.36	151.82	0.77	270.59	170.01	0.36	232.54	181.82	0.61
	C.albicans	1.80	2.49	0.40	4.42	4.17	0.91	18.10	19.66	0.88	31.15	22.41	0.52	26.23	47.43	0.25

2.3.1 Geometric mean cytokine levels analysed by sex

			5 days of age			6 weeks of age (pre-EPI1)			6 weeks of age (5d post-EPI1 +/-BCG)			10 weeks of age (pre-EPI2)		
			BCG at birth	**BCG at 6wks**	**p-value**	**BCG at birth**	**BCG at 6wks**	**p-value**	**BCG at birth**	**BCG at 6wks**	**p-value**	**BCG at birth**	**BCG at 6wks**	**p-value**
TNFα	**Medium**	Male	4.49	4.49	0.76	4.49	4.49	**0.04**	6.96	4.49	0.12	7.56	8.78	0.68
		Female	4.49	4.49	0.62	4.49	4.49	0.59	4.49	4.49	0.48	6.77	4.49	0.55
		Test for interaction			0.40			**0.04**			0.37			0.43
	PPD	Male	92.65	112.50	0.55	210.78	25.90	**<0.0001**	213.26	22.83	**<0.0001**	193.03	55.98	**0.003**
		Female	60.33	49.88	0.66	138.93	46.43	**0.01**	145.58	42.36	**0.004**	109.63	75.57	0.37
		Test for interaction			0.48			0.08			0.06			0.12
	Poly I:C	Male	1716.14	1039.93	0.06	1027.78	768.56	0.43	1181.10	823.36	0.09	1119.99	1041.86	0.82
		Female	965.39	1601.07	0.08	1107.83	802.33	0.22	815.67	1019.54	0.30	1090.54	1098.19	0.98
		Test for interaction			**0.01**			0.93			**0.05**			0.84
	S.pneumoniae	Male	1872.88	2010.88	0.72	1636.18	1613.47	0.96	2024.07	1295.92	**0.03**	1700.10	2140.46	0.17
		Female	1681.95	1705.63	0.95	1640.53	1693.07	0.86	1685.84	1502.51	0.58	1311.37	1387.99	0.83
		Test for interaction			0.84			0.89			0.25			0.58
	S.aureus	Male	226.78	271.59	0.54	363.86	248.95	0.28	302.23	162.05	0.06	214.09	337.98	0.17
		Female	267.61	237.43	0.74	320.62	324.72	0.96	224.12	173.53	0.45	238.99	260.78	0.78

IL-6		Test for interaction			0.50			0.39			0.41			0.44
	E.coli	Male	3435.29	2685.19	0.31	2335.57	3169.45	0.32	3034.73	2396.09	0.27	2593.91	2379.52	0.74
		Female	2755.18	2952.27	0.82	1965.06	2222.34	0.59	2550.95	2331.15	0.64	1720.39	2004.59	0.38
		Test for interaction			0.41			0.64			0.61			0.45
	C.albicans	Male	92.59	131.90	0.51	147.72	76.94	0.21	169.40	104.02	0.21	193.09	174.08	0.83
		Female	82.50	113.11	0.60	111.62	123.38	0.87	63.71	112.16	0.33	99.73	123.01	0.71
		Test for interaction			0.95			0.32			0.13			0.67
	Medium	Male	83.28	250.18	0.31	106.85	48.75	0.61	48.75	48.75	0.35	172.87	48.75	0.95
		Female	276.60	48.75	0.24	160.62	48.75	0.57	125.55	117.71	0.90	357.22	115.85	0.07
		Test for interaction			0.10			0.90			0.62			0.27
	PPD	Male	10442.84	11165.75	0.82	9930.1	3523.58	**0.0002**	12854.77	3482.46	**<0.0001**	11522.4	6307.1	**0.02**
		Female	7454.12	6967.35	0.83	9058.38	3027.33	**0.002**	9135.62	3725.21	**0.002**	6963.13	6458.14	0.76
		Test for interaction			0.75			0.88			0.29			0.14
	Poly I:C	Male	40741.14	40691.52	0.99	24942.61	23613.1	0.76	32686.78	25370.07	0.22	34103.9	30986.14	0.59
		Female	38856.87	44371.84	0.54	30992.66	22262.94	0.14	29259.28	30853.9	0.77	29332.51	28388.82	0.86
		Test for interaction			0.64			0.33			0.26			0.81
	S.pneumoniae	Male	87481.60	90836.3	0.78	56322.29	53028.68	0.73	65598.1	54620.22	0.18	54716.3	60063.03	0.50
		Female	80002.27	85687.84	0.67	52127.09	46674.34	0.51	66093.54	65821.56	0.97	49294.52	49258.95	0.99
		Test for interaction			0.88			0.84			0.34			0.68

	S.aureus	Male	3848.28	6211.43	0.18	4065.90	3551.22	0.63	5181.39	2786.03	**0.01**	4994.92	4137.74	0.41
		Female	5430.42	4140.03	0.39	4540.65	3672.11	0.48	4982.68	4720.62	0.83	3446.47	4959.19	0.28
		Test for interaction			0.11			0.85			0.11			0.17
	E.coli	Male	87452.74	82255.97	0.68	62842.23	57788.87	0.64	80919.38	70063.46	0.32	60048.08	76084.55	0.09
		Female	92176.84	96254.14	0.76	53736.61	51001.05	0.78	76867.04	75000.47	0.83	62518.8	57319.28	0.52
		Test for interaction			0.61			0.90			0.51			0.09
	C.albicans	Male	9385.39	16303.33	0.35	9830.0	6173.22	0.44	10753.60	7894.54	0.58	18503.04	12663.46	0.47
		Female	10321.18	10676.67	0.95	8880.23	8438.02	0.91	7677.78	7992.73	0.94	12547.70	13733.48	0.82
		Test for interaction			0.52			0.54			0.44			0.48
IL-1β	**Medium**	Male	26.46	13.34	0.77	15.15	2.24	**0.05**	2.24	2.24	0.62	34.44	10.91	0.34
		Female	36.23	7.98	0.09	21.56	8.49	0.29	2.24	11.92	0.71	31.36	17.68	0.22
		Test for interaction			0.15			0.53			0.70			0.92
	PPD	Male	88.45	130.19	0.42	100.69	53.30	0.19	89.28	44.67	0.19	128.48	64.95	0.06
		Female	119.85	75.21	0.34	66.63	81.93	0.67	101.55	57.94	0.27	94.02	133.57	0.51
		Test for interaction			0.21			0.21			0.85			0.10
	Poly I:C	Male	1574.79	1652.46	0.84	835.05	820.98	0.95	1159.89	826.66	0.24	1803.78	1311.41	0.19
		Female	1900.92	1563.83	0.48	926.34	737.03	0.54	759.70	1080.29	0.20	1415.06	1392.34	0.95
		Test for interaction			0.51			0.66			0.08			0.37
	S.pneumoniae	Male	1930.82	1513.95	0.29	1660.50	1442.58	0.55	1398.24	1252.03	0.73	2003.33	1638.74	0.29

		Female	1297.70	1255.16	0.89	1424.91	1546.57	0.75	1584.53	1740.80	0.65	1308.46	1420.77	0.69
		Test for interaction			0.53			0.51			0.61			0.31
	S.aureus	Male	688.59	746.41	0.70	639.13	702.94	0.60	661.80	565.43	0.52	873.71	718.56	0.19
		Female	748.46	635.35	0.49	635.67	717.30	0.67	630.75	634.09	0.98	671.56	658.18	0.92
		Test for interaction			0.44			0.94			0.58			0.47
	E.coli	Male	4914.11	3694.74	0.21	3826.99	3949.78	0.88	6655.90	4270.75	**0.02**	5328.02	4510.73	0.39
		Female	3673.48	4394.69	0.46	2912.23	3937.49	0.25	4748.84	4771.23	0.98	4579.32	4434.84	0.88
		Test for interaction			0.16			0.42			0.07			0.63
	C.albicans	Male	144.18	170.21	0.74	141.26	102.39	0.50	72.97	70.72	0.95	223.91	250.26	0.86
		Female	162.16	133.15	0.63	137.79	151.14	0.83	69.07	110.63	0.42	158.77	210.40	0.60
		Test for interaction			0.57			0.53			0.54			0.80
IL-10	**Medium**	Male	4.49	7.56	0.07	4.49	4.49	0.65	10.38	4.49	0.36	7.56	8.15	0.77
		Female	8.18	4.49	0.29	7.35	4.49	0.51	6.76	4.49	0.36	14.89	6.64	0.31
		Test for interaction			**0.03**			0.86			0.82			0.55
	PPD	Male	154.55	186.21	0.55	92.57	60.27	0.20	61.05	103.46	0.06	102.35	98.98	0.92
		Female	186.81	186.64	0.98	96.09	107.86	0.76	73.94	78.69	0.84	110.41	86.13	0.38
		Test for interaction			0.64			0.27			0.24			0.56
	Poly I:C	Male	642.54	623.17	0.94	208.20	186.19	0.74	238.59	295.24	0.48	465.83	285.03	**0.03**
		Female	415.71	535.56	0.48	448.58	345.05	0.44	329.28	390.49	0.44	375.71	431.29	0.65

		Test for interaction			0.56			0.75			0.93			0.09
	S.pneumoniae	Male	453.57	480.43	0.82	241.08	208.90	0.69	331.00	310.06	0.73	415.63	390.54	0.72
		Female	311.61	382.31	0.55	334.53	342.66	0.92	420.50	347.37	0.34	316.31	325.34	0.94
		Test for interaction			0.77			0.71			0.65			0.79
	S.aureus	Male	65.96	79.14	0.63	39.50	46.00	0.65	50.41	42.17	0.54	68.09	50.37	0.30
		Female	69.47	57.02	0.59	76.01	76.29	0.99	62.85	65.24	0.90	69.45	64.60	0.82
		Test for interaction			0.47			0.73			0.61			0.59
	E.coli	Male	1807.62	1445.94	0.41	791.83	980.27	0.51	1160.89	1220.27	0.74	1239.45	1124.70	0.42
		Female	1177.05	1499.17	0.35	974.32	992.36	0.92	1368.10	1232.40	0.41	970.17	1129.69	0.60
		Test for interaction			0.21			0.61			0.43			0.41
	C.albicans	Male	41.41	53.77	0.61	46.14	27.79	0.22	40.28	50.74	0.59	103.71	77.57	0.46
		Female	40.28	38.44	0.89	42.44	38.01	0.81	36.86	48.23	0.55	77.55	57.32	0.48
		Test for interaction			0.63			0.54			0.94			0.99
IFNγ	**Medium**	Male	8.57	9.18	0.58	6.79	2.69	0.93	10.89	9.26	0.54	9.07	7.68	0.62
		Female	7.39	10.71	0.97	7.75	2.69	0.30	17.16	10.31	0.51	6.15	3.31	0.41
		Test for interaction			0.52			0.93			0.59			0.64
	PPD	Male	25.37	26.22	0.94	1660.74	21.26	**<0.0001**	1581.85	20.99	**<0.0001**	1389.01	576.94	**0.005**
		Female	23.18	16.32	0.46	861.94	14.48	**<0.0001**	761.83	26.83	**<0.0001**	600.74	396.87	0.33
		Test for interaction			0.54			0.65			0.16			0.37

	Poly I:C	Male	121.20	84.93	0.40	204.32	272.23	0.54	175.11	123.58	0.46	486.86	416.17	0.63
		Female	83.12	162.79	0.18	138.51	109.24	0.64	58.61	83.88	0.49	223.69	231.71	0.93
		Test for interaction			0.12			0.45			0.31			0.73
	S.pneumoniae	Male	209.01	118.13	0.17	168.39	136.51	0.69	155.97	137.00	0.79	253.53	197.99	0.47
		Female	134.13	174.27	0.62	92.86	81.74	0.81	113.30	104.13	0.87	99.83	106.29	0.90
		Test for interaction			0.19			0.92			0.93			0.60
	S.aureus	Male	109.40	65.53	0.24	189.05	122.17	0.44	91.29	140.85	0.41	243.35	222.28	0.83
		Female	73.40	70.72	0.95	127.01	81.47	0.39	79.48	60.58	0.63	123.82	98.78	0.71
		Test for interaction			0.47			0.96			0.36			0.86
	E.coli	Male	295.58	76.93	**0.008**	247.19	298.60	0.73	477.81	276.70	0.33	518.58	382.68	0.46
		Female	173.20	243.49	0.57	215.87	176.63	0.74	265.54	221.63	0.76	225.20	183.05	0.71
		Test for interaction			**0.03**			0.63			0.65			0.87
	C.albicans	Male	16.65	12.02	0.49	31.31	69.28	0.16	74.91	73.17	0.99	99.05	87.11	0.81
		Female	11.56	21.61	0.20	51.36	27.55	0.24	58.25	30.38	0.24	25.10	66.26	0.09
		Test for interaction			0.16			0.07			0.40			0.14

2.3.2 Medium subtracted geometric mean cytokine levels, analysed by sex

			5 days of age			6 weeks of age (pre-EPI1)			6 weeks of age (5d post-EPI1 +/-BCG)			10 weeks of age (pre-EPI2)		
			BCG at birth	**BCG at 6wks**	**p-value**	**BCG at birth**	**BCG at 6wks**	**p-value**	**BCG at birth**	**BCG at 6wks**	**p-value**	**BCG at birth**	**BCG at 6wks**	**p-value**
TNFα	**PPD**	Male	50.06	62.06	0.65	158.94	12.44	**<0.0001**	193.02	9.42	**<0.0001**	166.07	30.52	**0.001**
		Female	40.61	23.19	0.32	109.63	22.51	**0.007**	97.53	24.21	**0.02**	76.63	51.45	0.44
		Test for interaction			0.29			0.21			**0.03**			0.07
	Poly I:C	Male	1658.46	750.65	0.06	772.05	709.97	0.87	1162.14	807.43	0.09	1009.56	872.77	0.75
		Female	672.15	1500.65	0.07	1090.33	752.44	0.17	796.80	1002.05	0.30	1056.20	1054.55	0.99
		Test for interaction			**0.009**			0.63			**0.05**			0.79
	S.pneumoniae	Male	1821.13	1901.38	0.83	1535.73	1602.77	0.89	2005.14	1276.69	**0.03**	1654.00	1644.63	0.99
		Female	1638.44	1276.42	0.49	1620.33	1649.43	0.92	1666.53	1480.82	0.57	1272.00	1364.29	0.80
		Test for interaction			0.47			0.95			0.25			0.87
	S.aureus	Male	176.20	177.98	0.98	234.99	216.03	0.88	284.10	139.29	**0.05**	157.43	230.51	0.45
		Female	218.77	128.64	0.28	302.79	239.18	0.55	203.81	124.74	0.30	179.76	228.56	0.56
		Test for interaction			0.40			0.82			0.70			0.83
	E.coli	Male	3355.95	2527.23	0.26	2276.12	3157.61	0.30	3018.57	2367.93	0.26	2512.74	1918.54	0.51
		Female	2695.25	2842.60	0.86	1943.36	2153.47	0.66	2526.22	2315.23	0.65	1667.42	1970.49	0.34

IL-6		Test for interaction			0.39			0.57			0.60			0.34
	C.albicans	Male	43.02	88.73	0.29	84.18	54.07	0.52	137.49	82.38	0.26	152.18	101.86	0.53
		Female	57.19	90.97	0.49	80.17	70.68	0.86	40.56	72.19	0.46	59.58	85.02	0.63
		Test for interaction			0.79			0.75			0.22			0.44
	PPD	Male	9602.12	8067.02	0.71	6864.84	3735.95	0.22	14203.42	3044.09	**<0.0001**	9801.04	4009.45	0.12
		Female	6592.62	6635.27	0.98	8775.75	1946.42	**0.005**	9223.97	2726.73	**0.01**	5210.31	4369.81	0.73
		Test for interaction			0.75			0.21			0.57			0.35
	Poly I:C	Male	39846.23	38290.22	0.84	24108.36	25074.2	0.83	32108.85	25041.85	0.25	30852.61	29683.59	0.85
		Female	38062.89	42415.27	0.63	30745.03	21195.18 (23)	0.10	28672.52	30484.75	0.74	28496.97	27458.07	0.86
		Test for interaction			0.62			0.15			0.27			0.99
	S.pneumoniae	Male	86034.48	88366.79	0.85	55237.69	55682.11	0.96	66228.54	54149.53	0.16	54429.49	62463.21	0.38
		Female	81281.16	84048.17	0.82	51869.4	45762.4	0.46	66099.50	65311.11	0.93	49107.13	48628.57	0.96
		Test for interaction			0.97			0.59			0.33			0.56
	S.aureus	Male	3196.86	5253.31	0.17	2789.70	2738.62	0.98	5298.42	2599.43	**0.005**	3595.87	2363.36	0.43
		Female	4826.88	3843.50	0.46	4235.52	3093.08	0.29	4666.40	3811.30	0.53	1809.63	4198.29	0.13
		Test for interaction			0.13			0.67			0.21			0.10
	E.coli	Male	87499.33	78196.93	0.51	61902.67	661259.16	0.95	83142.21	69583.32	0.23	59332.58	82104.57	**0.03**
		Female	90747.88	94311.53	0.80	53084.49	49962.92	0.75	77835.28	74662.09	0.72	62850.07	56620.69	0.46

IL-1β		Test for interaction			0.50			0.85			0.46			**0.04**
	C.albicans	Male	8068.42	12819.49	0.51	8472.16	6762.43	0.73	9732.37	7454.33	0.66	14679.37	10551.97	0.62
		Female	8443.87	10117.5	0.78	8510.44	7451.88	0.79	6484.33	4797.94	0.71	11261.07	10236.45	0.88
		Test for interaction			0.77			0.11			0.97			0.79
	PPD	Male	50.14	83.80	0.39	53.56	36.76	0.53	58.96	26.10	0.22	56.92	32.80	0.33
		Female	62.26	50.19	0.72	23.49	51.03	0.25	52.63	25.18	0.28	42.80	78.13	0.40
		Test for interaction			0.39			0.20			0.93			0.20
	Poly I:C	Male	1499.17	1542.31	0.91	681.84	806.05	0.68	1140.27	797.04	0.22	1575.56	1264.57	0.43
		Female	1794.87	1503.06	0.53	879.72	696.27	0.54	559.16	1044.67	0.12	1331.27	1351.55	0.95
		Test for interaction			0.58			0.47			**0.05**			0.52
	S.pneumoniae	Male	1857.21	1415.85	0.23	1603.51	1420.75	0.61	1332.43	1217.97	0.83	1887.66	1583.57	0.37
		Female	1213.25	1219.26	0.98	1388.14	1505.58	0.74	1482.62	168.17	0.56	1224.65	1376.41	0.57
		Test for interaction			0.41			0.55			0.64			0.31
	S.aureus	Male	593.17	635.42	0.75	472.70	687.42	0.26	645.18	532.03	0.42	765.01	544.89	0.29
		Female	634.04	594.98	0.79	582.31	674.68	0.60	425.20	579.42	0.36	548.18	619.02	0.58
		Test for interaction			0.68			0.61			0.23			0.25
	E.coli	Male	4815.86	3471.91	0.17	3760.68	3927.46	0.84	6638.50	4221.95	**0.02**	5225.97	4421.81	0.40
		Female	3465.25	4306.96	0.39	2529.08	3884.95	0.22	4678.19	4724.36	0.95	4442.49	4370.24	0.94
		Test for interaction			0.11			0.33			0.07			0.61

IL-10	***C.albicans***	Male	88.82	115.79	0.66	64.74	64.42	0.99	48.35	54.14	0.87	132.87	170.42	0.70
		Female	98.40	87.49	0.81	62.62	118.99	0.29	38.87	49.75	0.76	75.82	140.37	0.36
		Test for interaction			0.63			0.48			0.90			0.70
	PPD	Male	141.59	129.70	0.81	75.28	45.16	0.22	32.92	76.93	0.06	85.41	70.86	0.59
		Female	133.64	173.38	0.49	79.65	96.28	0.63	60.80	67.57	0.77	82.62	61.02	0.44
		Test for interaction			0.51			0.22			0.19			0.82
	Poly I:C	Male	622.88	533.00	0.66	183.64	166.01	0.81	192.97	281.02	0.32	440.73	267.56	**0.03**
		Female	373.15	488.56	0.53	434.97	332.72	0.44	318.02	381.40	0.42	337.53	404.83	0.57
		Test for interaction			0.44			0.76			0.66			0.08
	S.pneumoniae	Male	434.68	392.75	0.75	214.41	184.28	0.73	301.54	294.41	0.91	385.19	369.44	0.82
		Female	254.76	367.47	0.35	322.08	326.89	0.95	409.44	336.88	0.34	270.39	287.56	0.85
		Test for interaction			0.35			0.75			0.55			0.78
	S.aureus	Male	51.03	41.84	0.66	23.13	27.58	0.74	29.05	21.75	0.54	39.39	27.70	0.45
		Female	49.75	40.66	0.65	64.86	59.46	0.77	49.46	52.04	0.90	48.51	33.56	0.45
		Test for interaction			0.99			0.67			0.59			0.98
	E.coli	Male	1781.56	1241.40	0.26	730.48	963.67	0.46	1144.57	1199.92	0.75	1202.25	1103.18	0.50
		Female	1083.84	1466.34	0.33	955.36	971.36	0.93	1354.95	1223.64	0.42	880.71	1107.66	0.51
		Test for interaction			0.14			0.55			0.44			0.37
	C.albicans	Male	23.98	25.66	0.92	24.05	15.17	0.49	18.12	33.27	0.31	116.71	50.84	0.47

		Female	19.50	23.22	0.77	21.88	23.61	0.91	19.40	34.43	0.33	70.03	32.70	0.54
		Test for interaction			0.91			0.58			0.97			0.99
IFNγ	**PPD**	Male	6.47	8.35	0.69	1581.35	7.40	**<0.0001**	1539.53	7.36	**<0.0001**	1363.99	524.17	**0.004**
		Female	8.73	4.30	0.31	803.57	4.03	**<0.0001**	571.75	9.49	**<0.0001**	411.79	370.63	0.86
		Test for interaction			0.31			0.83			0.14			0.19
	Poly I:C	Male	65.84	46.63	0.58	147.08	228.17	0.44	97.51	86.04	0.85	461.59	351.60	0.48
		Female	54.28	84.96	0.50	109.57	65.30	0.44	22.86	43.48	0.36	128.65	163.89	0.73
		Test for interaction			0.38			0.28			0.42			0.50
	S.pneumoniae	Male	107.62	71.95	0.52	97.11	68.25	0.63	89.48	89.97	0.99	204.24	156.18	0.54
		Female	87.88	122.00	0.59	56.67	32.47	0.48	59.30	47.69	0.76	43.14	65.41	0.58
		Test for interaction			0.40			0.85			0.82			0.42
	S.aureus	Male	74.04	24.49	0.08	124.91	58.62	0.30	50.34	91.11	0.39	188.28	189.27	0.99
		Female	45.66	35.26	0.71	84.27	39.35	0.31	46.58	24.91	0.40	70.94	54.57	0.74
		Test for interaction			0.36			1.0			0.23			0.77
	E.coli	Male	185.55	45.91	**0.04**	190.95	223.28	0.81	363.58	238.39	0.52	414.17	295.16	0.54
		Female	115.05	138.31	0.81	158.62	106.66	0.62	188.51	126.00	0.60	126.65	100.13	0.77
		Test for interaction			0.12			0.59			0.98			0.91
	C.albicans	Male	6.29	2.53	0.21	13.99	39.36	0.16	39.18	42.78	0.90	68.48	55.08	0.74
		Female	3.07	6.94	0.29	25.39	10.21	0.24	23.48	12.46	0.40	9.19	39.44	0.06

	Test for interaction	0.10	0.07	0.49	0.10

2.4.1 Cross sectional comparison of medium subtracted geometric mean cytokine levels: combined analyses

			5 days of age			6 weeks of age (pre-EPI1)			6 weeks of age (5d post-EPI1 +/-BCG)			10 weeks of age (pre-EPI2)		
			BCG at birth	BCG at 6wks	p-value	BCG at birth	BCG at 6wks	p-value	BCG at birth	BCG at 6wks	p-value	BCG at birth	BCG at 6wks	p-value
TNFα	All pathogens	All participants	7557.62	7123.56	0.68	6135.23	6116.89	0.98	6617.59	5565.90	0.14	5674.53	6287.23	0.41
		Male	8119.47	7014.08	0.45	6355.89	6892.98	0.74	7300.26	5219.33	0.06	6496.78	6817.97	0.80
		Female	7054.08	7248.20	0.99	5841.39	5482.48	0.71	5869.31	5879.22	0.99	4956.35	5716.67	0.37
		Test for interaction			0.54			0.64			0.15			0.70
	Bacteria only	All participants	5612.98	5121.48	0.55	4601.60	4885.99	0.71	5128.60	4238.77	0.13	4030.27	4615.00	0.30
		Male	5944.60	5164.59	0.51	4768.11	5643.61	0.52	5620.07	4077.02	0.09	4677.22	5100.27	0.67
		Female	5311.57	5073.61	0.84	4379.94	4281.19	0.89	4585.90	4381.61	0.78	3472.81	4103.91	0.29
		Test for interaction			0.76			0.55			0.28			0.76
IL-6	All pathogens	All participants	239872.3	251042.2	0.67	167193.9	152970.7	0.46	205429.2	188088.9	0.31	170475.2	186217.9	0.43
		Male	246753.2	251079	0.91	170744.3	165542.6	0.86	213209.1	174762.7	0.16	175956.4	213388.6	0.20
		Female	233183.4	251000.5	0.62	162764.6	142372.1	0.43	197069.4	200722.0	0.87	165472.4	165419.5	1.0
		Test for interaction			0.80			0.67			0.23			0.39
	Bacteria only	All participants	178034.2	182159.00	0.82	120442.5	110098.1	0.45	155639.1	140249.0	0.20	118595.7	133259.0	0.28

		Male	183141.3	178840.3	0.87	124490.4	121784.6	0.90	159191.9	129785.5	0.11	119710.0	157970.6	**0.05**
		Female	173069.4	185984.9	0.60	115461.2	100851.5	0.41	151762.0	150206.1	0.92	117556.5	114935.3	0.86
		Test for interaction			0.63			0.64			0.24			0.15
IL-1β	**All pathogens**	All participants	9055.47	8407.00	0.58	7529.21	7682.05	0.86	9600.45	8230.17	0.18	9423.58	8692.11	0.51
		Male	9829.34	8147.68	0.33	7759.33	7665.39	0.94	11052.64	7505.95	**0.03**	10583.18	8797.15	0.28
		Female	8368.89	8696.35	0.84	7220.87	7697.36	0.71	8082.07	8901.97	0.51	8391.03	8570.39	0.91
		Test for interaction			0.40			0.74			**0.04**			0.40
	Bacteria only	All participants	6717.35	6287.82	0.64	6148.47	6388.54	0.74	8310.66	6841.48	0.09	7295.25	6850.98	0.61
		Male	7626.60	5947.96	0.22	6448.65	6400.27	0.96	9585.85	6304.59	**0.02**	8180.29	6946.88	0.34
		Female	5945.47	6676.71	0.56	5754.59	6377.80	0.54	6980.14	7334.74	0.73	6505.97	6740.10	0.85
		Test for interaction			0.20			0.64			**0.04**			0.42
IL-10	**All pathogens**	All participants	2819.21	3035.14	0.48	1836.56	1907.93	0.74	2132.40	2067.81	0.75	1949.50	1946.11	0.99
		Male	3148.45	3348.43	0.70	1678.31	1816.41	0.62	1998.69	1947.54	0.85	2326.01	1855.31	0.12
		Female	2500.28	2739.92	0.47	2071.01	1991.78	0.82	2308.02	2176.10	0.67	1619.57	2045.59	0.47
		Test for interaction			0.89			0.61			0.87			0.19
	Bacteria only	All participants	2124.14	2196.08	0.72	1380.29	1454.28	0.65	1712.30	1624.93	0.58	1365.22	1493.51	0.66
		Male	2360.59	2393.10	0.93	1322.41	1496.45	0.45	1600.83	1563.37	0.86	1672.81	1471.84	0.34

		Female	1893.91	2008.08	0.59	1461.43	1418.35	0.85	1859.15	1679.28	0.44	1102.92	1516.45	0.41
		Test for interaction			0.81			0.51			0.68			0.27
IFNγ	**All pathogens**	All participants	863.76	555.40	0.15	940.02	786.97	0.62	734.88	729.13	0.98	1347.72	1179.70	0.86
		Male	1117.73	389.33	**0.006**	1074.76	1268.99	0.70	938.22	1003.00	0.89	1784.57	1593.94	0.74
		Female	667.49	815.14	0.68	792.14	498.76	0.42	545.21	537.13	0.98	971.28	801.17	0.93
		Test for interaction			**0.04**			0.38			0.91			0.90
	Bacteria only	All participants	653.40	402.62	0.12	662.31	493.52	0.44	507.29	550.51	0.83	807.93	663.44	0.54
		Male	871.80	278.24	**0.003**	733.69	606.38	0.71	641.73	632.98	0.98	1101.72	895.14	0.56
		Female	489.71	600.08	0.69	576.66	401.67	0.55	380.60	478.78	0.69	562.63	442.77	0.67
		Test for interaction			**0.03**			0.83			0.76			0.96

			5 days of age			6 weeks of age (pre-EPI1)			6 weeks of age (5d post-EPI1 +/-BCG)			10 weeks of age (pre-EPI2)		
			BCG at birth	**BCG at 6wks**	**p-value**	**BCG at birth**	**BCG at 6wks**	**p-value**	**BCG at birth**	**BCG at 6wks**	**p-value**	**BCG at birth**	**BCG at 6wks**	**p-value**
All pro-inflammatory cytokines: TNF*α*, IL-6, IL-1β, IFN	**All pathogens**	All participants	263557.1	269284	0.83	186180.2	171722.5	0.48	225664.5	203560.6	0.25	189652.4	207691	0.41
		Male	271462.0	268232.4	0.94	191840.3	186938.3	0.88	235661.8	190999.9	0.14	198050.0	234244.7	0.25
		Female	255882.4	270431.7	0.69	179488.5	159025.9	0.45	214992.2	215844.7	0.97	182074.2	186172.6	0.89
		Test for interaction			0.74			0.68			0.23			0.51
	Bacteria only	All participants	195819.8	196437.8	0.97	134651.1	125473.0	0.53	172646.2	152873.1	0.14	132853	149364.3	0.27
		Male	202782.5	192230.9	0.70	139847.9	139281.0	0.98	177913.8	142850.9	0.09	135900.2	174231.5	0.07
		Female	189096.2	201115.0	0.63	128561.0	114654.7	0.45	166943.2	162713.4	0.80	130047.5	129851.5	0.99
		Test for interaction			0.54			0.63			0.24			0.23
TNF*α*, IL-6	**All pathogens**	All participants	250047.2	258928.3	0.74	174087	161019.1	0.74	213088.2	194265.3	0.30	176952.1	193088.3	0.43
		Male	259330.2	259123.4	1.0	178225.7	174626.6	1.0	221432.6	180399.0	0.15	183840.7	220698.3	0.22
		Female	241096.4	258707.8	0.63	168938.2	149571.0	0.63	204134.3	207417.6	0.89	170704.7	171903.2	0.97

		Test for interaction			0.74			0.74			0.22			0.43
	Bacteria only	All participants	185850.5	188114.3	0.68	125794.1	116827.6	0.55	161801.3	145102.3	**0.02**	161801.3	138484.3	0.28
		Male	193108.3	184940.6	0.61	130298.5	129730.6	0.64	165653.6	132307.3	**0.02**	125462.8	163631.7	0.46
		Female	178865.5	191767.7	0.95	120264.4	106655.4	0.79	157601.7	155372.8	0.48	121211.0	119780.4	0.42
		Test for interaction			0.77			0.84			0.14			0.95
TNFα:IL-10 ratio	**All pathogens**	All participants	2.56	2.17	0.25	3.36	3.08	0.62	3.10	2.69	0.34	2.88	3.16	0.65
		Male	2.58	1.93	0.17	3.83	3.51	0.73	3.65	2.68	0.14	2.79	3.56	0.34
		Female	2.55	2.46	0.87	2.82	2.75	0.92	2.54	2.70	0.78	2.97	2.79	0.86
		Test for interaction			0.38			0.85			0.22			0.47
	Bacteria only	All participants	2.53	2.14	0.26	3.36	3.22	0.81	3.0	2.61	0.38	2.90	3.01	0.87
		Male	2.52	1.98	0.26	3.66	3.47	0.84	3.51	2.61	0.17	2.80	3.34	0.48
		Female	2.54	2.33	0.68	3.0	3.02	0.98	2.47	2.61	0.81	3.02	2.71	0.78
		Test for interaction			0.60			0.87			0.26			0.54

2.5 Within-infant fold-change overtime, unadjusted and adjusted for baseline levels

2.5.1 TNFα

			5 days : Cord Blood				6 weeks (pre-EPI1) : Cord blood				6 weeks of age (5d post-EPI1 +/- BCG) : Cord blood				10 weeks (pre-EPI2) : Cord blood			
			BCG at birth	**BCG at 6wks**	**p-value**	**Adj p-value**	**BCG at birth**	**BCG at 6wks**	**p-value**	**Adj p-value**	**BCG at birth**	**BCG at 6wks**	**p-value**	**Adj p-value**	**BCG at birth**	**BCG at 6wks**	**p-value**	**Adj p-value**
TNFα	**Medium**	All participants	1	1	0.34	0.30	1	1	0.47	0.68	1	1	0.39	0.98	1	1	0.70	0.82
		Male	1	1	0.82	0.20	1	1	0.20	0.29	1	1	0.48	0.60	1.68	1	0.70	0.75
		Female	1	1	0.10	0.56	1	1	0.83	0.28	1	1	0.71	0.53	1.51	1	0.27	0.44
		Test for interaction			0.98	0.94			0.14	0.15			0.36	0.43			0.42	0.45
	PPD	All participants	4.34	4.89	0.69	0.19	19.64	2.73	**0.0001**	**0.001**	9.35	1.22	**<0.0001**	**<0.0001**	11.25	4.95	0.36	0.11
		Male	7.96	6.80	0.75	0.41	20.27	1.95	**0.0002**	**0.005**	14.25	1.12	**<0.0001**	**0.001**	16.94	4.65	0.25	0.11
		Female	2.93	3.72	0.57	0.31	15.29	5.50	**0.06**	0.09	7.40	1.71	**0.0009**	**0.01**	7.40	5.20	0.86	0.47
		Test for interaction			0.45	0.52			0.53	0.48			0.37	0.50			0.73	0.61
	Poly I:C	All participants	5.13	5.61	0.78	0.29	3.76	3.33	0.46	0.52	3.59	2.72	0.83	**0.05**	5.25	4.61	0.98	0.84
		Male	6.52	4.25	0.14	0.15	5.00	2.83	0.31	0.34	3.80	2.25	0.33	0.59	5.57	4.89	0.69	0.41
		Female	3.77	6.48	0.06	0.41	3.44	3.60	0.96	0.67	2.78	3.15	0.43	0.07	4.84	3.86	0.72	0.35

	S.pneumoniae	Test for interaction			0.51	0.51			0.83	0.99			0.20	0.12			0.21	0.23
		All participants	1.66	2.07	0.73	0.15	2.04	2.64	0.38	0.63	1.74	1.49	0.43	0.96	2.06	2.42	0.56	0.31
		Male	1.66	2.32	0.58	0.11	2.13	1.36	0.33	0.24	2.59	1.24	0.50	0.43	2.36	3.02	0.57	0.67
		Female	1.65	1.90	0.99	0.41	1.42	3.90	**0.04**	0.69	1.61	1.55	0.80	0.45	1.35	1.87	0.97	0.32
		Test for interaction			1.0	0.84			0.96	0.44			0.32	0.36			0.34	0.32
	S.aureus	All participants	0.31	0.80	0.38	0.59	4.83	5.60	0.86	0.61	3.70	2.21	0.19	0.32	1.86	3.38	0.24	0.35
		Male	3.11	4.22	0.30	0.38	6.99	4.65	0.21	0.97	2.01	2.02	0.33	0.48	1.92	3.96	0.39	0.79
		Female	2.95	3.19	0.88	0.37	2.93	8.46	0.24	0.60	4.58	2.36	0.27	0.52	1.80	3.33	0.45	0.23
		Test for interaction			0.20	0.24			0.57	0.53			0.91	0.86			0.95	0.77
	E.coli	All participants	3.72	3.07	0.94	0.25	2.44	2.83	0.53	0.37	3.06	2.27	0.58	0.63	1.89	3.11	0.08	0.38
		Male	3.72	2.84	0.35	0.81	3.09	2.99	0.69	0.14	3.55	2.35	0.41	0.90	2.03	2.90	0.57	0.42
		Female	2.99	3.66	0.43	0.27	2.42	2.83	0.51	0.64	2.21	2.19	0.91	0.62	1.75	3.64	0.06	0.44
		Test for interaction			0.34	0.38			0.67	0.81			0.62	0.75			0.32	0.29
	C.albicans	All participants	0.93	1	0.41	0.60	1.93	0.92	0.15	0.56	1.03	1.32	0.80	0.43	1.90	1.36	0.57	0.67
		Male	0.48	1.0	0.17	0.60	1.30	0.80	0.19	0.71	1.03	0.66	1.0	0.22	2.13	1.78	0.76	0.50

		Female	1.21	0.69	0.92	0.81	3.79	0.92	0.42	0.14	1.11	1.46	0.91	0.84	0.92	1.08	0.74	0.92
		Test for interaction			0.99	0.80			0.21	0.17			0.51	0.31			0.71	0.65
			6 weeks (pre EPI1) : 5 days				**6 weeks of age (5d post-EPI1 +/-BCG) : 5 days**				**10 weeks (pre-EPI2) : 5 days**							
			BCG at birth	**BCG at 6wks**	**p-value**	**Adj p-value**	**BCG at birth**	**BCG at 6wks**	**p-value**	**Adj p-value**	**BCG at birth**	**BCG at 6wks**	**p-value**	**Adj p-value**				
TNFα	**Medium**	All participants	1	1	0.43	0.36	1	1	0.35	0.31	1	1	0.86	0.76				
		Male	1	1	0.70	0.33	1	1	0.69	0.39	1	0.96	0.89	0.46				
		Female	1	0.61	0.41	0.53	0.83	1	**0.04**	0.25	1	1	0.34	0.80				
		Test for interaction			0.42	0.42			0.55	0.93			0.93	0.86				
	PPD	All participants	2.05	0.56	**0.008**	0.10	1.56	0.67	0.11	0.24	3.25	1.32	0.12	0.72				
		Male	2.45	0.52	**0.03**	0.11	1.56	0.17	**0.003**	**0.04**	3.25	1.04	**0.05**	0.09				
		Female	1.74	0.61	0.13	0.32	1.43	1.55	1.0	0.11	3.35	4.2	1.0	0.79				
		Test for interaction			0.62	0.67			0.64	0.29			0.38	0.49				
	Poly I:C	All participants	0.84	0.93	0.92	0.74	0.81	0.56	0.54	0.35	1.08	0.79	0.82	0.39				
		Male	0.84	1.23	0.33	0.98	0.78	0.88	0.38	0.28	0.92	0.93	0.57	0.46				
		Female	0.88	0.36	0.25	**0.04**	0.87	0.44	0.16	0.67	1.08	0.63	0.49	0.58				

		Test for interaction			**0.05**	**0.02**			0.19	0.32			0.47	0.51
	S.pneumoniae	All participants	0.88	1.42	0.25	0.65	0.97	0.71	0.08	0.66	0.88	0.78	0.66	0.51
		Male	1.11	1.41	1.0	0.68	1.02	0.70	0.08	0.31	0.64	1.00	0.26	0.17
		Female	0.61	1.44	**0.04**	0.16	0.91	0.76	0.39	0.77	0.95	0.70	0.64	0.57
		Test for interaction			0.28	0.61			0.97	0.96			0.16	0.13
	S.aureus	All participants	1.32	1.50	0.53	0.44	1.27	0.80	0.11	0.32	1.17	1.22	0.86	0.40
		Male	1.36	1.43	0.81	0.49	1.27	0.31	0.12	0.12	0.69	1.16	0.57	0.67
		Female	1.04	1.51	0.35	0.43	1.31	1.01	0.52	0.61	1.34	1.22	1.0	0.26
		Test for interaction			0.18	0.27			0.35	0.19			0.31	0.31
	E.coli	All participants	0.90	1.09	0.32	0.24	0.92	0.96	0.80	0.44	0.87	0.75	1.0	1.0
		Male	0.90	1.36	0.41	0.20	0.90	0.98	0.63	0.26	0.49	0.81	0.40	0.91
		Female	0.88	1.06	0.73	0.65	1.06	0.92	0.33	0.28	0.88	0.27	0.42	0.78
		Test for interaction			0.21	0.17			0.15	0.17			0.68	0.69
	C.albicans	All participants	0.70	1.29	0.47	0.45	0.85	0.59	0.45	0.20	2.18	1.34	0.38	0.09
		Male	1.60	1.55	0.81	0.52	0.85	0.54	0.28	0.54	7.79	2.21	0.26	0.11

		Female	0.54	0.86	0.42	0.37	0.73	1.05	0.91	0.28	1.01	1.27	0.82	0.22
		Test for interaction			0.27	0.24			0.53	0.87			0.19	0.20
			6 weeks (5d post-EPI1 +/-BCG) : 6 weeks (pre- EPI1)				**10 weeks (pre-EPI2) : 6 weeks (pre-EPI1)**				**10 weeks (pre-EPI2) 6 weeks (5d post-EPI1+/-BCG)**			
			BCG at birth	**BCG at 6wks**	**p-value**	**Adj p-value**	**BCG at birth**	**BCG at 6wks**	**p-value**	**Adj p-value**	**BCG at birth**	**BCG at 6wks**	**p-value**	**Adj p-value**
TNFα	**Medium**	All participants	1	1	0.74	0.66	1	1	1.0	0.30	1	1	0.27	0.45
		Male	1	1	0.92	0.60	2.61	6.81	0.49	0.37	1.40	1.48	0.74	0.44
		Female	0.74	0.78	0.73	0.65	1.51	1	0.57	0.47	9.17	0.49	0.14	0.32
		Test for interaction			0.58	0.84			0.35	0.43			0.17	0.22
	PPD	All participants	0.88	0.96	0.81	0.25	1.02	1.71	0.75	0.44	0.46	1.84	**0.01**	0.41
		Male	1.50	0.96	0.42	0.14	1.03	2.88	0.27	0.38	0.28	2.52	**0.03**	0.70
		Female	0.72	0.88	0.73	0.77	0.69	1.59	0.46	0.52	0.71	1.14	0.29	0.52
		Test for interaction			0.46	0.48			0.26	0.80			0.98	0.86
	Poly I:C	All participants	0.97	1.24	0.96	0.12	0.95	1.06	0.82	0.37	1.51	1.89	0.81	0.07
		Male	2.46	0.67	0.11	0.40	0.53	0.58	0.56	0.92	1.32	1.61	0.66	0.58
		Female	0.55	1.57	0.06	0.54	2.30	1.13	0.22	0.42	2.13	1.98	0.48	0.18

		Test for interaction			0.13	0.52			0.12	0.34			0.26	0.61
	S.pneumoniae	All participants	0.99	0.82	0.29	0.22	1.13	1.18	0.52	0.85	1.00	1.45	0.37	0.43
		Male	1.25	0.60	0.15	0.34	1.0	1.39	0.27	0.97	0.94	1.3	0.23	0.33
		Female	0.87	0.89	0.73	0.97	1.17	1.09	0.57	0.84	1.32	1.53	0.91	0.90
		Test for interaction			0.20	0.49			0.70	0.92			0.63	0.68
	S.aureus	All participants	1.07	0.34	0.32	0.75	1.05	1.24	0.27	0.47	1.25	1.16	0.39	0.35
		Male	1.43	0.33	0.20	0.47	1.02	2.19	0.10	0.58	1.22	1.45	0.33	0.41
		Female	0.46	0.41	0.87	0.74	2.22	1.10	0.68	0.87	1.76	1.14	0.91	0.66
		Test for interaction			0.34	0.55			0.24	0.54			0.33	0.46
	E.coli	All participants	1.50	0.90	1.0	0.50	0.87	0.87	0.85	0.37	1.01	1.11	0.14	0.60
		Male	1.50	0.71	0.15	0.77	0.79	1.35	0.31	0.51	1.05	1.26	0.39	0.91
		Female	1.25	0.93	0.50	0.64	1.14	0.86	0.17	0.38	0.96	1.0	0.35	0.22
		Test for interaction			0.94	0.79			0.07	0.12			0.98	0.67
	C.albicans	All participants	1.03	1.50	0.45	0.39	2.01	0.82	0.15	0.75	0.43	1.39	0.24	0.49
		Male	6.20	7.38	0.63	0.55	1.57	1.1	0.96	0.21	0.40	1.75	0.13	0.42

	Female	0.14	0.96	0.23	0.59	11.68	0.51	0.06	0.32	0.81	1.08	0.72	0.44
	Test for interaction			0.87	0.91			0.06	0.12			0.22	0.34

2.5.2 IL-6

			5 days : Cord Blood				6 weeks (pre-EPI1) : Cord blood				6 weeks of age (5d post-EPI1 +/- BCG) : Cord blood				10 weeks (pre-EPI2) : Cord blood			
			BCG at birth	**BCG at 6wks**	**p-value**	**Adj p-value**	**BCG at birth**	**BCG at 6wks**	**p-value**	**Adj p-value**	**BCG at birth**	**BCG at 6wks**	**p-value**	**Adj p-value**	**BCG at birth**	**BCG at 6wks**	**p-value**	**Adj p-value**
IL6	**Medium**	All participants	9.73	2.15	0.44	0.10	5.18	1.28	0.57	0.25	18.12	17.40	0.44	0.29	4.59	9.94	0.86	0.36
		Male	3.69	7.02	0.65	**0.05**	7.24	1.97	0.48	0.23	18.12	1.97	0.22	0.45	4.21	6.03	0.91	0.52
		Female	17.76	1	0.10	0.97	3.67	1.05	0.73	0.15	17.40	18.12	0.95	0.35	7.16	10.41	0.89	0.54
		Test for interaction			0.13	0.15			0.12	0.12			0.72	0.76			0.86	0.84
	PPD	All participants	1.99	1.96	0.85	0.31	2.65	0.57	**0.0001**	0.41	2.18	0.71	**0.0001**	**0.01**	1.49	1.74	0.64	0.39
		Male	2.28	1.92	0.99	0.75	2.65	0.53	**0.0008**	**0.009**	2.54	0.76	**0.002**	**0.02**	1.98	1.62	0.18	0.73
		Female	1.73	1.98	0.95	0.28	2.31	0.68	**0.01**	0.44	1.91	0.69	**0.01**	0.27	0.96	2.35	0.06	0.32
		Test for interaction			0.30	0.27			0.38	0.41			0.21	0.17			0.09	0.22
	Poly I:C	All participants	2.37	2.73	0.34	0.35	1.76	1.36	0.20	0.43	1.89	1.45	0.93	0.38	1.95	1.71	0.53	0.76
		Male	2.45	2.61	0.76	0.87	1.40	1.60	1.0	0.55	1.91	1.37	0.44	0.61	2.58	2.00	0.28	0.43
		Female	2.03	3.29	0.38	0.22	2.18	1.36	**0.04**	0.55	1.87	1.66	0.64	**0.03**	1.52	1.37	0.86	0.27
		Test for interaction			0.41	0.23			0.54	0.74			0.29	0.08			0.26	0.17
	S.pneumoniae	All participants	1.06	1.18	0.68	0.28	0.70	0.72	0.70	0.26	0.79	0.73	0.90	0.63	0.82	0.80	0.99	0.40

	Male	1.16	1.20	0.69	0.49	0.82	0.68	0.15	0.59	0.79	0.68	0.70	0.83	0.70	0.89	0.31	0.29
	Female	1.04	1.00	0.89	0.27	0.54	0.75	0.39	0.09	0.81	0.92	0.96	0.42	0.95	0.55	0.35	0.95
	Test for interaction			0.55	0.41			0.99	0.22			0.68	0.40			0.24	0.43
S.aureus	All participants	2.16	2.99	0.34	0.75	2.46	1.68	0.29	0.44	2.74	1.60	0.24	0.63	2.74	2.20	0.77	0.07
	Male	1.57	3.07	0.09	0.15	3.30	1.07	0.07	0.46	1.68	1.12	0.27	0.10	3.53	1.89	0.40	0.23
	Female	2.33	2.71	0.78	0.21	2.34	2.08	0.66	0.19	3.15	3.05	0.69	0.23	0.71	2.26	0.12	0.13
	Test for interaction			**0.05**	0.06			0.24	0.12			0.06	**0.04**			0.53	0.30
E.coli	All participants	1.62	1.52	0.88	0.40	1.13	0.99	0.32	0.73	1.40	1.28	0.79	0.48	0.99	1.26	0.07	0.46
	Male	1.50	1.47	0.75	0.47	1.10	0.70	0.27	0.49	1.37	1.07	0.38	0.07	1.0	1.35	**0.01**	**0.03**
	Female	1.71	2.04	0.46	0.24	1.16	1.12	0.64	0.96	1.44	1.34	0.86	0.37	0.96	1.21	0.84	0.75
	Test for interaction			0.17	0.18			0.32	0.66			0.08	**0.05**			0.45	0.29
C.albicans	All participants	0.24	0.44	0.17	0.67	0.42	0.22	0.09	0.38	0.33	0.34	0.99	0.91	0.63	0.53	0.72	0.89
	Male	0.16	0.64	**0.03**	0.45	0.42	0.22	0.07	0.53	0.10	0.37	0.48	0.67	0.42	0.72	0.63	0.76
	Female	0.55	0.37	0.72	0.23	0.69	0.28	0.41	0.49	0.41	0.34	0.55	0.63	0.75	0.48	0.34	0.89
	Test for interaction			0.08	0.15			0.70	0.89			0.94	0.68			0.72	0.79

			6 weeks (pre-EPI1) : 5 days				6 weeks of age (5d post-EPI1 +/-BCG) : 5 days				10 weeks (pre-EPI2) : 5 days			
			BCG at birth	**BCG at 6wks**	**p-value**	**Adj p-value**	**BCG at birth**	**BCG at 6wks**	**p-value**	**Adj p-value**	**BCG at birth**	**BCG at 6wks**	**p-value**	**Adj p-value**
IL6	**Medium**	All participants	0.81	0.96	0.47	0.39	1	0.93	0.98	0.27	1.03	2.30	0.37	0.13
		Male	1	1.03	0.46	0.28	1	0.12	0.22	0.49	1.03	2.16	0.85	0.50
		Female	0.30	0.06	0.85	0.26	0.05	0.97	0.24	0.52	1	4.44	0.47	0.15
		Test for interaction			0.23	0.20			0.28	0.43			0.24	0.32
	PPD	All participants	0.69	0.33	0.23	0.08	1.24	0.43	**0.02**	**0.01**	1.35	0.85	0.08	0.26
		Male	0.73	0.30	0.27	0.08	0.95	0.49	0.11	0.08	1.53	0.71	0.16	0.84
		Female	0.68	0.37	0.42	0.23	1.51	0.28	0.23	**0.02**	1.35	1.02	0.35	0.14
		Test for interaction			0.79	0.91			0.74	0.29			0.60	0.48
	Poly I:C	All participants	0.75	0.54	0.45	0.22	0.59	0.72	0.81	0.55	0. 69	0.81	0.15	0.38
		Male	0.51	0.63	0.83	0.49	0.64	0.99	0.20	0.32	1.15	0.83	0.40	**0.05**
		Female	0.77	0.34	0.42	0.33	0.56	0.57	0.91	0.11	0.57	0.49	0.56	0.51
		Test for interaction			0.81	0.61			0.09	0.06			0.56	0.74
	S.pneumoniae	All participants	0.56	0.44	0.75	0.89	0.56	0.59	0.38	0.47	0.65	0.54	0.14	0.23
		Male	0.60	0.39	0.83	0.94	0.53	0.66	0.25	0.31	0.98	0.54	0.09	0.14
		Female	0.50	0.49	1.0	0.76	0.59	0.58	1.0	**0.05**	0.52	0.41	0.35	0.51

	Test for interaction			0.42	0.96			**0.04**	**0.05**			0.55	0.64
S.aureus	All participants	0.47	0.81	0.56	0.64	1.51	0.61	0.09	0.47	0.91	0. 99	0.82	0.97
	Male	0.37	0.54	0.87	0.39	1.78	0.32	**0.05**	0.15	0.97	0.91	0.40	0.50
	Female	0.49	0.93	0.57	0.28	1.19	1.02	0.46	0.80	0.87	1.15	0.64	0.65
	Test for interaction			0.28	0.23			0.27	0.59			0.49	0.59
E.coli	All participants	0.72	0.54	0.52	0.51	0.83	0.64	0.78	0.49	0.75	0.58	0.45	0.15
	Male	0.77	0.55	0.83	0.99	0.85	0.63	0.42	0.28	0.71	0.71	0.78	0.73
	Female	0.67	0.54	0.42	0.43	0.83	1.15	0.28	0.09	0.75	0.44	0.08	0.10
	Test for interaction			0.57	0.89			0.17	0.52			0.65	0.62
C.albicans	All participants	0.70	1.0	0.52	0.24	0.64	0.93	0.75	0.47	1.18	0.78	0.25	0.53
	Male	1.59	1.0	0.27	0.35	0.35	0.56	0.73	0.43	3.11	1.38	0.57	0.68
	Female	0.53	1	0.73	0.47	1.03	1.28	0.83	0.99	1.14	0.65	0.20	0.30
	Test for interaction			0.36	0.60			0.54	0.39			0.31	0.27

			6 weeks (5d post-EPI1 +/-BCG) : 6 weeks (pre- EPI1)				10 weeks (pre-EPI2) : 6 weeks (pre-EPI1)				10 weeks (pre-EPI2) 6 weeks (5d post EPI1+/-BCG)			
			BCG at birth	**BCG at 6wks**	**p-value**	**Adj p-value**	**BCG at birth**	**BCG at 6wks**	**p-value**	**Adj p-value**	**BCG at birth**	**BCG at 6wks**	**p-value**	**Adj p-value**
IL6	**Medium**	All participants	2.58	1	0.19	0.55	2.98	2.01	0.97	0.94	2.84	3.57	0.76	0.55
		Male	18.12	0.55	**0.005**	**0.03**	8.82	3.76	0.83	0.30	2.84	1.83	0.50	0.96
		Female	0.75	1	0.39	0.38	2.98	2.01	0.81	0.35	3.35	5.41	0.31	0.52
		Test for interaction			0.21	0.21			0.19	0.15			0.34	0.47
	PPD	All participants	1.06	1.45	0.41	0.68	0.91	1.36	0.11	0.45	1.05	2.30	**0.02**	0.27
		Male	1.31	1.01	0.75	0.59	0.91	1.33	0.27	0.48	0.88	2.73	0.09	0.45
		Female	0.63	1.84	0.13	0.80	0.93	1.51	0.37	0.64	1.33	2.02	0.20	0.48
		Test for interaction			0.36	0.53			0.37	0.52			0.99	0.78
	Poly I:C	All participants	1.01	1.32	0.35	0.40	1.03	1.10	0.85	0.98	1.23	1.96	0.28	0.87
		Male	1.62	0.82	0.15	0.42	0.83	0.69	0.71	0.68	1.20	2.09	0.33	0.57
		Female	0.51	1.82	**0.01**	0.11	1.36	1.74	0.81	0.76	1.45	1.77	0.70	0.42
		Test for interaction			**0.009**	0.08			0.46	0.55			0.97	0.30
	S.pneumoniae	All participants	1.08	0.97	0.60	0.69	0.90	1.18	0.52	0.74	0.96	0.79	0.42	0.70
		Male	1.12	0.75	0.34	0.98	0.81	1.57	0.56	**0.03**	0.96	1.20	0.39	0.73

	Female	1.08	1.13	1.0	0.87	1.31	1.13	0.57	0.54	0.95	0.48	**0.04**	0.07
	Test for interaction			0.68	0.73			0.06	0.08			**0.04**	0.13
S.aureus	All participants	1.18	0.46	0.08	0.28	1.20	1.63	0.73	0.11	0.53	0.79	0.54	0.81
	Male	1.50	0.38	0.11	0.06	1.45	1.70	0.96	0.43	0.46	1.15	0.09	0.45
	Female	0.86	0.72	0.73	0.82	0.54	1.55	0.34	0.30	0.61	0.40	0.56	0.38
	Test for interaction			0.32	0.15			0.79	0.90			0.09	0.24
E.coli	All participants	0.97	1.29	0.56	0.26	0.87	1.23	0.31	0.78	0.76	1.16	0.07	0.09
	Male	0.96	0.99	1.0	0.73	0.82	1.44	0.19	**0.01**	0.76	1.32	0.07	0.16
	Female	1.16	1.42	0.61	0.10	1.17	1.08	0.46	0.66	0.79	0.99	0.61	0.46
	Test for interaction			0.45	0.18			0.07	0.08			0.26	0.48
C.albicans	All participants	0.91	1.31	0.68	0.75	2.17	0.86	0.12	0.14	1.10	1.30	0.82	0.44
	Male	0.26	0.87	1.0	0.08	1.20	0.87	0.56	0.10	1.10	1.44	0.54	0.54
	Female	1.73	1.69	0.50	0.53	3.21	0.57	0.06	0.48	0.91	1.16	1.0	0.59
	Test for interaction			0.33	0.15			0.34	0.41			0.31	0.53

2.5.3 IL-1β

			5 days : Cord Blood				6 weeks (pre-EPI1) : Cord blood				6 weeks of age (5d post-EPI1 +/- BCG): Cord blood				10 weeks (pre-EPI2) : Cord blood			
			BCG at birth	BCG at 6wks	p-value	Adj p-value	BCG at birth	BCG at 6wks	p-value	Adj p-value	BCG at birth	BCG at 6wks	p-value	Adj p-value	BCG at birth	BCG at 6wks	p-value	Adj p-value
IL-1β	**Medium**	All participants	3.22	1	0.12	0.51	3.00	1	0.09	0.97	1	1	0.46	0.31	5.28	1	**0.02**	0.24
		Male	1.46	1.07	0.86	0.52	3.74	1	0.10	**0.04**	1	1	0.52	0.32	4.32	1	**0.02**	0.28
		Female	4.35	1	**0.04**	0.74	2.24	1	0.47	0.53	1	1.45	0.77	0.86	5.36	3.12	0.36	0.55
		Test for interaction			0.86	0.85			0.15	0.18			0.36	0.47			0.53	0.63
	PPD	All participants	1.81	1.69	0.45	0.25	2.33	1.93	0.59	0.37	2.45	1.21	0.56	0.59	2.55	1.94	0.60	0.13
		Male	1.48	2.85	0.79	0.26	2.33	2.17	0.38	0.34	2.56	2	0.80	0.46	2.71	1	0.07	0.06
		Female	1.97	0.84	0.21	0.65	2.39	1.19	0.82	0.55	2.23	1	0.24	0.99	1.50	2.93	0.25	0.55
		Test for interaction			0.38	0.36			0.96	0.91			0.89	0.55			0.07	**0.05**
	Poly I:C	All participants	6.29	6.36	0.63	0.14	4.57	4.09	0.90	0.57	3.08	4.78	0.43	0.22	6.93	4.23	0.13	0.85
		Male	6.70	6.22	0.40	0.17	4.57	4.38	0.83	0.30	5.98	4.74	0.96	0.69	10.58	4.17	**0.04**	0.31
		Female	6.17	6.49	0.85	0.59	4.26	3.04	0.96	0.17	2.03	4.82	0.27	0.23	5.29	5.30	0.92	0.21
		Test for			0.38	0.40			0.15	0.08			0.35	0.24			0.13	0.10

	interaction																
S.pneumoniae	All participants	0.89	0.85	0.80	0.25	0.84	1.06	0.54	0.65	1.16	0.85	0.71	0.73	1.11	0.97	0.25	0.28
	Male	0.97	0.84	0.59	0.89	1.05	0.70	0.50	0.80	0.97	0.67	0.80	0.72	1.11	0.83	0.30	0.74
	Female	0.83	0.85	0.85	0.26	0.72	1.37	0.13	0.19	1.75	1.23	0.32	0.94	1.08	1.03	0.58	0.28
	Test for interaction			0.30	0.30			0.94	0.37			0.76	0.76			0.26	0.22
S.aureus	All participants	2.32	1.97	0.63	0.26	1.91	2.61	0.29	0.36	2.0	1.61	0.49	0.15	1.65	1.74	0.60	0.46
	Male	2.32	1.84	0.64	0.71	1.71	2.94	0.15	0.20	1.52	1.52	0.87	0.76	1.66	1.72	0.39	0.74
	Female	2.25	2.24	0.94	0.09	2.04	2.52	0.96	0.42	3.60	1.67	0.34	0.13	1.46	1.76	0.99	0.49
	Test for interaction			0.41	0.15			0.37	0.35			0.33	0.20			0.49	**0.46**
E.coli	All participants	2.31	2.72	0.72	0.85	1.95	2.08	0.52	0.42	4.06	2.34	0.22	0.40	2.74	2.01	0.13	0.26
	Male	1.75	2.24	0.49	0.60	2.34	1.83	0.86	0.24	3.43	2.11	0.11	0.18	2.63	1.92	0.08	0.68
	Female	2.54	3.15	0.28	0.52	1.71	2.83	0.37	0.75	4.46	2.81	0.75	0.40	2.85	2.63	0.66	0.27
	Test for interaction			0.50	0.34			0.32	0.25			0.40	0.38			0.33	0.37
C.albicans	All participants	1	0.46	0.59	0.29	1	0.58	0.66	0.72	0.45	0.60	0.95	0.85	2.94	0.84	0.34	0.65
	Male	0.5	0.38	0.76	0.52	0.91	0.23	0.17	0.16	0.23	0.29	0.86	0.22	2.79	0.81	0.36	0.14
	Female	1	0.51	0.25	0.18	1	1.17	0.58	0.98	0.79	0.90	0.82	0.56	3.09	0.87	0.65	0.60
	Test for interaction			0.81	0.98			0.47	0.52			0.40	0.25			0.14	0.19

			6 weeks (pre-EPI1) : 5 days				6 weeks of age (5d post-EPI1 +/- BCG) : 5 days				10 weeks (pre-EPI2) : 5 days			
			BCG at birth	**BCG at 6wks**	**p-value**	**Adj p-value**	**BCG at birth**	**BCG at 6wks**	**p-value**	**Adj p-value**	**BCG at birth**	**BCG at 6wks**	**p-value**	**Adj p-value**
IL-1β	**Medium**	All participants	0.61	0.18	0.21	0.22	0.37	1	0.33	0.40	0.44	2.07	0.13	0.38
		Male	1	0.59	0.33	0.35	0.45	0.16	0.96	0.55	0.77	1	0.78	0.84
		Female	0.40	0.18	0.56	0.43	0.27	1	0.16	**0.05**	0.27	6.56	0.11	0.24
		Test for interaction			0.83	0.96			0.21	0.18			0.27	0.37
	PPD	All participants	0.71	0.53	0.64	0.19	0.67	0.99	0.96	0.30	0.90	3.48	0.08	0.68
		Male	2.15	0.39	0.13	0.49	0.81	0.07	0.44	0.66	1.33	2.32	0.78	0.68
		Female	0.25	1	0.30	0.23	0.27	1.07	0.66	0.10	0.90	4.30	**0.01**	0.65
		Test for interaction			0.94	0.85			0.70	0.88			0.76	0.33
	Poly I:C	All participants	0.44	0.45	0.56	0.44	0.60	0.64	0.69	0.06	0.80	1.38	0.43	0.24
		Male	0.42	0.45	1.0	0.85	0.60	0.68	0.85	0.07	1.36	1.39	0.57	0.33
		Female	0.51	0.37	0.42	0.34	0.56	0.57	0.59	0.42	0.58	1.38	0.35	0.33
		Test for interaction			0.33	0.43			0.73	0.44			0.19	0.61
	S.pneumoniae	All participants	0.82	1.66	0.28	0.47	0.92	1.15	0.77	0.68	1.37	1.11	0.66	0.15

		Male	0.82	1.14	0.93	0.91	0.65	1.02	1.0	0.56	1.53	1.12	0.32	0.70
		Female	0.83	1.67	0.13	0.38	1.91	1.30	0.52	**0.04**	0.97	0.94	1.0	0.31
		Test for interaction			0.70	0.47			0.20	0.11			0.38	0.62
	S.aureus	All participants	0.70	1.04	0.33	0.11	1.20	0.55	0.15	0.69	0.96	1.50	0.79	0.28
		Male	0.79	0.66	0.72	0.46	1.14	0.82	0.44	0.83	1.09	1.29	0.89	0.18
		Female	0.65	1.23	0.08	0.18	1.22	0.51	0.45	0.64	0.82	1.76	0.56	0.73
		Test for interaction			0.66	0.54			0.99	0.69			0.95	0.50
	E.coli	All participants	0.79	0.90	0.94	0.25	1.17	0.80	0.19	0.66	1.76	1.20	0.56	0.46
		Male	0.76	0.86	0.72	0.85	1.08	1.0	0.85	0.31	1.42	1.11	1.0	0.46
		Female	0.80	0.95	0.73	0.25	3.12	0.78	**0.04**	0.35	1.78	1.33	0.42	0.65
		Test for interaction			0.40	0.27			**0.02**	0.12			0.52	0.94
	C.albicans	All participants	0.73	1.22	0.26	0.17	0.30	1.02	0.30	0.38	1.15	0.87	0.60	0.34
		Male	0.83	2.06	0.42	0.27	0.30	1.02	0.73	0.62	3.43	0.89	0.12	0.17
		Female	0.60	0.88	0.42	0.31	0.23	1.51	0.12	0.28	0.90	0.81	0.91	0.44
		Test for interaction			0.45	0.48			0.09	0.14			0.08	0.10

			6 weeks (5d post-EPI1 +/- BCG) : 6 weeks (pre- EPI1)				10 weeks (pre EPI2) : 6 weeks (pre-EPI1)				10 weeks (pre EPI2) 6 weeks (5d post EPI1+/-BCG)			
			BCG at birth	**BCG at 6wks**	**p-value**	**Adj p-value**	**BCG at birth**	**BCG at 6wks**	**p-value**	**Adj p-value**	**BCG at birth**	**BCG at 6wks**	**p-value**	**Adj p-value**
IL-1β	**Medium**	All participants	1	1	0.35	0.29	3.99	1	0.12	0.61	4.99	1	**0.01**	**0.02**
		Male	1.34	1	0.51	**0.05**	5.91	1.34	0.56	0.57	3.29	1	0.10	0.20
		Female	0.52	5.54	0.09	0.19	3.06	7.14	0.14	0.54	9.2	2.21	0.10	**0.05**
		Test for interaction			0.14	0.07			0.44	0.51			0.63	0.49
	PPD	All participants	1.25	0.51	0.18	0.40	1.56	1.72	1.0	0.64	1.32	1.10	0.90	0.85
		Male	1.87	0.67	0.15	0.15	1.71	0.23	0.49	0.20	0.86	1.19	0.55	0.44
		Female	0.35	0.38	0.87	0.93	0.73	1.94	0.57	0.34	6.25	1.10	0.48	1.0
		Test for interaction			0.38	0.14			0.12	0.91			0.94	0.40
	Poly I:C	All participants	1.27	0.76	0.52	0.82	1.19	1.46	0.96	0.51	1.75	2.15	0.57	0.57
		Male	1.59	0.76	**0.05**	0.26	1.29	1.32	0.71	0.20	2.10	1.77	0.74	0.91
		Female	0.68	1.26	0.23	0.28	1.01	1.59	0.46	0.32	1.40	2.60	0.81	0.48
		Test for interaction			0.07	0.11			0.35	0.12			0.81	0.57
	S.pneumoniae	All participants	0.99	0.73	0.27	0.19	1.18	1.04	0.33	0.18	0.87	1.06	0.84	0.26
		Male	0.99	0.46	0.08	0.20	1.38	0.78	0.27	0.12	0.87	1.07	0.74	0.09

		Female	1.13	1.00	1.0	0.85	0.86	1.18	0.81	0.79	1.06	0.81	0.81	0.74
		Test for interaction			0.27	0.24			0.47	0.36			0.21	0.11
	S.aureus	All participants	1.08	0.59	0.14	0.45	0.94	0.86	0.65	0.20	1.05	0.98	0.47	0.62
		Male	1.08	0.42	0.11	0.74	0.97	0.66	0.12	**0.03**	1.79	0.98	0.23	0.31
		Female	1.19	0.79	0.61	0.29	0.58	1.68	0.37	0.83	0.91	0.98	0.81	0.34
		Test for interaction			0.53	0.43			0.10	0.29			0.20	0.15
	E.coli	All participants	1.74	0.97	0.08	0.31	1.03	1.07	0.89	0.79	0.93	0.82	0.49	1.0
		Male	1.91	0.88	**0.04**	0.22	1.02	1.14	0.63	0.95	0.90	0.85	0.52	0.57
		Female	1.11	1.07	0.73	0.27	1.79	0.99	0.57	0.82	0.98	0.82	0.72	0.30
		Test for interaction			0.34	0.19			0.47	0.64			0.59	0.27
	C.albicans	All participants	0.86	0.54	0.91	0.50	3.36	0.91	0.27	0.39	2.65	2.53	0.75	0.16
		Male	0.86	0.71	0.87	0.71	1.93	1.11	0.71	0.84	2.65	2.62	0.91	0.53
		Female	0.83	0.52	0.73	0.69	3.63	0.71	0.17	0.30	4.73	2.38	0.81	0.22
		Test for interaction			0.99	0.71			0.96	0.84			0.20	0.22

2.5.4 IL-10

			5 days : Cord Blood				6 weeks (pre-EPI1) : Cord blood				6 weeks of age (5d post-EPI1 +/- BCG) : Cord blood				10 weeks (pre-EPI2) : Cord blood			
			BCG at birth	**BCG at 6wks**	**p-value**	**Adj p-value**	**BCG at birth**	**BCG at 6wks**	**p-value**	**Adj p-value**	**BCG at birth**	**BCG at 6wks**	**p-value**	**Adj p-value**	**BCG at birth**	**BCG at 6wks**	**p-value**	**Adj p-value**
IL10	**Medium**	All participants	1	1.05	0.16	0.22	1	1	0.69	0.71	1.29	1	0.21	0.87	1	1	0.53	0.16
		Male	1	1.56	**0.04**	0.13	1	1	0.70	0.61	1.63	1	0.55	0.60	1	1	0.58	0.22
		Female	1	1	0.82	0.58	1	1	0.76	0.99	1	1	0.42	0.30	2.07	1	0.29	0.51
		Test for interaction			0.13				0.84				0.40				0.56	
	PPD	All participants	1.78	2.66	0.19	**0.04**	1.66	0.85	**0.04**	0.64	0.82	0.95	0.31	0.15	1.15	1.26	0.31	0.57
		Male	1.78	3.67	0.29	**0.02**	1.73	0.49	**0.01**	0.09	0.78	0.97	0.07	0.21	1.18	1.20	0.40	0.39
		Female	2.09	2.22	0.66	0.74	1.23	1.27	0.70	0.61	1.24	0.93	0.76	0.59	1.03	1.32	0.50	0.11
		Test for interaction			0.16				0.38				0.33				0.31	
	Poly I:C	All participants	1.94	2.10	0.23	0.72	1.11	0.99	0.38	0.21	0.78	1.16	0.11	0.14	1.91	1.27	0.14	0.83
		Male	2.32	2.07	0.90	0.56	0.85	0.54	0.61	0.35	0.73	1.04	0.30	0.71	2.22	1.30	0.06	0.96
		Female	1.67	2.36	0.12	0.23	2.12	1.28	0.15	0.30	0.95	1.33	0.38	0.11	1.61	1.23	0.75	0.75
		Test for interaction			0.16				0.65				0.34				0.89	

	S.pneumoniae	All participants	0.56	0.66	0.36	0.55	0.54	0.52	0.92	0.43	0.45	0.49	0.87	0.68	0.62	0.58	0.50	0.68
		Male	0.56	0.72	0.34	0.61	0.54	0.34	0.37	0.59	0.37	0.37	0.52	0.42	0.62	0.66	0.66	0.87
		Female	0.59	0.56	0.87	0.85	0.54	0.74	0.42	0.32	0.64	0.56	0.44	**0.05**	0.66	0.39	0.14	0.09
		Test for interaction			0.86				0.71				0.09				0.17	
	S.aureus	All participants	1.44	1.33	0.51	0.14	1.42	1.88	0.50	0.25	0.99	1.10	0.81	0.52	2.22	1.57	0.61	0.11
		Male	1.44	1.25	0.56	0.15	1.65	1.89	0.85	0.09	0.86	0.84	0.98	0.43	2.68	1.88	0.43	0.18
		Female	1.51	1.46	0.63	0.71	1.36	1.0	0.40	0.83	2.95	1.43	0.42	0.85	1.69	1.40	0.96	0.28
		Test for interaction			0.30				0.27				0.47				0.78	
	E.coli	All participants	1.30	1.38	0.67	0.42	0.93	1.02	0.51	0.62	1.02	1.20	0.23	0.70	1.30	1.04	0.23	0.52
		Male	1.39	1.39	0.90	0.98	0.87	1.02	0.51	0.61	1.01	1.07	0.32	0.68	1.42	1.07	0.45	0.18
		Female	1.24	1.38	0.72	0.25	1.03	1.01	0.98	0.72	1.19	1.29	0.53	0.76	1.23	0.98	0.29	0.73
		Test for interaction			0.37				0.99				0.70				0.61	
	C.albicans	All participants	0.23	0.21	0.95	0.93	0.32	0.16	0.09	0.32	0.18	0.33	0.42	0.98	1	0.34	0.08	0.12
		Male	0.13	0.23	0.39	0.34	0.32	0.13	0.09	0.58	0.15	0.29	0.07	0.17	0.86	0.64	0.75	0.88
		Female	0.40	0.21	0.43	0.63	0.64	0.22	0.39	**0.04**	0.88	0.34	0.26	0.13	1.05	0.27	**0.04**	0.11
		Test for			0.27				0.10				**0.02**				0.09	

			6 weeks (pre-EPI1) : 5 days				6 weeks of age (5d post-EPI1 +/-BCG) : 5 days				10 weeks (pre-EPI2) : 5 days			
			BCG at birth	BCG at 6wks	p-value	Adj p-value	BCG at birth	BCG at 6wks	p-value	Adj p-value	BCG at birth	BCG at 6wks	p-value	Adj p-value
IL10	Medium	All participants	1	1	0.89	0.87	1.57	0.98	0.13	0.38	0.84	1	0.20	0.16
		Male	1	1	0.93	0.92	2.42	0.14	0.08	0.70	0.66	0.66	0.57	0.57
		Female	0.71	1	0.95	0.89	0.71	1	0.70	0.89	0.84	1	0.10	0.07
		Test for interaction			0.94				0.45				0.07	
	PPD	All participants	0.51	0.39	0.65	0.30	0.76	0.47	0.26	0.22	0.66	0.55	0.96	0.98
		Male	0.51	0.44	0.93	0.31	0.83	0.38	0.17	0.26	0.60	0.54	0.89	0.90
		Female	0.45	0.30	0.82	0.96	0.76	0.52	0.91	0.13	0.66	0.55	0.73	0.84
		Test for interaction			0.37				0.39				1.0	
	Poly I:C	All participants	0.49	0.29	0.15	0.57	0.49	0.59	0.91	0.38	0.95	0.53	**0.05**	0.35
		Male	0.49	0.20	0.12	0.48	0.44	0.34	0.70	0.65	1.15	0.46	0.09	**0.02**
		Female	0.49	0.49	0.64	0.66	0.69	0.71	0.91	0.15	0.95	0.54	0.30	0.18
		Test for interaction			0.49				0.21				0.33	

	S.pneumoniae	All participants	0.66	0.50	0.76	0.36	0.89	0.64	0.80	0.35	0.95	0.69	0.17	0.22
		Male	0.64	0.39	0.29	0.91	0.57	0.45	0.77	1.0	1.28	0.78	0.32	**0.04**
		Female	0.72	1.32	0.42	0.21	1.17	0.75	0.33	0.13	0.95	0.68	0.42	0.38
		Test for interaction			0.38				0.17				0.45	
	S.aureus	All participants	0.45	0.61	0.58	0.72	1.08	1.04	0.82	0.86	0.95	1.53	0.40	0.54
		Male	0.36	0.68	0.85	0.55	1.01	0.28	0.28	0.66	0.86	1.49	0.40	0.75
		Female	0.66	0.54	0.57	0.16	1.89	1.78	0.64	0.57	1.09	1.53	0.56	0.46
		Test for interaction			0.40				0.44				0.49	
	E.coli	All participants	0.67	0.71	0.76	0.26	0.90	0.77	0.80	0.29	0.65	0.59	0.11	**0.03**
		Male	0.61	0.55	1.0	0.37	0.71	0.82	0.33	0.39	0.68	0.60	0.26	0.08
		Female	0.68	0.82	0.42	0.93	1.38	0.73	0.16	0.33	0.65	0.59	0.20	0.67
		Test for interaction			0.79				0.17				0.63	
	C.albicans	All participants	1	0.77	0.39	0.81	0.79	1.18	0.64	0.59	1.50	1.82	0.91	0.27
		Male	0.92	0.20	0.37	0.69	0.79	1.18	0.92	0.61	9.09	2.84	0.78	0.93
		Female	1.02	1	0.60	0.45	0.82	1.15	0.52	0.26	1.03	1.68	0.69	0.41
		Test for			0.32				0.42				0.79	

			6 weeks (5d post-EPI1 +/-BCG) : 6 weeks (pre- EPI1)				10 weeks (pre EPI2) : 6 weeks (pre-EPI1)				10 weeks (pre-EPI2): 6 weeks (5d post EPI1+/-BCG)			
			BCG at birth	BCG at 6wks	p-value	Adj p-value	BCG at birth	BCG at 6wks	p-value	Adj p-value	BCG at birth	BCG at 6wks	p-value	Adj p-value
IL10	Medium	All participants	1	1	0.33	0.19	2.02	1	0.18	0.44	0.89	1.02	0.87	0.14
		Male	1	1	1.0	0.48	2.92	1	0.36	0.39	0.46	1.24	0.19	**0.04**
		Female	1	0.99	0.20	0.15	1.16	0.66	0.57	0.78	3.06	1	0.35	0.06
		Test for interaction			0.84				0.40				0.06	
	PPD	All participants	0.44	1.11	0.38	0.95	0.90	0.88	0.96	0.25	1.22	1.23	0.31	0.44
		Male	0.56	2.00	0.26	0.70	0.96	0.56	0.43	0.28	0.93	1.56	0.74	0.32
		Female	0.37	0.65	0.73	0.34	0.60	1.39	0.57	0.51	2.99	0.89	0.16	0.06
		Test for interaction			0.76				0.27				0.08	
	Poly I:C	All participants	0.53	0.91	1.0	0.39	1.51	1.26	0.52	0.62	0.98	0.94	0.84	0.06
		Male	2.90	0.91	0.20	0.58	1.70	1.16	0.12	0.45	0.71	1.39	0.23	0.83
		Female	0.26	0.84	0.23	0.21	0.73	1.45	0.46	0.80	1.76	0.90	0.35	**0.04**
		Test for interaction			0.19				0.51				0.12	
	S.pneumoniae	All	1.36	0.90	0.41	0.09	1.21	1.03	0.41	0.09	1.17	0.94	0.54	0.54

	participants												
	Male	2.53	0.98	0.42	0.17	1.30	0.91	0.56	0.10	1.04	1.47	0.23	0.73
	Female	0.72	0.84	0.87	0.15	1.01	1.06	0.81	0.87	1.29	0.56	**0.01**	**0.003**
	Test for interaction			0.40				0.31				**0.04**	
S.aureus	All participants	1.29	0.67	0.08	0.14	2.86	1.15	0.20	0.65	0.65	0.79	0.84	0.70
	Male	1.95	0.88	0.15	0.25	3.53	0.93	0.27	0.83	0.71	0.89	0.32	0.46
	Female	1.08	0.49	0.40	0.24	0.76	1.37	0.81	0.25	0.65	0.79	0.64	0.28
	Test for interaction			0.34				0.73				0.14	
E.coli	All participants	1.09	0.93	0.27	0.53	1.35	1.06	0.36	0.74	0.92	1.09	0.97	0.38
	Male	1.09	0.85	0.11	0.84	1.58	1.33	0.31	0.45	0.78	1.24	0.16	0.50
	Female	1.14	1.02	0.87	0.71	0.64	1.06	0.57	0.44	1.11	0.67	0.24	**0.03**
	Test for interaction			0.34				0.67				0.10	
C.albicans	All participants	1.17	2.51	0.95	0.33	2.89	0.95	0.36	0.72	2.61	0.86	0.42	0.28
	Male	6.07	2.51	1.0	0.11	2.97	1.28	0.96	0.39	1.42	1.94	0.66	0.74
	Female	1.10	2.53	1.0	0.65	2.81	0.64	0.22	0.72	10.32	0.80	0.10	**0.03**
	Test for interaction			0.31				0.39				0.10	

2.5.5 IFNγ

			5 days : Cord Blood				6 weeks (pre-EPI1) : Cord blood				6 weeks of age (5d post-EPI1 +/- BCG) : Cord blood				10 weeks (pre-EPI2) : Cord blood			
			BCG at birth	**BCG at 6wks**	**p-value**	**Adj p-value**	**BCG at birth**	**BCG at 6wks**	**p-value**	**Adj p-value**	**BCG at birth**	**BCG at 6wks**	**p-value**	**Adj p-value**	**BCG at birth**	**BCG at 6wks**	**p-value**	**Adj p-value**
IFNγ	**Medium**	All participants	1	1	0.26	0.37	0.91	1	0.86	0.33	1	1	0.79	0.75	1.21	1	0.28	0.76
		Male	1	1	0.65	0.86	1	1	0.70	0.70	1	1	0.73	0.73	1.93	1	0.30	0.34
		Female	1	0.73	0.28	0.23	0.70	1	0.38	0.39	1	1.13	0.95	0.49	1	1	0.66	0.39
		Test for interaction			0.62				0.34				0.77				0.28	
	PPD	All participants	1.45	1.45	0.55	0.28	157.11	1.36	**<0.0001**	**<0.0001**	80.57	3.79	**<0.0001**	**0.001**	157.08	50.20	**0.01**	**0.02**
		Male	1.38	1.35	0.67	0.66	403.31	1	**<0.0001**	**0.003**	105.21	3.77	**<0.0001**	**0.005**	221.26	19.16	**0.004**	**0.05**
		Female	1.45	1.50	0.68	0.30	55.81	1.45	**0.0001**	**0.008**	74.21	4.30	**0.0007**	0.14	118.44	75.14	0.61	0.09
		Test for interaction			0.37				**0.01**				0.10				0.68	
	Poly I:C	All participants	6.92	11.89	0.35	0.82	11.04	11.82	1.0	0.59	5.41	7.31	0.84	0.36	37.20	31.44	0.57	0.36
		Male	8.43	13.31	0.86	0.65	27.18	15.15	0.43	0.37	13.71	5.85	0.86	0.52	71.10	31.84	0.17	0.27
		Female	3.67	11.44	0.12	0.49	3.80	9.64	0.19	0.75	3.00	8.77	0.52	0.56	21.25	31.04	0.48	0.86
		Test for			0.31				0.54				0.77				0.35	

		interaction															
	S.pneumoniae	All participants	15.94	22.38	0.80	0.39	15.67	10.99	0.43	0.52	16.51	6.42	0.08	0.24	19.25	8.73	0.14	0.06
		Male	23.21	17.55	0.33	0.77	23.44	7.35	0.07	0.50	20.27	6.25	0.17	0.30	26.56	7.96	0.06	0.06
		Female	12.45	28.20	0.19	0.43	7.35	15.16	0.36	0.81	15.38	7.04	0.26	0.44	15.57	9.81	0.98	0.43
		Test for interaction			0.45				0.81				0.39				0.31	
	S.aureus	All participants	10.53	9.97	0.33	0.61	27.20	10.49	0.18	0.53	10.47	8.43	0.59	0.29	14.10	8.99	0.28	0.36
		Male	11.14	8.29	0.20	0.51	32.04	9.58	**0.05**	0.31	9.67	11.44	0.95	0.24	28.70	10.04	0.21	0.48
		Female	9.26	18.33	0.94	0.99	15.81	14.82	0.82	0.60	32.76	8.35	0.52	0.72	10.56	7.60	0.73	0.73
		Test for interaction			0.67				0.17				0.22				0.45	
	E.coli	All participants	13.41	5.72	0.22	0.77	10.79	10.13	0.95	0.41	14.40	7.71	0.14	0.30	20.23	15.33	0.16	0.13
		Male	20.56	1.68	**0.02**	0.46	15.51	3.79	0.09	0.81	18.62	3.69	0.07	0.40	20.01	5.46	0.11	0.15
		Female	7.06	16.40	0.41	0.98	6.06	31.75	0.25	0.19	12.42	16.38	0.75	0.53	20.44	23.95	0.76	0.57
		Test for interaction			0.74				0.28				0.80				0.14	
	C.albicans	All participants	2.17	1.68	0.93	0.67	3.49	11.15	0.16	0.81	8.03	4.98	0.54	0.91	6.39	8.12	0.74	0.45
		Male	1	1.60	1.0	0.45	2.75	18.56	0.26	0.08	7.13	6.60	0.89	0.38	20.21	11.66	0.54	0.83
		Female	2.17	1.68	0.81	0.92	4.91	8.85	0.52	0.23	16.51	2.97	0.37	0.65	5.39	5.17	0.42	0.47

		Test for interaction			0.61				0.12				0.50			0.48
			6 weeks (pre-EPI1) : 5 days				6 weeks of age (5d post-EPI1 +/- BCG) : 5 days				10 weeks (pre- EPI2) : 5 days			
			BCG at birth	BCG at 6wks	p-value	Adj p-value	BCG at birth	BCG at 6wks	p-value	Adj p-value	BCG at birth	BCG at 6wks	p-value	Adj p-value
IFNγ	Medium	All participants	0.65	1	0.63	0.97	2.54	1.26	0.43	0.07	1	0.50	0.71	0.15
		Male	1	0.97	0.81	0.84	1.46	1.68	0.85	0.12	1	1.45	0.67	0.35
		Female	0.53	1	0.22	0.61	3.66	1	0.19	0.12	1.37	0.25	0.20	0.68
		Test for interaction			0.78				0.13				0.30	
	PPD	All participants	54.72	2.34	**0.001**	**0.05**	95.71	2.08	**0.0002**	**0.003**	52.81	31.41	0.30	0.88
		Male	79.49	0.48	**0.005**	0.12	115.14	2.11	**0.003**	**0.01**	85.67	40.55	0.64	0.49
		Female	31.38	3.16	0.13	**0.03**	69.79	2.04	**0.02**	0.17	9.38	27.97	0.48	0.80
		Test for interaction			0.17				0.36				0.70	
	Poly I:C	All participants	2.17	1.31	0.84	0.42	0.54	1.40	0.39	0.93	2.87	2.76	0.86	0.42
		Male	2.17	1.61	0.70	0.94	0.28	3.02	0.06	0.30	6.72	3.32	0.67	0.64
		Female	1.87	1.31	0.91	0.44	1.05	1.08	0.41	0.18	2.13	2.76	0.82	0.44
		Test for interaction			0.42				0.08				0.36	

	S.pneumoniae	All participants	1.12	0.79	0.80	0.50	0.63	0.62	0.91	0.96	0.80	1.61	0.33	0.56
		Male	1.12	0.58	0.63	0.79	0.40	0.75	0.38	0.47	1.0	1.86	0.16	0.96
		Female	1.34	1.92	0.64	0.50	1.60	0.60	0.29	0.16	0.80	0.30	0.91	0.41
		Test for interaction			0.73				0.07				0.36	
	S.aureus	All participants	3.15	9.70	0.32	0.22	0.94	1.67	0.91	0.34	0.93	3.14	**0.04**	0.53
		Male	3.15	1.98	0.92	0.30	0.76	2.12	0.10	0.64	2.18	2.43	0.48	0.88
		Female	2.57	15.14	0.20	0.54	7.28	0.66	0.13	0.43	0.61	9.10	0.16	0.36
		Test for interaction			0.57				0.55				0.26	
	E.coli	All participants	1.98	4.85	0.72	0.40	1.04	1.38	0.62	0.83	1.22	7.47	0.06	0.22
		Male	0.47	2.85	0.50	0.83	0.67	1.94	0.14	0.43	1.22	10.53	0.20	0.44
		Female	4.16	4.88	0.82	0.45	3.05	1.24	0.29	0.12	1.12	2.16	0.20	0.38
		Test for interaction			0.40				0.15				0.60	
	C.albicans	All participants	1.97	11.87	**0.002**	0.07	5.62	1.37	0.28	0.45	2.50	7.71	0.35	0.21
		Male	1	23.56	**0.002**	0.08	2.84	2.53	0.49	0.40	15.28	10.65	0.89	0.72
		Female	4.65	8.90	0.49	0.68	10.21	1	**0.01**	0.65	2.27	3.24	0.56	0.17
		Test for			0.16				0.47				0.26	

			6 weeks (5d post-EPI1 +/-BCG) : 6 weeks (pre- EPI1)				10 weeks (pre EPI2) : 6 weeks (pre-EPI1)				10 weeks (pre EPI2) 6 weeks (5d post EPI1+/-BCG)			
			BCG at birth	**BCG at 6wks**	**p-value**	**Adj p-value**	**BCG at birth**	**BCG at 6wks**	**p-value**	**Adj p-value**	**BCG at birth**	**BCG at 6wks**	**p-value**	**Adj p-value**
IFNγ	**Medium**	All participants	0.83	1	0.68	0.50	1.60	1	0.50	0.21	1.47	1	0.78	0.93
		Male	1.72	0.64	0.69	0.74	2.17	1.70	0.63	0.51	1.88	2.56	0.59	0.61
		Female	0.65	1.06	0.09	0.45	1.29	1	0.60	0.21	1	0.53	0.46	0.59
		Test for interaction			0.37				0.88				0.28	
	PPD	All participants	1.53	2.88	0.33	0.39	0.67	72.90	**0.0006**	**0.009**	0.67	25.04	**<0.0001**	0.19
		Male	1.43	3.64	0.34	0.30	0.63	70.67	**0.02**	0.11	0.67	63.64	**0.001**	0.27
		Female	2.89	2.60	0.87	0.59	0.81	75.14	**0.02**	**0.04**	0.67	17.67	**0.01**	0.1
		Test for interaction			0.29				0.45				0.13	
	Poly I:C	All participants	0.64	1.19	0.22	0.65	1.13	0.97	0.68	0.51	2.69	6.77	0.81	0.41
		Male	1.14	2.97	0.34	0.68	1.25	0.93	0.87	0.34	1.57	7.31	0.10	0.87
		Female	0.64	1.07	0.31	0.43	1.07	1	0.75	0.52	31.71	5.05	0.32	0.78
		Test for interaction			0.94				0.39				0.38	

	S.pneumoniae	All participants	1.71	2.17	0.64	0.97	0.99	1.21	0.38	0.60	1.62	1.32	0.81	0.88
		Male	2.69	1.57	1.0	0.36	0.66	1.19	0.92	0.47	1.19	1.07	1.0	0.55
		Female	1.71	2.69	0.50	0.22	1.12	1.23	0.15	0.42	3.40	1.80	0.74	0.57
		Test for interaction			0.20				0.16				0.94	
	S.aureus	All participants	0.90	1.87	0.73	0.42	1.16	1.67	0.50	0.13	4.18	2.46	0.20	0.08
		Male	1.13	2.75	0.75	0.39	0.53	2.98	0.22	0.08	2.56	0.99	0.66	0.16
		Female	0.29	1.33	0.40	0.76	2.85	1.63	0.25	0.19	5.88	2.80	0.26	0.55
		Test for interaction			0.37				0.07				0.42	
	E.coli	All participants	2.62	1.86	0.91	0.17	0.74	1.10	0.64	0.84	2.54	0.72	0.38	0.98
		Male	2.62	3.07	0.87	0.34	0.65	1.21	0.37	**0.03**	1.69	0.69	0.66	0.72
		Female	4.50	0.98	0.87	0.31	2.01	1	0.60	0.25	2.91	1.07	0.46	0.57
		Test for interaction			0.91				0.06				0.98	
	C.albicans	All participants	1.03	1.26	0.58	0.47	0.50	0.92	1.0	0.36	1.29	2.21	0.32	0.15
		Male	0.84	1.57	0.52	0.51	0.36	1	0.63	0.86	1.07	3.04	0.66	0.44
		Female	1.03	1.26	0.87	0.48	1.42	0.83	0.46	0.28	2.66	2.21	0.59	0.21
		Test for			0.61				0.23				0.50	

	interaction		

2.6 Within-infant fold change over time by BCG status, medium subtracted levels

2.6.1 TNFα

			5 days : Cord Blood				6 weeks (pre-EPI1) : Cord blood				6 weeks of age (5d post-EPI1 +/-BCG) : Cord blood				10 weeks (pre-EPI2) : Cord blood			
			BCG at birth	**BCG at 6wks**	**p-value**	**Adj p-value**	**BCG at birth**	**BCG at 6wks**	**p-value**	**Adj p-value**	**BCG at birth**	**BCG at 6wks**	**p-value**	**Adj p-value**	**BCG at birth**	**BCG at 6wks**	**p-value**	**Adj p-value**
TNFα	**PPD**	All participants	10.60	3.00	0.09	0.42	8.29	2.14	**0.006**	**0.03**	5.16	1	**0.0001**	0.17	6.26	4.87	0.82	0.36
		Male	14.59	5.64	0.28	0.58	10.30	2.33	0.06	0.16	4.56	1.20	**0.02**	0.16	7.52	6.41	1.0	0.69
		Female	2.78	0.79	0.12	0.49	7.99	1.62	**0.05**	0.11	5.60	0.95	**0.001**	0.25	4.76	4.87	0.66	0.28
		Test for interaction			0.90	0.92			0.89	0.97			0.23	0.36			0.56	0.75
	Poly I:C	All participants	4.77	5.51	0.61	0.29	3.62	3.37	0.57	0.85	3.59	2.70	0.43	0.28	4.72	3.86	1.0	0.76
		Male	5.81	4.21	0.24	0.94	4.47	2.87	0.47	0.45	3.81	2.58	0.40	0.44	5.50	5.08	0.95	0.59
		Female	5.58	3.35	**0.05**	0.19	3.31	3.65	0.92	0.77	2.61	2.84	0.98	0.24	4.69	3.31	0.89	0.57
		Test for interaction			0.34	0.22			0.33	0.49			0.53	0.73			0.45	0.47
	S.pneumoniae	All participants	1.59	1.86	0.65	0.60	1.77	1.72	0.64	0.17	1.68	1.33	0.23	0.74	2.12	2.29	0.76	0.69
		Male	1.56	2.02	0.43	0.24	2.12	1.34	0.28	0.63	2.47	1.21	0.39	0.35	2.37	2.64	0.65	0.72
		Female	1.59	1.74	0.87	0.69	1.34	3.03	0.06	0.22	1.54	1.44	0.51	0.95	1.31	1.75	0.85	0.75

		Test for interaction			0.49	0.35			0.97	0.39			0.62	0.69			0.95	0.90
	S.aureus	All participants	1.82	3.62	0.69	0.36	2.61	5.20	0.60	0.16	1.58	2.32	0.62	0.94	1.82	2.59	0.33	0.41
		Male	2.11	3.79	0.85	0.34	3.77	4.81	0.62	0.21	1.53	1.21	0.76	0.66	1.63	2.28	0.48	0.48
		Female	1.78	3.27	0.78	0.36	2.49	6.75	0.25	0.19	3.81	2.37	0.59	0.57	1.84	3.48	0.47	0.78
		Test for interaction			0.30	0.32			0.47	0.49			0.62	0.53			0.48	0.54
	E.coli	All participants	3.32	3.03	0.93	0.28	2.47	2.52	0.67	0.94	3.06	2.38	0.69	0.41	1.87	3.13	0.06	0.11
		Male	3.46	2.71	0.33	0.82	3.16	3.01	0.67	0.12	3.56	2.61	0.51	0.86	2.04	3.13	0.38	0.40
		Female	2.66	3.09	0.38	0.27	2.46	2.52	0.77	0.53	2.25	2.19	0.83	0.50	1.78	3.02	0.07	0.11
		Test for interaction			0.38	0.10			0.34	0.22			0.43	0.48			0.87	0.70
	C.albicans	All participants	0.49	0.71	0.26	0.32	1.08	0.57	0.18	**0.05**	0.58	1.04	0.81	0.75	0.76	1.15	0.90	0.25
		Male	0.08	0.95	**0.02**	0.60	1.09	0.63	0.39	0.34	0.32	0.63	0.33	0.54	2.10	1.29	0.57	0.48
		Female	1.16	0.41	0.53	0.27	1	0.52	0.37	0.06	1.23	1.17	0.54	0.85	0.21	0.88	0.46	0.36
		Test for interaction			0.67	0.86			0.43	0.39			0.76	0.57			0.73	0.77

			6 weeks (pre EPI1) : 5 days				6 weeks of age (5d post-EPI1 +/-BCG) : 5 days				10 weeks (pre EPI2) : 5 days			
			BCG at birth	**BCG at 6wks**	**p-value**	**Adj p-value**	**BCG at birth**	**BCG at 6wks**	**p-value**	**Adj p-value**	**BCG at birth**	**BCG at 6wks**	**p-value**	**Adj p-value**
TNFα	**PPD**	All participants	2.36	0.57	**0.004**	0.28	1.65	0.24	0.13	0.39	4.02	1.04	0.15	0.99
		Male	2.94	0.57	**0.02**	**0.03**	1.65	0.06	**0.003**	**0.03**	3.43	0.57	0.12	0.18
		Female	1.79	0.36	0.09	0.38	1.53	2.01	0.90	0.11	4.61	4.95	0.95	0.60
		Test for interaction			0.39	0.26			0.53	0.24			0.30	0.37
	Poly I:C	All participants	0.88	0.94	0.88	0.59	0.84	0.56	0.25	0.15	1.13	0.84	0.91	0.35
		Male	0.88	1.45	0.31	0.97	0.80	0.84	0.90	0.38	0.92	0.95	0.57	0.46
		Female	0.88	0.91	0.49	0.07	0.85	0.45	0.27	0.86	1.13	0.63	0.75	0.34
		Test for interaction			0.06	**0.02**			0.92	0.63			0.58	0.51
	S.pneumoniae	All participants	0.84	1.41	0.21	0.71	1.01	0.71	0.09	0.70	0.88	0.82	0.66	0.53
		Male	0.95	1.41	0.74	0.80	1.04	0.71	0.12	0.48	0.64	1.0	0.26	0.16
		Female	0.61	1.34	0.07	0.25	0.93	0.74	0.39	0.78	0.96	0.69	0.56	0.54
		Test for interaction			0.43	0.21			0.94	0.99			0.13	0.12
	S.aureus	All	1.20	1.54	0.13	0.29	1.25	0.67	0.07	0.24	1.24	0.98	0.59	0.56

		participants											
		Male	1.20	1.56	0.37	0.96	1.25	0.51	0.12	0.21	0.69	0.98	0.64	0.29
		Female	1.02	1.52	0.24	0.36	1.59	0.91	0.23	0.47	1.37	1.06	0.44	0.76
		Test for interaction			0.12	0.21			0.27	0.33			0.69	0.54
	E.coli	All participants	0.97	1.09	0.27	0.22	0.96	0.93	0.77	0.48	0.87	0.74	1.0	0.96
		Male	0.97	1.36	0.37	0.19	0.91	1.25	0.49	0.24	0.49	0.80	0.40	0.93
		Female	0.88	1.06	0.56	0.95	1.09	0.90	0.33	0.28	0.88	0.26	0.42	0.84
		Test for interaction			0.21	0.16			0.14	0.16			0.63	0.74
	C.albicans	All participants	0.56	1.31	0.45	0.58	0.43	0.42	0.53	0.12	1.39	1.17	0.19	0.13
		Male	0.90	1.95	0.29	0.91	0.33	0.31	0.67	0.42	3.41	1.09	0.23	0.37
		Female	0.54	0.74	0.95	0.26	0.74	0.96	0.51	**0.05**	1.19	1.20	0.67	0.27
		Test for interaction			0.62	0.62			0.50	0.72			0.42	0.42

			6 weeks (post-EPI1 +/-BCG) : 6 weeks (pre- EPI1)				10 weeks (pre EPI2) : 6 weeks (pre-EPI1)				10 weeks (pre EPI2) 6 weeks (post EPI1+/-BCG)			
			BCG at birth	**BCG at 6wks**	**p-value**	**Adj p-value**	**BCG at birth**	**BCG at 6wks**	**p-value**	**Adj p-value**	**BCG at birth**	**BCG at 6wks**	**p-value**	**Adj p-value**
TNFα	**PPD**	All participants	1.00	0.92	0.36	0.95	0.63	1.51	0.37	0.56	0.42	1.26	**0.03**	0.58
		Male	1.47	0.64	0.67	0.81	0.63	2.04	0.48	0.45	0.24	2.98	0.08	0.79
		Female	0.73	0.94	0.85	0.98	0.47	1.44	0.45	0.97	0.62	0.98	0.26	0.39
		Test for interaction			0.41	0.38			0.37	0.42			0.50	0.58
	Poly I:C	All participants	0.99	1.30	0.95	0.10	0.94	1.18	0.75	0.42	1.52	1.69	0.97	0.08
		Male	2.47	0.67	0.06	0.38	0.39	0.56	0.49	1.0	1.33	1.51	0.81	0.55
		Female	0.54	1.59	0.11	0.51	2.28	1.26	0.37	0.56	1.80	2.00	0.83	0.21
		Test for interaction			0.12	0.51			0.20	0.50			0.29	0.65
	S.pneumoniae	All participants	0.98	0.81	0.27	0.21	1.05	1.16	0.43	0.90	0.93	1.37	0.33	0.40
		Male	1.30	0.60	0.15	0.35	0.93	1.12	0.49	0.94	0.93	1.41	0.23	0.32
		Female	0.86	0.91	0.73	0.96	1.17	1.21	0.94	0.75	1.21	1.37	0.81	0.85
		Test for interaction			0.19	0.48			0.86	0.82			0.70	0.75
	S.aureus	All	1.42	0.37	0.38	0.83	0.81	0.95	0.84	0.53	1.12	1.19	0.42	0.32

		participants											
		Male	1.45	0.32	0.27	0.29	0.63	1.01	0.64	0.92	1.21	1.45	0.28	0.39
		Female	0.46	0.43	1.0	0.71	2.25	0.95	0.47	0.16	0.89	1.19	1.0	0.88
		Test for interaction			0.33	0.48			0.25	0.48			0.28	0.41
	E.coli	All participants	1.55	0.90	0.13	0.49	0.78	1.00	0.78	0.39	1.01	1.11	0.12	0.57
		Male	1.56	0.71	0.15	0.75	0.75	1.15	0.43	0.54	1.05	1.27	0.39	0.90
		Female	1.27	0.93	0.50	0.63	1.12	0.86	0.29	0.39	0.95	1.00	0.24	0.19
		Test for interaction			0.94	0.79			0.08	0.14			0.93	0.63
	C.albicans	All participants	1.08	1.06	0.84	0.34	1.33	0.67	0.33	0.73	0.42	1.39	0.31	0.41
		Male	8.16	15.53	0.83	0.40	1.33	1.16	0.87	0.91	0.39	1.77	0.19	0.55
		Female	0.13	0.97	0.49	0.61	10.95	0.35	0.27	0.24	0.56	1.16	0.67	0.33
		Test for interaction			0.70	0.69			0.21	0.50			0.11	0.21

2.6.2 IL-6

			5 days : Cord Blood				6 weeks (pre-EPI1) : Cord blood				6 weeks of age (5d post-EPI1 +/- BCG) : Cord blood				10 weeks (pre-EPI2) : Cord blood			
			BCG at birth	**BCG at 6wks**	**p-value**	**Adj p-value**	**BCG at birth**	**BCG at 6wks**	**p-value**	**Adj p-value**	**BCG at birth**	**BCG at 6wks**	**p-value**	**Adj p-value**	**BCG at birth**	**BCG at 6wks**	**p-value**	**Adj p-value**
IL6	**PPD**	All participants	1.86	2.02	0.73	0.98	2.75	0.54	**<0.0001**	**0.001**	2.43	0.71	**<0.0001**	**0.02**	1.07	1.64	0.58	0.28
		Male	2.35	1.94	0.95	0.68	2.80	0.51	**0.001**	**0.02**	2.75	0.70	**0.0002**	**0.01**	2.16	0.92	0.15	0.90
		Female	1.68	2.09	0.75	0.84	1.58	0.59	**0.006**	**0.01**	1.88	0.71	**0.04**	0.74	0.76	1.96	0.06	0.30
		Test for interaction			0.68	0.80			0.37	0.14			0.06	**0.05**			0.11	0.28
	Poly I:C	All participants	2.28	2.64	0.54	0.19	1.77	1.48	0.24	0.56	1.90	1.45	0.94	0.35	1.82	1.70	0.79	0.87
		Male	2.51	2.63	0.93	0.88	1.40	1.58	0.85	1.0	1.92	1.39	0.43	0.70	2.19	2.06	0.52	0.42
		Female	2.02	2.92	0.45	0.09	2.49	1.39	**0.05**	0.52	1.88	1.67	0.68	0.14	1.41	1.36	1.0	0.37
		Test for interaction			0.26	0.11			0.48	0.49			0.29	0.13			0.32	0.24
	S.pneumoniae	All participants	1.04	1.18	0.59	0.26	0.59	0.72	0.94	0.26	0.82	0.71	0.75	0.73	0.83	0.79	0.84	0.71
		Male	1.09	1.20	0.54	0.53	0.76	0.70	0.33	0.80	0.79	0.63	0.46	0.29	0.91	0.54	0.52	0.58
		Female	1.04	0.99	0.80	0.21	0.51	0.80	0.45	0.08	0.90	0.95	0.94	0.51	0.71	0.84	0.51	0.97
		Test for interaction			0.38	0.25			0.87	0.18			0.44	0.33			0.51	0.67

	S.aureus	All participants	1.87	2.50	0.19	0.35	2.37	2.43	0.66	0.30	2.86	1.42	0.11	0.48	1.97	1.96	0.54	0.29
		Male	1.48	2.46	0.06	0.19	3.38	1.28	0.18	0.97	1.46	1.16	0.22	0.08	3.19	1.68	0.52	0.67
		Female	2.31	2.93	1.0	0.38	2.25	2.64	0.43	0.11	3.14	2.89	0.43	0.37	0.67	2.14	0.09	0.31
		Test for interaction			0.34	0.36			0.28	0.15			0.08	0.06			0.74	0.47
	E.coli	All participants	1.57	1.52	0.90	0.24	1.16	0.90	0.24	0.56	1.47	1.28	0.43	0.62	0.99	1.26	0.16	0.65
		Male	1.48	1.46	0.78	0.76	1.17	0.81	0.32	0.60	1.31	1.04	0.29	0.08	1.09	1.35	**0.05**	**0.06**
		Female	1.70	2.04	0.49	0.14	1.10	0.99	0.52	0.70	1.52	1.36	0.72	0.52	0.89	1.16	0.73	0.55
		Test for interaction			0.16	0.16			0.52	0.98			0.13	0.13			0.52	0.24
	C.albicans	All participants	0.23	0.44	0.24	0.54	0.38	0.27	0.32	0.71	0.32	0.33	0.92	0.66	0.50	0.57	0.96	0.91
		Male	0.16	0.65	**0.04**	0.67	0.38	0.25	0.29	0.38	0.09	0.29	0.51	0.39	0.31	0.98	0.53	0.97
		Female	0.53	0.36	0.65	0.25	0.27	0.38	0.48	0.96	0.41	0.36	0.68	0.52	0.66	0.48	0.40	0.82
		Test for interaction			0.10	0.23			0.90	0.97			0.62	0.33			0.96	0.86

			6 weeks (pre-EPI1) : 5 days				6 weeks of age (5d post-EPI1 +/-BCG) : 5 days				10 weeks (pre-EPI2) : 5 days			
			BCG at birth	**BCG at 6wks**	**p-value**	**Adj p-value**	**BCG at birth**	**BCG at 6wks**	**p-value**	**Adj p-value**	**BCG at birth**	**BCG at 6wks**	**p-value**	**Adj p-value**
	PPD	All participants	0.70	0.32	0.34	0.11	1.02	0.44	**0.04**	**0.02**	1.36	0.83	**0.05**	0.07
		Male	0.88	0.29	0.34	0.12	0.94	0.51	0.20	0.14	1.60	0.66	0.06	0.13
		Female	0.70	0.34	0.65	0.16	1.59	0.28	0.18	0.06	1.36	0.97	0.39	0.26
		Test for interaction			0.81	0.90			0.83	0.39			0.91	0.76
	Poly I:C	All participants	0.76	0.52	0.55	0.21	0.58	0.65	0.98	0.68	0.58	0.84	0.88	0.46
		Male	0.67	0.59	0.85	0.33	0.63	0.99	0.25	0.26	1.16	0.84	0.34	0.06
		Female	0.76	0.33	0.41	0.43	0.58	0.57	0.81	0.06	0.55	0.70	0.89	0.80
		Test for interaction			0.96	0.84			**0.05**	**0.03**			0.21	0.31
	S.pneumoniae	All participants	0.44	0.44	0.78	0.96	0.56	0.59	0.25	0.88	0.54	0.54	0.37	0.35
		Male	0.53	0.39	0.85	0.95	0.53	0.63	0.30	0.34	0.97	0.53	0.19	0.26
		Female	0.44	0.49	0.95	0.59	0.58	0.57	0.71	0.20	0.50	0.52	0.69	0.61
		Test for interaction			0.42	0.99			0.10	0.11			0.70	0.82
	S.aureus	All participants	0.48	0.80	0.58	0.36	1.69	0.50	0.08	0.42	0.88	0.96	0.84	0.84
		Male	0.48	0.53	0.82	0.33	2.00	0.41	**0.05**	0.15	0.90	0.89	0.57	0.25

		Female	0.48	0.82	0.48	0.18	1.49	0.69	0.44	0.89	0.83	1.06	0.67	0.90
		Test for interaction			0.34	0.28			0.27	0.50			0.72	0.81
	E.coli	All participants	0.72	0.54	0.66	0.54	0.84	0.64	0.98	0.87	0.73	0.65	0.98	0.28
		Male	0.64	0.56	1.0	0.58	0.83	1.15	0.35	0.70	0.71	1.08	0.57	0.41
		Female	0.72	0.53	0.41	0.62	0.86	0.64	0.54	0.28	0.73	0.48	0.25	0.23
		Test for interaction			0.60	0.98			0.11	0.33			0.71	0.70
	C.albicans	All participants	0.76 (14)	0.92 (16)	0.65	0.59	0.39 (13)	0.70 (17)	0.95	0.55	1.07 (11)	0.76 (12)	0.36	0.77
		Male	1.24 (7)	1.01 (9)	0.56	0.75	0.34 (8)	0.54 (7)	0.64	0.42	3.16 (4)	1.63 (6)	0.39	0.78
		Female	0.43 (7)	0.83 (7)	0.75	0.43	0.87 (5)	0.95 (10)	0.90	0.92	0.99 (7)	0.76 (6)	0.48	0.62
		Test for interaction			0.77	0.89			0.43	0.32			0.70	0.59
			6 weeks (5d post-EPI1 +/-BCG) : 6 weeks (pre-EPI1)				**10 weeks (pre-EPI2) : 6 weeks (pre-EPI1)**				**10 weeks (pre-EPI2) 6 weeks (5d post-EPI1+/-BCG)**			
			BCG at birth	**BCG at 6wks**	**p-value**	**Adj p-value**	**BCG at birth**	**BCG at 6wks**	**p-value**	**Adj p-value**	**BCG at birth**	**BCG at 6wks**	**p-value**	**Adj p-value**
IL6	**PPD**	All participants	1.25	1.62	0.33	0.80	0.82	1.11	0.43	0.93	0.81	2.11	**0.02**	0.35
		Male	1.33	1.02	0.72	0.42	0.70	0.76	0.39	1.0	0.86	2.51	0.24	0.66
		Female	0.62	2.37	0.09	0.83	0.82	1.46	0.58	0.81	0.58	1.95	**0.04**	0.43
		Test for interaction			0.33	0.48			0.91	0.68			0.98	0.75

	Poly I:C	All participants	0.90	1.40	0.27	0.51	1.22	0.81	0.97	0.65	1.26	1.91	0.28	0.79
		Male	1.60	0.82	0.20	0.46	0.83	0.56	0.83	0.93	1.66	2.06	0.31	0.35
		Female	0.52	2.05	**0.01**	0.16	1.35	1.66	0.81	0.65	1.02	1.78	0.87	0.22
		Test for interaction			**0.01**	0.11			0.62	0.60			0.39	0.10
		All participants	1.10	0.96	0.33	0.51	0.94	1.17	0.92	0.84	0.88	0.80	0.72	0.85
		Male	1.22	0.75	0.27	0.90	0.86	1.09	1.0	0.19	0.96	1.15	0.40	0.57
		Female	1.09	0.96	0.71	0.68	1.29	1.17	0.57	0.55	0.87	0.48	0.09	0.11
		Test for interaction			0.74	0.67			0.16	0.18			0.09	0.16
	S.aureus	All participants	1.17	0.50	0.08	0.20	0.70	1.30	0.32	0.24	0.31	0.85	0.24	0.38
		Male	1.17	0.38	0.10	0.07	1.02	0.72	1.0	0.94	0.32	1.37	0.14	0.57
		Female	1.08	0.71	0.57	0.98	0.47	1.53	0.26	0.29	0.30	0.36	1.0	0.70
		Test for interaction			0.38	0.20			0.38	0.32			0.34	0.41
	E.coli	All participants	1.01	1.31	0.82	0.15	1.01	1.25	0.92	0.52	0.69	1.16	0.09	0.22
		Male	1.01	0.99	0.72	0.51	0.82	1.34	0.67	0.18	0.63	1.32	0.18	0.27
		Female	1.16	1.38	0.71	0.12	1.15	1.15	0.57	0.63	0.81	0.98	0.50	0.69
		Test for interaction			0.46	0.23			0.20	0.20			0.36	0.51
	C.albicans	All participants	1.51	1.77	0.39	0.87	2.43	0.66	**0.02**	0.62	0.91	1.22	0.81	0.72

	Male	1.94	2.43	0.75	0.15	1.81	0.76	0.09	0.34	1.10	1.34	0.87	0.53
	Female	0.26	0.94	0.34	0.49	3.32	0.57	0.12	0.69	0.79	1.08	0.87	0.76
	Test for interaction			0.31	0.18			0.50	0.48			0.43	0.51

2.6.3 IL-1β

			5 days : Cord Blood				6 weeks (pre EPI1) : Cord blood				6 weeks of age (5d post-EPI1 +/- BCG) : Cord blood				10 weeks (pre EPI2) : Cord blood			
			BCG at birth	**BCG at 6wks**	**p-value**	**Adj p-value**	**BCG at birth**	**BCG at 6wks**	**p-value**	**Adj p-value**	**BCG at birth**	**BCG at 6wks**	**p-value**	**Adj p-value**	**BCG at birth**	**BCG at 6wks**	**p-value**	**Adj p-value**
IL-1β	**PPD**	All participants	1.37	1	0.63	0.62	1.33	1.31	0.23	0.35	1.72	0.86	0.33	0.29	0.77	1.72	0.85	0.60
		Male	1.44	2.33	0.88	0.23	1.26	2.69	0.46	0.63	2.23	1	0.60	0.52	0.76	0.92	0.91	0.58
		Female	1.35	0.78	0.40	0.92	1.36	1.12	0.37	0.43	1.36	0.72	0.47	0.36	1.29	2.70	0.74	0.83
		Test for interaction			0.81	0.75			0.73	0.77			0.44	0.77			0.39	0.54
	Poly I:C	All participants	5.49	7.01	0.93	0.10	3.89	3.13	0.98	0.69	2.96	5.21	0.58	0.94	6.60	4.38	0.24	0.67
		Male	5.43	6.67	0.81	0.74	3.01	4.14	0.64	0.75	6.02	5.04)	0.79	0.23	9.70	4.60	0.28	0.53
		Female	5.77	7.02	0.95	0.08	3.96	2.75	0.67	0.62	2.39	5.98	0.28	0.25	5.16	3.55	0.49	0.78
		Test for interaction			0.29	0.27			0.30	0.54			0.23	0.08			0.77	0.47
	S.pneumoniae	All participants	0.83	0.84	0.93	0.97	0.82	1.11	0.48	0.66	1.08	0.77	0.80	0.74	1.07	0.95	0.41	0.14
		Male	0.85	0.83	0.63	0.99	1.05	0.70	0.58	0.79	0.98	0.67	0.87	0.72	1.11	0.83	0.38	0.83
		Female	0.77	0.86	0.47	0.95	0.74	1.35	0.13	0.20	1.63	1.28	0.46	0.49	0.99	1.04	0.81	0.06
		Test for interaction			1.0	0.95			0.92	0.37			0.74	0.43			0.38	0.17

	S.aureus	All participants	2.14	1.92	0.80	0.41	1.95	2.60	0.34	0.55	1.92	1.50	0.30	0.46	1.92	1.50	0.42	0.24
		Male	2.22	1.80	0.72	0.89	1.66	2.90	0.13	0.64	1.53	1.50	0.76	0.33	1.62	1.21	0.22	0.32
		Female	2.12	2.14	0.98	0.29	2.01	2.47	0.83	0.88	3.53	1.51	0.29	0.63	1.43	1.62	0.94	0.31
		Test for interaction			0.81	0.36			0.79	0.70			0.52	0.28			0.49	0.46
	E.coli	All participants	2.16	2.65	0.63	0.56	1.94	2.08	0.50	0.41	4.06	2.41	0.17	0.32	2.63	2.01	0.11	0.49
		Male	1.83	2.24	0.53	0.57	2.29	1.83	0.90	0.23	3.43	1.97	0.09	0.18	2.54	1.92	0.10	0.81
		Female	2.52	3.20	0.22	0.30	1.71	2.78	0.37	0.75	4.47	2.75	0.67	0.78	2.67	2.61	0.47	0.50
		Test for interaction			0.30	0.17			0.31	0.24			0.37	0.21			0.70	0.56
	C.albicans	All participants	0.49	0.29	0.73	0.34	0.32	0.37	0.80	0.33	0.22	0.30	0.90	0.28	0.73	0.62	0.95	0.18
		Male	0.27	0.30	0.61	0.69	0.31	0.23	0.60	0.99	0.10	0.21	0.45	0.70	0.58	0.65	0.85	0.81
		Female	0.83	0.28	0.29	0.26	0.38	0.82	0.43	0.36	0.74	0.55	0.52	0.30	0.77	0.62	0.98	0.06
		Test for interaction			0.21	0.28			0.31	0.33			0.21	0.32			0.50	0.36

			6 weeks (pre EPI1) : 5 days				6 weeks of age (5d post-EPI1 +/-BCG) : 5 days				10 weeks (pre EPI2) : 5 days			
			BCG at birth	**BCG at 6wks**	**p-value**	**Adj p-value**	**BCG at birth**	**BCG at 6wks**	**p-value**	**Adj p-value**	**BCG at birth**	**BCG at 6wks**	**p-value**	**Adj p-value**
IL-1β	**PPD**	All participants	0.41	0.49	0.98	0.27	0.45	0.76	0.66	0.76	0.58	2.39	0.11	0.40
		Male	1.33	0.32	0.29	0.44	0.71	0.07	0.87	0.57	0.55	0.97	0.73	0.33
		Female	0.33	1	0.30	0.15	0.08	0.96	0.85	0.12	0.60	3.82	**0.04**	0.36
		Test for interaction			0.57	0.66			0.30	0.14			0.35	0.40
	Poly I:C	All participants	0.44	0.46	0.97	0.34	0.61	0.64	0.56	0.06	0.82	1.37	0.51	0.26
		Male	0.45	0.47	0.79	0.79	0.61	0.86	0.63	0.06	1.40	1.40	0.57	0.51
		Female	0.43	0.46	0.73	0.34	0.58	0.57	0.52	0.49	0.57	1.37	0.35	0.33
		Test for interaction			0.35	0.42			0.56	0.38			0.17	0.57
	S.pneumoniae	All participants	0.87	1.67	0.26	0.52	0.97	1.33	0.72	0.76	1.30	1.11	0.54	0.14
		Male	0.85	1.10	1.0	0.90	0.65	1.33	0.92	0.53	1.57	1.12	0.26	0.78
		Female	0.87	1.70	0.11	0.43	1.93	1.29	0.45	0.09	0.99	0.94	0.91	0.25
		Test for interaction			0.71	0.50			0.20	0.15			0.39	0.57
	S.aureus	All participants	0.74	1.09	0.31	0.12	1.31	0.88	0.28	0.65	0.99	1.41	0.89	0.44
		Male	0.79	0.64	0.66	0.55	1.21	1.34	0.77	0.83	0.98	1.35	0.89	0.20
		Female	1.66	0.69	0.08	0.17	1.55	0.48	0.83	0.69	0.99	1.54	0.73	0.87

		Test for interaction			0.51	0.42			0.84	0.77			0.73	0.46
	E.coli	All participants	0.82	0.95	0.97	0.25	1.17	0.86	0.23	0.54	1.76	1.19	0.54	0.47
		Male	0.80	0.91	0.93	0.85	1.08	1.00	0.70	0.33	1.39	1.12	1.0	0.49
		Female	0.82	0.95	0.82	0.24	4.21	0.79	**0.04**	0.28	1.78	1.34	0.42	0.64
		Test for interaction			0.41	0.25			**0.008**	0.11			0.51	0.98
	C.albicans	All participants	0.52	0.99	0.27	0.07	0.07	1.23	0.48	0.65	1.46	0.85	0.62	0.71
		Male	0.51	0.38	0.87	0.25	0.34	0.73	0.72	0.54	2.46	0.88	0.31	0.81
		Female	0.75	1.19	0.19	0.31	0.06	1.52	0.12	0.58	0.71	0.16	0.70	0.76
		Test for interaction			0.98	1.0			0.11	0.18			0.79	0.72
			6 weeks (5d post-EPI1 +/-BCG) : 6 weeks (pre- EPI1)				**10 weeks (pre EPI2) : 6 weeks (pre-EPI1)**				**10 weeks (pre EPI2) : 6 weeks (5d post EPI1+/-BCG)**			
			BCG at birth	**BCG at 6wks**	**p-value**	**Adj p-value**	**BCG at birth**	**BCG at 6wks**	**p-value**	**Adj p-value**	**BCG at birth**	**BCG at 6wks**	**p-value**	**Adj p-value**
IL-1β	**PPD**	All participants	0.65	0.09	0.15	0.53	0.68	0.45	0.93	0.25	0.52	0.75	0.98	0.29
		Male	1.19	0.66	0.27	0.15	1.05	0.04	**0.05**	0.11	0.32	1.10	0.12	0.36
		Female	0.31	0.00	0.28	0.59	0.58	9.95	0.29	0.42	5.41	0.39	0.23	0.06
		Test for interaction			0.18	0.18			0.29	0.30			**0.04**	**0.03**
	Poly I:C	All participants	1.32	0.76	0.52	0.91	1.10	1.45	0.85	0.55	1.68	2.16	0.44	0.44

		Male	1.58	0.76	**0.05**	0.26	1.10	1.32	0.96	0.23	1.26	2.61	0.66	0.84
		Female	0.67	1.22	0.23	0.31	1.01	1.60	0.46	0.20	1.76	2.10	0.81	0.36
		Test for interaction			0.06	0.12			0.28	0.11			0.86	0.51
	S.pneumoniae	All participants	1.04	0.75	0.32	0.17	1.15	1.02	0.41	0.20	0.65	1.04	0.75	0.71
		Male	1.03	0.46	0.08	0.20	1.35	0.79	0.22	0.12	0.65	1.06	0.33	0.64
		Female	1.15	1.05	0.87	0.72	0.85	1.16	0.68	0.84	1.01	0.90	0.81	0.52
		Test for interaction			0.28	0.25			0.39	0.31			0.81	0.81
	S.aureus	All participants	1.11	0.54	0.16	0.38	0.74	0.66	0.52	0.18	1.0	0.98	0.90	0.70
		Male	1.11 (6)	0.42 (6)	0.11	0.64	0.78 (9)	0.37 (7)	0.06	0.07	1.78 (6)	0.98	0.33	0.33
		Female	1.21 (4)	0.73 (8)	0.61	0.23	0.49 (5)	1.64 (7)	0.29	0.99	0.69 (6)	0.98	0.72	0.25
		Test for interaction			0.33	0.22			0.08	0.40			0.20	0.14
	E.coli	All participants	1.75	1.02	0.08	0.49	1.02	1.07	0.82	0.82	0.93 (12)	0.82	0.63	0.95
		Male	1.94	0.89	**0.04**	0.23	1.02	1.14	0.71	0.95	0.90 (6)	0.85	0.59	0.60
		Female	1.11	1.11	0.73	0.25	1.75	0.99	0.57	0.85	0.95 (6)	0.82	0.91	0.27
		Test for interaction			0.15	0.09			0.51	0.66			0.58	0.27
	C.albicans	All participants	0.64	0.40	0.48	0.54	1.79	0.71	0.61	0.66	0.65	2.46	0.32	0.95
		Male	0.63	0.51	0.92	0.56	1.23	0.70	0.70	0.70	0.92	2.83	0.22	0.42

	Female	0.85	0.18	0.49	0.42	3.78	0.71	0.57	0.79	0.39	2.22	0.68	0.75
	Test for interaction			0.31	0.46			0.93	0.81			0.38	0.49

2.6.4 IL-10

			5 days : Cord Blood				6 weeks (pre-EPI1) : Cord blood				6 weeks of age (5d post-EPI1 +/- BCG) : Cord blood				10 weeks (pre-EPI2) : Cord blood			
			BCG at birth	**BCG at 6wks**	**p-value**	**Adj p-value**	**BCG at birth**	**BCG at 6wks**	**p-value**	**Adj p-value**	**BCG at birth**	**BCG at 6wks**	**p-value**	**Adj p-value**	**BCG at birth**	**BCG at 6wks**	**p-value**	**Adj p-value**
IL-10	**PPD**	All participants	1.86	2.35	0.35	0.08	1.60	0.89	0.07	0.91	0.80	1.00	0.28	0.52	1.12	1.31	0.46	0.46
		Male	1.86	3.16	0.50	0.13	1.75	0.43	**0.03**	0.12	0.73	1.08	**0.05**	0.33	1.14	1.18	0.34	0.46
		Female	2.17	2.17	0.64	0.40	1.24	1.32	0.84	0.76	1.01	0.96	0.66	0.43	0.86	1.44	0.97	0.41
		Test for interaction			0.65	0.53			0.96	0.92			0.18	0.23			0.47	0.43
	Poly I:C	All participants	1.95	2.21	0.30	0.78	1.11	0.96	0.32	0.42	0.80	1.22	0.08	0.11	1.93	1.15	0.15	0.11
		Male	2.36	2.19	0.94	0.27	0.77	0.49	0.57	0.59	0.74	0.97	0.30	0.81	2.17	1.12	0.06	**0.01**
		Female	1.69	2.40	0.11	0.22	2.10	1.29	0.13	0.29	0.90	1.55	0.20	0.08	1.62	1.15	0.75	0.76
		Test for interaction			0.08	0.12			0.47	0.39			0.27	0.08			0.42	0.28
	S.pneumoniae	All participants	0.57	0.61	0.49	0.46	0.53	0.51	0.89	0.28	0.44	0.48	0.97	0.26	0.63	0.54	0.58	0.96
		Male	0.57	0.66	0.61	0.94	0.38	0.32	0.39	0.47	0.37	0.37	0.68	0.33	0.61	0.66	0.58	0.78
		Female	0.58	0.53	0.75	0.49	0.54	0.73	0.39	0.25	0.64	0.56	0.53	0.74	0.66	0.45	0.17	0.08
		Test for interaction			0.35	0.38			0.36	0.58			0.46	0.44			0.18	0.35

	S.aureus	All participants	1.75	1.27	0.74	0.65	1.63	1.69	0.56	0.26	0.84	1.10	0.71	0.76	1.03	1.31	0.51	0.43
		Male	1.64	1.22	0.89	0.49	1.51	0.99	0.74	0.19	0.73	0.72	0.93	0.74	1.29	1.21	0.60	0.50
		Female	1.75	1.76	0.53	0.49	1.86	2.12	0.33	0.54	1.99	1.52	0.93	0.94	1.03	1.42	0.71	0.69
		Test for interaction			0.34	0.37			0.63	0.35			0.88	0.87			0.54	0.70
	E.coli	All participants	1.30	1.39	0.74	0.45	0.91	1.01	0.36	0.16	1.01	1.20	0.11	0.62	1.27	1.07	0.29	0.59
		Male	1.31	1.39	0.91	0.73	0.84	1.01	0.33	0.13	1.01	1.07	0.20	0.23	1.42	1.12	0.55	0.22
		Female	1.24	1.39	0.65	0.30	1.00	1.04	0.90	0.74	1.17	1.39	0.44	0.80	1.25	0.92	0.30	0.68
		Test for interaction			0.29	0.19			0.40	0.40			0.53	0.50			0.63	0.18
	C.albicans	All participants	0.12	0.10	0.85	0.68	0.19	0.11	0.56	0.85	0.10	0.21	0.12	0.95	0.48	0.26	**0.05**	0.12
		Male	0.11	0.16	0.64	0.54	0.20	0.09	0.39	0.33	0.07	0.17	0.10	0.36	0.66	0.32	0.26	0.51
		Female	0.15	0.07	0.85	0.58	0.15	0.19	0.75	**0.04**	0.15	0.30	0.83	0.19	0.28	0.22	0.15	0.17
		Test for interaction			0.34	0.40			0.18	0.11			0.10	0.11			0.28	0.24
			6 weeks (pre-EPI1) : 5 days				**6 weeks of age (post-EPI1 +/-BCG) : 5 days**				**10 weeks (pre-EPI2) : 5 days**							
			BCG at birth	**BCG at 6wks**	**p-value**	**Adj p-value**	**BCG at birth**	**BCG at 6wks**	**p-value**	**Adj p-value**	**BCG at birth**	**BCG at 6wks**	**p-value**	**Adj p-value**				
IL-10	**PPD**	All participants	0.46	0.41	0.80	0.36	0.42	0.48	0.72	0.47	0.65	0.53	0.96	0.85				
		Male	0.49	0.40	0.74	0.33	0.34	0.32	0.92	0.27	0.60	0.54	0.89	0.98				

	Female	0.35	0.41	0.95	0.91	0.72	0.50	0.81	0.81	0.65	0.53	0.91	0.96
	Test for interaction			0.40	0.38			0.51	0.25			0.80	0.92
Poly I:C	All participants	0.48	0.29	0.24	0.59	0.46	0.59	0.56	0.76	0.96	0.46	**0.02**	**0.007**
	Male	0.48	0.18	0.12	0.50	0.42	0.39	0.92	0.53	1.16	0.46	0.09	**0.01**
	Female	0.48	0.51	0.91	0.89	0.67	0.71	0.46	0.58	0.96	0.47	0.09	0.23
	Test for interaction			0.53	0.46			0.67	0.75			0.53	0.14
S.pneumoniae	All participants	0.79	0.50	0.78	0.45	0.86	0.75	0.64	0.75	0.97	0.68	0.23	0.29
	Male	0.70	0.35	0.25	0.97	0.55	0.64	0.49	0.34	1.28	0.78	0.26	**0.04**
	Female	0.81	1.33	0.35	0.23	0.95	0.76	0.46	0.09	0.97	0.48	0.48	0.91
	Test for interaction			0.31	0.34			0.17	0.08			0.45	0.22
S.aureus	All participants	0.40	0.59	0.55	0.62	0.43	0.79	0.65	0.14	1.00	1.12	0.98	0.51
	Male	0.36	0.68	1.0	0.47	0.26	0.10	0.27	0.82	0.80	1.12	0.64	0.72
	Female	0.64	0.50	0.41	0.20	1.91	2.01	0.76	0.35	1.45	0.97	0.77	0.68
	Test for interaction			0.37	0.32			0.47	0.75			0.65	0.88
E.coli	All participants	0.68	0.77	0.56	0.22	0.81	0.85	0.72	0.65	0.65	0.59	0.10	**0.03**
	Male	0.66	0.61	1.0	0.38	0.70	1.06	0.12	0.33	0.68	0.60	0.26	0.08
	Female	0.69	0.95	0.25	0.89	1.36	0.73	0.22	0.10	0.65	0.59	0.20	0.69

	C.albicans	Test for interaction			0.70	0.83			0.13	0.22			0.56	0.90
		All participants	0.92	0.43	0.47	0.40	0.34	0.71	0.09	0.35	1.76	0.34	0.23	0.62
		Male	0.92	0.11	0.53	0.81	0.35	0.56	0.60	0.24	3.11	0.34	0.30	0.68
		Female	0.84	0.86	0.63	0.39	0.25	1.33	0.09	0.43	1.3	0.66	0.34	0.42
		Test for interaction			0.23	0.25			0.42	0.26			0.99	0.69
			6 weeks (5d post-EPI1 +/- BCG) : 6 weeks (pre-EPI1)				**10 weeks (pre-EPI2) : 6 weeks (pre-EPI1)**				**10 weeks (pre-EPI2) 6 weeks (5d post-EPI1+/-BCG)**			
			BCG at birth	**BCG at 6wks**	**p-value**	**Adj p-value**	**BCG at birth**	**BCG at 6wks**	**p-value**	**Adj p-value**	**BCG at birth**	**BCG at 6wks**	**p-value**	**Adj p-value**
IL-10	**PPD**	All participants	0.37	1.22	0.10	**0.04**	0.65	0.60	0.90	0.32	2.37	1.26	0.12	0.61
		Male	0.37	1.89	**0.04**	0.09	0.84	0.55	0.48	0.29	2.18	1.66	0.66	0.31
		Female	0.33	0.74	0.73	0.23	0.61	1.54	0.52	0.29	2.98	0.91	0.06	0.07
		Test for interaction			0.21	0.27			0.29	0.34			0.17	0.11
	Poly I:C	All participants	0.60	0.91	0.61	0.50	1.45	1.25	0.58	0.75	1.04	0.94	0.97	0.08
		Male	2.97	0.91	0.36	1.0	1.71	1.16	0.12	0.43	0.75	1.43	0.23	0.78
		Female	0.24	0.86	0.17	0.22	0.68	1.45	0.37	0.51	1.75	0.89	0.24	0.06
		Test for interaction			0.16	0.15			0.34	0.29			0.14	0.20
	S.pneumoniae	All participants	0.93	0.92	0.75	**0.03**	1.05	1.04	0.71	0.09	1.09	0.91	0.63	0.70

	Male	1.79	0.98	0.72	0.15	1.15	0.91	0.94	0.10	1.05	1.46	0.28	0.80
	Female	0.72	0.84	1.0	0.13	0.93	1.06	0.87	0.88	1.13	0.49	**0.01**	**0.004**
	Test for interaction			0.54	0.62			0.33	0.41			0.06	0.15
S.aureus	All participants	1.36	0.68	0.15	0.11	1.65	1.39	0.40	0.88	0.68	0.62	0.73	0.98
	Male	1.36	0.88	0.27	0.21	2.58	0.59	0.41	0.63	0.90	0.76	0.67	0.55
	Female	1.17	0.51	0.40	0.07	0.73	1.48	0.57	0.99	0.65	0.59	0.55	0.33
	Test for interaction			0.29	0.24			0.86	0.57			0.23	0.31
E.coli	All participants	1.00	0.93	0.45	0.93	1.35	1.12	0.43	0.80	0.91	1.09	1.0	0.47
	Male	1.00	0.85	0.20	0.26	1.60	1.33	0.31	0.47	0.79	1.23	0.23	0.51
	Female	1.14	1.02	0.87	0.69	0.64	1.06	0.57	0.40	1.02	0.65	0.19	**0.05**
	Test for interaction			0.75	0.75			0.61	0.49			0.10	**0.04**
C.albicans	All participants	1.14	3.13	0.70	0.43	1.78	0.84	0.32	0.45	6.85	0.74	0.26	**0.02**
	Male	1.14	5.80	0.60	0.42	2.07	1.28	0.48	0.62	0.94	1.43	0.70	0.51
	Female	1.10	1.24	0.71	0.63	0.44	0.49	0.87	0.60	15.49	0.52	0.07	0.07
	Test for interaction			0.45	0.42			0.60	0.65			0.09	0.17

2.6.5 IFNγ

			5 days : Cord Blood				6 weeks (pre-EPI1) : Cord blood				6 weeks of age (5d post-EPI1 +/- BCG) : Cord blood				10 weeks (pre-EPI2) : Cord blood			
			BCG at birth	**BCG at 6wks**	**p-value**	**Adj p-value**	**BCG at birth**	**BCG at 6wks**	**p-value**	**Adj p-value**	**BCG at birth**	**BCG at 6wks**	**p-value**	**Adj p-value**	**BCG at birth**	**BCG at 6wks**	**p-value**	**Adj p-value**
IFNγ	**PPD**	All participants	0.06	0.33	0.96	0.27	135.36	0.08	**<0.0001**	**0.004**	86.41	0.11	**<0.0001**	**0.02**	99.56	28.42	0.20	0.48
		Male	0.70	0.55	1.0	0.28	782.55	0.06	**0.0005**	**0.01**	239.29	0.28	**0.003**	**0.09**	511.67	20.58	**0.04**	0.16
		Female	0.01	0.005	0.93	0.85	9.03	0.09	**0.04**	0.13	31.14	0.09	**0.04**	**0.009**	24.91	58.02	0.52	0.44
		Test for interaction			0.35	0.39			0.23	0.33			0.19	0.21			0.10	0.13
	Poly I:C	All participants	6.88	5.12	0.34	0.59	6.16	8.51	0.64	0.09	1.42	1.69	0.69	0.87	41.36	35.21	0.94	0.61
		Male	8.01	3.05	0.69	0.64	26.20	0.62	0.07	0.15	7.74	5.14	0.58	0.69	59.38	35.21	0.47	0.55
		Female	5.74	5.12	0.39	0.18	1.47	10.54	0.10	0.68	1.01	1.17	0.68	0.81	24.49	34.77	0.58	0.42
		Test for interaction			0.39	0.20			**0.04**	0.14			0.51	0.49			0.26	0.35
	S.pneumoniae	All participants	18.55	9.66	0.46	0.63	9.64	6.41	0.31	0.71	8.17	4.99	0.29	0.84	17.39	11.94	0.94	0.79
		Male	46.40	8.12	**0.03**	0.23	41.40	0.87	**0.03**	0.20	13.80	1.44	0.37	0.72	6.88	9.11	0.86	0.37
		Female	13.40	38.85	0.19	0.08	5.27	47.00	0.36	0.87	5.33	5.32	0.82	0.97	25.91	42.01	0.50	0.36
		Test for interaction			**0.02**	**0.04**			0.09	0.15			0.89	0.88			0.10	0.15
	S.aureus	All participants	11.00	4.93	0.07	0.26	40.18	2.87	0.07	0.22	2.48	1.71	0.40	0.26	4.10	5.67	0.97	0.59

		Male	6.16	9.14	0.74	0.47	46.65	1.90	0.08	0.27	1.29	3.03	0.88	0.38	10.55	4.02	0.85	0.79
		Female	22.77	1.20	**0.05**	0.28	29.29	16.38	0.65	0.60	4.30	0.14	0.14	0.25	2.61	5.96	0.88	0.51
		Test for interaction			0.09	0.13			0.41	0.43			0.59	0.56			0.43	0.55
	E.coli	All participants	6.71	4.45	0.70	0.23	2.91	2.81	0.47	0.07	18.14	3.24	0.11	0.37	14.10	7.91	0.98	0.39
		Male	6.51	1.71	0.60	0.82	9.94	2.39	0.46	0.16	18.75	2.55	0.10	0.77	8.82	3.32	0.40	0.42
		Female	10.24	10.31	0.86	0.32	0.68	32.71	0.06	0.18	8.66	5.13	0.65	0.48	34.57	55.11	0.41	0.61
		Test for interaction			0.24	0.46			0.98	0.67			0.32	0.42			0.93	0.70
		All participants	0.09	0	0.34	0.33	1.00	2.03	0.75	0.12	1.00	0.56	0.57	0.36	3.74	6.37	0.44	0.25
		Male	0.14	0	0.11	0.35	2.09	0.01	0.45	0.47	2.25	0.78	0.40	0.33	1.76	4.98	0.95	0.81
	C.albicans	Female	0	0	0.87	0.76	0.12	45.04	0.18	0.35	0.55	0.10	0.84	0.50	10.18	8.71	0.43	0.38
		Test for interaction			0.20	0.25			0.09	0.21			0.13	0.24			0.14	0.18

			6 weeks (pre-EPI1) : 5 days				**6 weeks of age (5d post-EPI1 +/- BCG) : 5 days**				**10 weeks (pre-EPI2) : 5 days**			
			BCG at birth	**BCG at 6wks**	**p-value**	**Adj p-value**	**BCG at birth**	**BCG at 6wks**	**p-value**	**Adj p-value**	**BCG at birth**	**BCG at 6wks**	**p-value**	**Adj p-value**
IFNγ	**PPD**	All participants	86.21	0.13	**0.001**	0.22	137.08	0.13	**0.004**	**0.008**	40.87	24.72	0.39	0.40
		Male	98.20	0.12	**0.04**	0.34	382.25	0.87	**0.05**	**0.006**	12.32	31.15	0.73	0.41
		Female	44.09	0.63	**0.04**	0.13	92.61	0.13	0.09	0.33	53.36	7.46	0.51	0.95

	Test for interaction			0.36	0.40			0.11	**0.03**			0.80	0.81
Poly I:C	All participants	1.14	1.25	0.75	0.47	0.31	1.25	0.58	0.55	3.55	2.64	0.88	0.93
	Male	1.06	1.20	0.44	0.48	0.26	2.09	0.54	0.65	6.93	2.04	0.22	0.83
	Female	2.49	1.32	0.70	0.36	0.43	1.11	0.73	0.24	2.14	3.23	0.77	0.96
	Test for interaction			0.20	0.26			0.20	0.28			0.66	0.93
S.pneumoniae	All participants	1.04	0.75	0.79	0.51	0.31	0.08	0.50	0.21	0.52	1.22	0.28	0.44
	Male	0.17	0.22	0.68	0.51	0.42	0.06	0.56	0.14	1.00	1.65	0.26	0.95
	Female	1.48	1.97	0.73	0.39	0.29	0.36	0.61	0.85	0.44	0.16	0.85	0.50
	Test for interaction			0.36	0.42			0.58	0.36			0.71	0.70
S.aureus	All participants	1.69	4.16	0.60	0.33	0.98	0.97	0.81	0.71	0.65	4.14	**0.03**	0.44
	Male	2.13	2.58	0.77	0.38	0.54	2.02	0.79	0.63	2.18	3.73	0.40	0.70
	Female	1.41	10.17	0.57	0.93	2.30	0.67	0.53	0.36	0.26	12.17	0.19	0.20
	Test for interaction			0.46	0.41			0.50	0.45			0.10	0.13
E.coli	All participants	1.47	1.41	0.57	0.31	0.83	1.21	0.61	0.55	1.20	5.15	0.24	0.20
	Male	0.37	3.44	0.09	0.12	0.32	1.45	0.26	0.62	1.20	8.09	0.28	0.31
	Female	5.03	1.41	0.46	0.42	1.78	1.12	0.73	0.99	1.25	2.22	0.88	0.85
	Test for interaction			0.24	0.31			0.86	0.68			0.23	0.17

	C.albicans	All participants	0.12	18.99	0.08	0.41	0.37	1.18	0.57	0.32	5.35	1.37	0.96	0.10
		Male	0	19.15	**0.02**	**0.03**	0.35	17.86	0.25	0.56	22.02	9.74	0.65	0.43
		Female	9.14	9.69	1.0	0.73	3.39	0.11	0.14	**0.05**	3.04	0.25	0.31	0.34
		Test for interaction			0.71	0.71			0.68	0.54			0.57	0.65
			6 weeks (5d post-EPI1 +/-BCG) : 6 weeks (pre-EPI1)				**10 weeks (pre-EPI2) : 6 weeks (pre-EPI1)**				**10 weeks (pre-EPI2) 6 weeks (5d post-EPI1+/-BCG)**			
			BCG at birth	**BCG at 6wks**	**p-value**	**Adj p-value**	**BCG at birth**	**BCG at 6wks**	**p-value**	**Adj p-value**	**BCG at birth**	**BCG at 6wks**	**p-value**	**Adj p-value**
IFNγ	**PPD**	All participants	1.57	0.47	0.13	0.53	0.65	18.64	**0.01**	**0.05**	0.33	36.20	**<0.0001**	0.18
		Male	1.46	1.00	1.0	0.28	0.63	18.41	**0.05**	0.16	0.67	68.78	**0.003**	0.26
		Female	3.11	0	0.08	**<0.0001**	0.81	91.74	0.12	0.29	0.05	15.62	**0.006**	0.06
		Test for interaction			0.11	0.14			0.72	0.65			0.13	0.17
	Poly I:C	All participants	0.57	0.90	0.34	0.39	1.16	0.93	0.87	0.37	2.44	6.02	0.37	0.11
		Male	1.06	3.45	0.34	0.56	1.43	0.93	0.79	0.29	1.72	8.28	0.13	0.09
		Female	0.55	0.79	0.65	0.36	1.04	1.31	0.81	0.61	2.97	3.24	0.73	1.0
		Test for interaction			0.99	0.87			0.36	0.33			0.40	0.11
	S.pneumoniae	All participants	0.61	2.30	0.37	0.37	0.61	1.11	0.25	0.62	0.63	1.13	0.69	0.17
		Male	0.40	7.84	0.36	0.72	0.56	0.93	0.46	0.50	0.63	1.08	0.71	0.49
		Female	0.82	1.10	1.0	0.40	0.72	1.23	0.26	0.94	8.22	1.34	0.71	0.64

		Test for interaction			0.52	0.36			0.59	0.55			0.34	0.51
	S.aureus	All participants	1.05	0.44	0.97	0.50	0.78	1.71	0.79	0.18	4.18	2.12	0.28	0.20
		Male	1.26	0.78	0.67	0.42	0.51	3.69	0.35	0.17	1.87	0.86	0.54	0.59
		Female	0.15	0.44	0.42	0.59	6.57	1.71	0.16	0.26	17.57	2.44	0.12	0.79
		Test for interaction			0.41	0.42			0.21	0.31			0.20	0.24
	E.coli	All participants	0.91	1.20	0.97	0.28	0.37	1.36	0.11	0.10	2.11	0.61	0.09	0.11
		Male	2.64	3.05	0.87	0.34	0.40	13.35	0.24	**0.02**	1.73	0.63	0.29	0.85
		Female	0.16	0.71	0.73	0.73	0.08	1.36	0.39	0.75	8.98	0.59	0.13	0.30
		Test for interaction			0.59	0.71			0.19	**0.04**			0.08	0.14
	C.albicans	All participants	10.6	0.24	0.51	0.89	0.11	0.66	0.32	0.84	1.00	2.11	0.32	0.33
		Male	0.76	0.09	0.28	0.35	0.16	0.76	0.70	0.65	1.00	1.05	0.91	0.89
		Female	1.06	1.15	1.0	0.45	0	0.66	0.28	0.97	1.70	6.38	0.09	0.20
		Test for interaction			0.28	0.22			0.74	0.62			0.18	0.14

3. Iron sub-study

3.1 Study numbers, per protocol analysis (numbers of female infants in brackets)

3.1.1 Cross-sectional analysis

	Cord		S1 5 days of age		S2 6 weeks of age (pre-EPI1)		S3 6 weeks +5 days (post-EPI1 and BCG in delayed group)		S4 10 weeks of age (pre-EPI2)	
	BCG at birth	**BCG at 6 wks**	**BCG at birth**	**BCG at 6 wks**	**BCG at birth**	**BCG at 6 wks**	**BCG at birth**	**BCG at 6 wks**	**BCG at birth**	**BCG at 6 wks**
IL-6	118 (58)	121 (59)	58 (28)	54 (28)	56 (27)	53 (22)	44 (21)	50 (27)	46 (22)	44 (25)
Hepcidin	117 (58)	120 (59)	57 (27)	54 (28)	57 (27)	52 (23)	44 (21)	50 (27)	46 (22)	44 (25)
Iron parameters	119 (58)	121 (59)	48 (22)	43 (22)	51 (25)	49 (23)	44 (21)	49 (26)	45 (22)	41 (22)
Erythrocyte parameters	113 (55)	118 (57)	56 (26)	52 (29)	54 (26)	51 (23)	39 (18)	47 (26)	43 (21)	39 (24)
Leucocyte differential counts	113 (55)	118 (57)	56 (26)	52 (29)	54 (26)	51 (23)	39 (18)	47 (26)	43 (21)	39 (24)

3.1.2 Within-infant changes over time

		S1		S2		S3		S4	
		BCG at birth	**BCG at 6 wks**	**BCG at birth**	**BCG at 6 wks**	**BCG at birth**	**BCG at 6 wks**	**BCG at birth**	**BCG at 6 wks**
Cord	**IL-6**	56 (27)	54 (28)	52 (24)	52 (22)	42 (20)	50 (27)	46 (22)	44 (25)
	Hepcidin	54 (26)	53 (28)	51 (24)	53 (23)	42 (20)	50 (27)	46 (22)	44 (25)
	Iron parameters	47 (21)	43 (22)	47 (22)	49 (23)	42 (20)	49 (26)	45 (22)	41 (22)
	Erythrocyte parameters	51 (23)	49 (27)	47 (21)	51 (23)	36 (25)	45 (20)	40 (20)	38 (23)
	Leucocyte differentials	51 (23)	49 (27)	47 (21)	51 (23)	36 (25)	45 (20)	40 (20)	38 (23)
S1	**IL-6**			22 (9)	15 (5)	14 (7)	19 (11)	16 (8)	13 (9)
	Hepcidin			21 (8)	15 (5)	14 (7)	19 (11)	16 (8)	13 (9)
	Iron parameters			17 (8)	9 (3)	12 (5)	15 (7)	11 (5)	11 (8)
	Erythrocyte parameters			21 (9)	13 (5)	11 (5)	18 (11)	14 (7)	12 (10)
	Leucocyte differentials			21 (9)	13 (5)	11 (5)	18 (11)	14 (7)	12 (10)
S2	**IL-6**					12 (6)	17 (8)	17 (8)	16 (6)
	Hepcidin					13 (6)	17 (8)	17 (8)	17 (7)
	Iron parameters					12 (6)	16 (8)	15 (8)	14 (5)
	Erythrocyte parameters					11 (6)	14 (7)	15 (7)	15 (7)
	Leucocyte differential counts					11 (6)	14 (7)	15 (7)	15 (7)
S3	**IL-6**							12 (6)	13 (8)
	Hepcidin							12 (6)	13 (8)
	Iron parameters							12 (6)	13 (8)
	Erythrocyte parameters							11 (5)	11 (7)
	Leucocyte differential counts							11 (5)	11 (7)

3.2 Cross-sectional comparisons by BCG status.

3.2.1 Inflammatory-iron parameters, geometric means

		Cord Blood			5 days of age			6 weeks of age (1 day post-EPI1)			6 weeks of age (5 days post-EPI1 +/-BCG)			10 weeks of age (1 day post-EPI2)		
		BCG at birth	**BCG at 6wks**	**p-value**	**BCG at birth**	**BCG at 6wks**	**p-value**	**BCG at birth**	**BCG at 6wks**	**p-value**	**BCG at birth**	**BCG at 6wks**	**p-value**	**BCG at birth**	**BCG at 6wks**	**p-value**
IL6	All participants	10.03	7.05	0.07	8.68	7.21	0.41	44.85	62.75	0.08	11.41	7.00	**0.04**	42.57	38.36	0.49
	Male	10.14	6.48	0.13	8.41	7.05	0.57	40.95	58.26	0.18	10.78	5.28	**0.04**	47.71	41.65	0.61
	Female	9.92	7.70	0.32	8.98	7.36	0.55	49.88	69.44	0.23	12.15	8.92	0.35	37.60	36.03	0.71
	Test for interaction			0.72			0.95			0.96			0.39			0.95
Hepcidin	All participants	61.38	56.70	0.92	91.22	104.45	0.48	192.50	189.71	0.83	87.85	71.18	0.18	201.17	189.55	0.68
	Male	55.64	53.78	0.82	84.19	86.25	0.60	183.75	173.52	0.95	90.25	48.06	**0.009**	190.96	170.05	0.95
	Female	68.08	59.89	0.93	100.06	124.78	0.20	203.63	213.12	0.67	85.29	99.46	0.40	212.94	205.85	0.51
	Test for interaction			0.82			0.19			0.72			**0.009**			0.69
Ferritin	All participants	138.17	131.44	0.58	248.02	251.75	0.89	219.36	191.16	0.17	235.65	187.67	0.07	153.46	128.91	0.26
	Male	131.47	123.66	0.65	224.80	257.25	0.40	217.93	172.52	0.10	248.39	153.33	**0.008**	134.16	112.77	0.40
	Female	145.88	140.15	0.75	280.11	246.37	0.36	221.07	216.03	0.81	222.39	224.41	0.96	176.62	144.70	0.39

	Test for interaction			0.91			0.22			0.35			**0.05**			0.96
TSAT	All participants	40.99	43.65	0.23	39.79	40.20	0.88	13.28	11.46	0.11	27.31	25.46	0.38	9.35	8.70	0.56
	Male	40.32	42.52	0.49	38.27	38.84	0.88	13.94	10.77	0.11	27.61	22.27	0.08	9.54	7.59	0.17
	Female	41.73	44.88	0.30	41.60	41.68	0.98	12.53	12.37	0.61	26.98	28.66	0.58	9.14	9.79	0.57
	Test for interaction			0.85			0.93			0.43			0.09			0.15
Iron	All participants	19.63	20.81	0.25	15.54	15.81	0.80	6.41	5.33	0.07	12.72	12.39	0.70	4.89	4.56	0.54
	Male	19.21	20.88	0.24	15.54	15.89	0.82	6.38	5.14	0.20	12.64	11.09	0.23	5.00	4.09	0.23
	Female	20.10	20.75	0.66	15.54	15.74	0.89	6.43	5.55	0.19	12.80	13.65	0.48	4.78	5.01	0.75
	Test for interaction			0.62			0.96			0.96			0.18			0.27
TIBC	All participants	47.89	47.68	0.90	38.69	39.97	0.40	46.16	46.76	0.68	46.56	48.65	0.17	52.35	52.42	1.0
	Male	47.64	49.10	0.49	39.89	40.90	0.67	45.82	47.69	0.39	45.79	49.81	0.06	52.43	53.90	0.60
	Female	48.18	46.23	0.43	37.36	39.01	0.39	46.55	45.64	0.65	47.43	47.65	0.92	52.27	51.17	0.61
	Test for interaction			0.29			0.81			0.36			0.22			0.46
sTFR	All participants	6.38	6.54	0.58	4.65	4.31	0.43	2.97	2.76	0.45	2.81	2.82	0.92	4.66	4.94	0.34
	Male	6.64	6.89	0.61	4.60	4.07	0.37	3.10	3.01	0.71	2.97	3.05	0.71	4.87	5.76	0.06
	Female	6.11	6.19	0.77	4.71	4.57	0.80	2.82	2.46	0.43	2.65	2.63	0.94	4.45	4.32	0.74
	Test for interaction			0.87			0.66			0.68			0.75			0.11

3.2.2 Erythrocyte and leucocyte parameters

		Cord Blood			5 days of age			6 weeks of age (1 day post-EPI1)			6 weeks of age (5 days post-EPI1 +/- BCG)			10 weeks of age (1 day post-EPI2)		
		BCG at birth	**BCG at 6wks**	**p-value**	**BCG at birth**	**BCG at 6wks**	**p-value**	**BCG at birth**	**BCG at 6wks**	**p-value**	**BCG at birth**	**BCG at 6wks**	**p-value**	**BCG at birth**	**BCG at 6wks**	**p-value**
Hb	All participants	15.44	15.23	0.40	15.99	15.93	0.88	10.74	10.85	0.69	10.97	11.05	0.81	10.53	10.22	0.15
	Male	15.49	15.44	0.82	16.02	15.86	0.80	10.91	10.86	0.74	10.88	11.00	0.85	10.54	9.79	**0.02**
	Female	15.38	15.00	0.31	15.97	15.98	0.96	10.55	10.84	0.35	11.09	11.09	0.92	10.53	10.49	0.90
	Test for interaction			0.60			0.82			0.36			0.96			0.11
Haematocrit	All participants	47.09	46.35	0.33	48.38	47.78	0.65	32.02	32.25	0.72	32.71	32.68	1.0	32.03	31.17	0.18
	Male	47.41	47.04	0.66	48.46	47.48	0.62	32.59	32.24	0.61	32.47	32.30	0.79	31.93	30.41	0.08
	Female	46.75	45.62	0.33	48.22	48.01	0.90	31.40	32.27	0.30	32.99	32.98	0.89	32.12	31.65	0.62
	Test for interaction			0.74			0.77			0.26			0.78			0.43
RBC	All participants	4.58	4.43	0.05	4.93	4.78	0.24	3.65	3.62	0.62	3.85	3.79	0.53	3.81	3.72	0.35
	Male	4.63	4.51	0.23	4.96	4.75	0.27	3.72	3.68	0.64	3.89	3.71	0.14	3.76	3.71	0.67
	Female	4.52	4.34	0.11	4.90	4.79	0.62	3.58	3.54	0.81	3.80	3.85	0.73	3.85	3.72	0.38
	Test for interaction			0.71			0.65			0.88			0.23			0.69
MCV	All participants	103.33	105.05	0.05	98.27	100.33	0.17	87.85	89.49	0.09	85.49	86.43	0.28	84.61	84.21	0.76

	Male	102.79	104.51	0.23	97.73	99.96	0.25	87.86	88.07	0.92	83.95	87.14	**0.02**	85.18	82.27	0.14
	Female	103.89	105.63	0.12	98.88	100.62	0.48	87.85	91.22	**0.02**	87.28	85.85	0.54	84.05	85.42	0.47
	Test for interaction			0.82			0.81			0.09			**0.05**			0.12
MCH	All participants	33.88	34.53	**0.04**	32.51	33.47	0.08	29.46	30.13	0.11	28.63	29.24	0.14	27.83	27.60	0.63
	Male	33.59	34.29	0.14	32.33	33.42	0.11	29.39	29.67	0.69	28.04	29.66	**0.007**	28.08	26.49	**0.02**
	Female	34.17	34.79	0.17	32.72	33.52	0.38	29.53	30.68	0.05	29.32	28.90	0.59	27.58		0.27
	Test for interaction			0.95			0.73			0.23			**0.02**			**0.01**
MCHC	All participants	32.80	32.88	0.37	33.09	32.95	0.73	33.52	33.66	0.61	33.53	33.83	0.15	32.90	32.77	0.44
	Male	32.70	32.84	0.22	33.09	32.52	0.50	33.44	33.68	0.23	33.47	34.04	**0.02**	33.00	32.17	**0.0006**
	Female	32.90	32.91	0.93	33.08	33.29	0.25	33.61	33.63	0.65	33.61	33.67	0.94	32.80	33.15	0.14
	Test for interaction			0.47			0.36			0.26			0.10			**<0.0001**
RDW	All participants	12.19	12.27	0.52	12.27	12.09	0.24	12.27	12.12	0.25	12.44	12.46	0.78	11.41	11.61	0.45
	Male	12.24	12.39	0.40	12.31	12.10	0.33	12.29	12.23	0.72	12.62	12.60	0.79	11.75	12.18	0.28
	Female	12.13	12.14	0.98	12.24	12.08	0.52	12.25	11.98	0.20	12.23	12.33	0.97	11.07	11.25	0.58
	Test for interaction			0.56			0.89			0.47			0.84			0.63
WBC	All participants	13.91	14.36	0.47	9.15	9.02	0.71	14.55	14.90	0.68	9.25	9.53	0.62	15.28	13.90	0.19
	Male	14.19	13.81	0.71	9.48	9.13	0.61	14.80	15.11	0.58	9.64	10.16	0.52	15.32	14.13	0.52
	Female	13.63	14.94	0.13	8.77	8.94	0.90	14.28	14.63	0.99	8.78	9.03	0.80	15.24	13.75	0.24
	Test for interaction			0.19			0.65			0.73			0.76			0.89

Neutrophils	All participants	6.58	6.72	0.55	2.75	2.66	0.69	6.62	7.20	0.38	2.13	2.10	0.85	7.04	5.61	**0.02**
	Male	6.40	6.48	0.92	2.82	2.88	0.89	6.68	7.48	0.28	2.23	2.41	0.64	7.21	5.72	0.14
	Female	6.76	6.97	0.45	2.66	2.49	0.57	6.56	6.86	0.90	2.00	1.86	0.50	6.88	5.54	0.11
	Test for interaction			0.64			0.63			0.53			0.43			0.91
Lymphocytes	All participants	5.44	5.83	0.36	4.62	4.65	0.96	5.99	5.79	0.55	5.82	6.12	0.40	6.17	6.53	0.45
	Male	5.84	5.59	0.72	4.79	4.59	0.59	6.11	5.65	0.39	5.98	6.4	0.36	6.03	6.62	0.48
	Female	5.03	6.09	0.07	4.42	4.70	0.44	5.85	5.97	0.99	5.63	5.89	0.66	6.31	6.47	0.77
	Test for interaction			0.13			0.36			0.60			0.81			0.66
Monocytes	All participants	1.30	1.28	0.95	1.12	1.16	0.72	1.53	1.51	0.96	0.90	0.93	0.67	1.43	1.28	0.31
	Male	1.28	1.21	0.46	1.16	1.11	0.69	1.59	1.59	0.81	0.98	0.92	0.68	1.44	1.32	0.64
	Female	1.32	1.35	0.50	1.08	1.20	0.45	1.47	1.41	0.64	0.81	0.94	0.23	1.42	1.25	0.38
	Test for interaction			0.32			0.40			0.65			0.27			0.88
Eosinophils	All participants	0.29	0.27	0.57	0.38	0.32	0.18	0.19	0.16	0.17	0.27	0.23	0.33	0.41	0.27	**0.007**
	Male	0.31	0.32	0.73	0.40	0.34	0.34	0.20	0.18	0.57	0.32	0.29	0.68	0.43	0.27	**0.05**
	Female	0.27	0.23	0.14	0.34	0.31	0.47	0.18	0.14	0.16	0.22	0.19	0.38	0.39	0.28	0.09
	Test for interaction			0.25			0.76			0.52			0.98			0.60
Basophils	All participants	0.128	0.28	0.92	0.30	0.23	0.10	0.22	0.23	0.51	0.14	0.15	0.42	0.22	0.20	0.50
	Male	0.31	0.26	0.36	0.32	0.21	0.16	0.22	0.23	0.56	0.14	0.15	0.85	0.21	0.20	0.82
	Female	0.25	0.31	0.24	0.28	0.24	0.43	0.21	0.23	0.74	0.13	0.15	0.31	0.24	0.21	0.42

	Test for interaction	0.14	0.49	0.84	0.60	0.72

3.3 Within-infant fold-changes over-time by BCG status

3.3.1 Iron

	5 days : Cord Blood				6 weeks of age (1 day post-EPI1) : Cord blood				6 weeks of age (5 days post-EPI1 +/- BCG) : Cord blood				10 weeks of age (1 day post-EPI2) : Cord Blood			
	BCG at birth	**BCG at 6wks**	**p-value**	**Adj p-value**	**BCG at birth**	**BCG at 6wks**	**p-value**	**Adj p-value**	**BCG at birth**	**BCG at 6wks**	**p-value**	**Adj p-value**	**BCG at birth**	**BCG at 6wks**	**p-value**	**Adj p-value**
All participants	0.79	0.81	0.92	0.68	0.29	0.23	0.11	0.09	0.65	0.56	0.11	0.61	0.25	0.21	0.11	0.42
Male	0.85	0.79	0.43	0.94	0.28	0.22	0.43	0.21	0.64	0.52	0.08	0.30	0.27	0.20	**0.04**	0.43
Female	0.77	0.85	0.59	0.64	0.31	0.23	0.14	0.24	0.65	0.61	0.64	0.70	0.22	0.22	0.95	0.97
Test for interaction			**0.01**	0.73			0.24	0.92			**0.003**	0.28			**0.003**	0.41

	6 weeks of age (1 day post-EPI1) : 5 days				6 weeks of age (5 days post-EPI1 +/-BCG) : 5 days				10 weeks of age (1 day post-EPI2) : 5 days			
	BCG at birth	**BCG at 6wks**	**p-value**	**Adj p-value**	**BCG at birth**	**BCG at 6wks**	**p-value**	**Adj p-value**	**BCG at birth**	**BCG at 6wks**	**p-value**	**Adj p-value**
All participants	0.29	0.45	0.20	0.82	0.86	0.84	0.92	0.17	0.29	0.37	0.97	0.86
Male	0.25	0.39	0.41	0.44	0.93	0.75	0.64	0.73	0.28	0.74	0.30	0.17
Female	0.30	0.83	0.10	0.15	0.81	1.01	0.37	0.14	0.71	0.35	0.24	0.15
Test for interaction			**<0.0001**	0.11			0.07	0.27			**<0.0001**	**0.04**

	6 weeks of age (5 days post-EPI1 +/- BCG) : 6 weeks of age (1 day post-EPI1)				10 weeks of age (1 day post-EPI2) : 6 weeks of age (1 day post-EPI1)				10 weeks of age (1 day post-EPI2) : 6 weeks of age (5 days post-EPI1 +/-BCG)			
	BCG at birth	**BCG at 6wks**	**p-value**	**Adj p-value**	**BCG at birth**	**BCG at 6wks**	**p-value**	**Adj p-value**	**BCG at birth**	**BCG at 6wks**	**p-value**	**Adj p-value**
All participants	1.94	3.01	0.06	0.42	0.52	0.85	0.34	079	0.37	0.40	0.50	1.0
Male	1.49	3.28	**0.04**	0.86	0.73	0.72	0.49	0.34	0.29	0.26	0.58	0.27
Female	2.98	2.65	1.0	0.33	0.46	0.89	**0.04**	0.14	0.37	0.47	0.42	0.60
Test for interaction			**<0.0001**	0.55			**<0.0001**	0.15			0.5	0.25

3.3.2 TSAT

	5 days : Cord Blood				6 weeks of age (1 day post-EPI1) : Cord blood				6 weeks of age (5 days post-EPI1 +/- BCG) : Cord blood				10 weeks of age (1 day post-EPI2) : Cord Blood			
	BCG at birth	BCG at 6wks	p-value	Adj p-value	BCG at birth	BCG at 6wks	p-value	Adj p-value	BCG at birth	BCG at 6wks	p-value	Adj p-value	BCG at birth	BCG at 6wks	p-value	Adj p-value
All participants	0.97	0.97	0.92	0.82	0.28	0.22	**0.04**	0.20	0.61	0.54	0.11	0.35	0.25	0.19	0.22	0.54
Male	0.95	0.89	0.89	0.91	0.27	0.22	0.18	0.07	0.58	0.45	0.08	0.08	0.30	0.17	**0.04**	0.51
Female	0.98	0.99	0.75	0.90	0.30	0.21	0.13	0.98	0.65	0.60	0.56	0.65	0.20	0.21	0.43	0.58
Test for interaction			0.36	0.97			0.47	0.15			**0.003**	0.10			**<0.0001**	0.26

	6 weeks of age (1 day post-EPI1) : 5 days				6 weeks of age (5 days post-EPI1 +/- BCG) : 5 days				10 weeks of age (1 day post-EPI2) : 5 days			
	BCG at birth	BCG at 6wks	p-value	Adj p-value	BCG at birth	BCG at 6wks	p-value	Adj p-value	BCG at birth	BCG at 6wks	p-value	Adj p-value
All participants	0.27	0.30	0.75	0.76	0.74	0.74	0.56	0.30	0.23	0.26	0.72	0.88
Male	0.28	0.30	0.89	0.72	0.73	0.63	0.91	0.89	0.22	0.35	0.44	0.33
Female	0.26	0.54	0.60	0.51	0.75	0.87	0.29	0.27	0.36	0.23	0.17	0.31

Test for interaction			**0.03**	0.46			0.26	0.35			**<0.0001**	0.10
	6 weeks of age (5 days post-EPI1 +/- BCG) : 6 weeks of age (1 day post-EPI1)				**10 weeks of age (1 day post-EPI2) : 6 weeks of age (1 day post-EPI1)**				**10 weeks of age (1 day post-EPI2) : 6 weeks of age (5 days post-EPI1 +/-BCG)**			
	BCG at birth	**BCG at 6wks**	**p-value**	**Adj p-value**	**BCG at birth**	**BCG at 6wks**	**p-value**	**Adj p-value**	**BCG at birth**	**BCG at 6wks**	**p-value**	**Adj p-value**
All participants	1.67	2.94	0.08	0.28	0.59	0.72	0.43	1.0	0.33	0.34	0.74	0.76
Male	1.47	3.03	**0.01**	0.92	0.71	0.66	0.37	0.34	0.25	0.22	0.47	0.29
Female	2.86	2.42	0.89	0.25	.2.86	2.42	**0.03**	0.17	0.46	0.85	0.56	0.88
Test for interaction			**<0.0001**	0.55			**<0.0001**	0.11			0.62	0.39

3.3.3 Hepcidin

	5 days : Cord Blood				6 weeks of age (1 day post-EPI1) : Cord blood				6 weeks of age (5 days post-EPI1 +/- BCG) : Cord blood				10 weeks of age (1 day post-EPI2) : Cord Blood			
	BCG at birth	BCG at 6wks	p-value	Adj p-value	BCG at birth	BCG at 6wks	p-value	Adj p-value	BCG at birth	BCG at 6wks	p-value	Adj p-value	BCG at birth	BCG at 6wks	p-value	Adj p-value
All participants	1.55	1.68	0.90	0.71	3.19	3.25	0.91	0.46	1.22	1.17	0.37	0.22	3.21	2.61	0.89	0.37
Male	1.41	1.62	0.97	0.84	3.53	3.56	0.76	0.56	1.18	0.54	0.10	**0.05**	4.03	3.52	0.84	0.24
Female	1.71	1.79	0.93	0.86	2.91	3.21	0.65	0.70	2.91	3.21	0.93	0.85	1.21	1.60	0.93	0.99
Test for interaction			0.28	0.95			0.10	0.88			0.15	0.11			0.21	0.45

	6 weeks of age (1 day post-EPI1) : 5 days				6 weeks of age (5 days post-EPI1 +/- BCG) : 5 days				10 weeks of age (1 day post-EPI2) : 5 days			
	BCG at birth	BCG at 6wks	p-value	Adj p-value	BCG at birth	BCG at 6wks	p-value	Adj p-value	BCG at birth	BCG at 6wks	p-value	Adj p-value
All participants	2.50	2.61	0.86	1.0	0.87	0.79	0.58	0.33	2.09	1.60	0.29	0.61
Male	2.89	3.42	0.58	0.45	0.69	0.89	1.0	0.54	1.92	0.85	0.06	0.56
Female	2.09	1.02	0.11	0.28	1.13	0.75	0.62	0.49	2.27	2.0	0.85	0.92
Test for			**0.02**	0.16			0.27	0.94			**<0.0001**	0.20

interaction												
	6 weeks of age (5 days post-EPI1 +/- BCG) : 6 weeks of age (1 day post-EPI1)				10 weeks of age (1 day post-EPI2) : 6 weeks of age (1 day post-EPI1)				10 weeks of age (1 day post-EPI2) : 6 weeks of age (5 days post-EPI1 +/-BCG)			
	BCG at birth	BCG at 6wks	p-value	Adj p-value	BCG at birth	BCG at 6wks	p-value	Adj p-value	BCG at birth	BCG at 6wks	p-value	Adj p-value
All participants	0.45	0.29	0.29	0.57	0.84	1.19	0.79	0.40	2.68	2.60	0.70	0.60
Male	0.50	0.20	0.10	0.28	0.60	1.56	**0.03**	0.21	3.67	8.73	0.58	**0.04**
Female	0.30	0.36	0.70	0.65	1.30	1.04	0.25	**0.03**	2.38	1.22	0.35	0.41
Test for interaction			0.16	0.93			**<0.0001**	**0.03**			**0.05**	**0.03**

3.3.4 IL-6

	5 days : Cord Blood				6 weeks of age (1 day post-EPI1) : Cord blood				6 weeks of age (5 days post-EPI1 +/- BCG) : Cord blood				10 weeks of age (1 day post-EPI2) : Cord Blood			
	BCG at birth	**BCG at 6wks**	**p-value**	**Adj p-value**	**BCG at birth**	**BCG at 6wks**	**p-value**	**Adj p-value**	**BCG at birth**	**BCG at 6wks**	**p-value**	**Adj p-value**	**BCG at birth**	**BCG at 6wks**	**p-value**	**Adj p-value**
All participants	1.19	1.0	0.54	0.20	4.85	11.39	0.11	0.06	1.13	1.35	0.80	**0.03**	3.28	9.20	0.09	0.83
Male	1.21	1.0	0.66	0.79	4.48	6.59	0.49	0.10	1.08	1.0	0.71	**0.03**	1.98	10.52	**0.02**	0.21
Female	1.18	1.05	0.61	0.09	6.35	12.83	0.06	0.20	1.36	1.92	0.98	0.34	6.52	7.43	0.83	0.69
Test for interaction			0.17	0.37			0.08	0.78			0.68	0.30			**<0.0001**	0.36

	6 weeks of age (1 day post-EPI1) : 5 days				6 weeks of age (5 days post-EPI1 +/- BCG) : 5 days				10 weeks of age (1 day post-EPI2) : 5 days			
	BCG at birth	**BCG at 6wks**	**p-value**	**Adj p-value**	**BCG at birth**	**BCG at 6wks**	**p-value**	**Adj p-value**	**BCG at birth**	**BCG at 6wks**	**p-value**	**Adj p-value**
All participants	6.04	3.03	0.62	0.87	2.27	1.02	0.26	0.15	5.59	5.58	0.83	0.46
Male	6.25	11.69	0.35	0.37	2.05	0.69	0.25	0.15	5.59	3.92	0.31	0.30
Female	1.49	2.38	0.55	0.17	2.50	2.16	0.62	0.54	6.49	8.32	1.0	0.83
Test for interaction			**0.003**	0.10			0.51	0.73			**0.05**	0.63

	6 weeks of age (5 days post-EPI1 +/- BCG) : 6 weeks of age (1 day post-EPI1)				10 weeks of age (1 day post-EPI2) : 6 weeks of age (1 day post-EPI1)				10 weeks of age (1 day post-EPI2) : 6 weeks of age (5 days post-EPI1 +/- BCG)			
	BCG at birth	**BCG at 6wks**	**p-value**	**Adj p-value**	**BCG at birth**	**BCG at 6wks**	**p-value**	**Adj p-value**	**BCG at birth**	**BCG at 6wks**	**p-value**	**Adj p-value**
All participants	0.17	0.13	0.18	0.64	0.82	0.85	0.94	0.81	4.74	6.64	0.34	0.67
Male	0.31	0.09	0.10	0.26	0.82	0.92	0.41	0.54	13.2	9.52	0.86	0.20
Female	0.12	0.16	1.0	0.65	0.80	0.64	0.52	0.36	2.9	4.56	0.25	0.41
Test for interaction			**<0.0001**	0.30			**<0.0001**	0.69			0.07	0.20

3.3.5 Transferrin

	5 days : Cord Blood				6 weeks of age (1 day post-EPI1) : Cord blood				6 weeks of age (5 days post-EPI1 +/- BCG) : Cord blood				10 weeks of age (1 day post-EPI2) : Cord Blood			
	BCG at birth	BCG at 6wks	p-value	Adj p-value	BCG at birth	BCG at 6wks	p-value	Adj p-value	BCG at birth	BCG at 6wks	p-value	Adj p-value	BCG at birth	BCG at 6wks	p-value	Adj p-value
All participants	0.85	0.80	0.08	0.08	0.95	1.02	0.06	0.35	1.02	1.11	0.70	0.25	1.10	1.13	0.64	0.63
Male	0.86	0.80	0.12	0.16	0.91	1.05	0.06	0.17	1.04	1.11	0.67	0.08	1.08	1.35	0.09	0.23
Female	0.83	0.81	0.38	0.37	0.95	0.98	0.57	0.88	0.99	1.04	0.79	0.92	1.15	1.05	0.26	0.69
Test for interaction			0.13	0.53			0.10	0.26			0.86	0.19			**<0.0001**	0.23

	6 weeks of age (1 day post-EPI1) : 5 days				6 weeks of age (5 days post-EPI1 +/- BCG) : 5 days				10 weeks of age (1 day post-EPI2) : 5 days			
	BCG at birth	BCG at 6wks	p-value	Adj p-value	BCG at birth	BCG at 6wks	p-value	Adj p-value	BCG at birth	BCG at 6wks	p-value	Adj p-value
All participants	1.02	**1.34**	**0.02**	0.52	1.33	1.21	0.22	0.46	1.48	1.29	0.14	0.39
Male	0.96	1.33	**0.01**	0.27	1.34	1.13	0.25	0.51	1.46	1.69	0.30	0.23
Female	1.17	1.36	0.54	0.71	1.17	1.24	0.94	0.83	1.58	1.25	0.06	**0.05**
Test for interaction			**0.01**	0.27			0..09	0.65			**<0.0001**	**0.02**

	6 weeks of age (5 days post-EPI1 +/- BCG) : 6 weeks of age (1 day post-EPI1)				10 weeks of age (1 day post-EPI2) : 6 weeks of age (1 day post-EPI1)				10 weeks of age (1 day post-EPI2) : 6 weeks of age (5 days post-EPI1 +/-BCG)			
	BCG at birth	**BCG at 6wks**	**p-value**	**Adj p-value**	**BCG at birth**	**BCG at 6wks**	**p-value**	**Adj p-value**	**BCG at birth**	**BCG at 6wks**	**p-value**	**Adj p-value**
All participants	1.14	1.10	0.49	0.96	1.22	1.18	0.63	0.68	1.11	1.24	0.06	0.09
Male	1.08	1.14	0.30	0.08	1.30	1.23	0.56	0.48	1.12	1.24	0.07	**0.05**
Female	1.16	1.06	**0.05**	**0.05**	1.14	1.09	0.38	0.49	1.07	1.15	0.25	0.38
Test for interaction			**<0.0001**	**0.02**			0.76	0.91			**0.05**	0.41

3.3.6 Ferritin

	5 days : Cord Blood				6 weeks of age (1 day post-EPI1) : Cord blood				6 weeks of age (5 days post-EPI1 +/- BCG) : Cord blood				10 weeks of age (1 day post-EPI2) : Cord Blood			
	BCG at birth	BCG at 6wks	p-value	Adj p-value	BCG at birth	BCG at 6wks	p-value	Adj p-value	BCG at birth	BCG at 6wks	p-value	Adj p-value	BCG at birth	BCG at 6wks	p-value	Adj p-value
All participants	1.65	1.83	0.60	0.95	1.39	1.25	0.16	**0.05**	1.74	1.31	0.09	0.07	1.03	0.84	0.21	0.21
Male	1.58	1.83	1.0	0.90	1.51	1.19	0.16	**0.03**	1.91	1.04	**0.05**	**0.03**	1.07	0.75	0.27	0.23
Female	1.65	1.82	0.57	0.84	1.35	1.28	0.50	0.57	1.70	1.41	0.72	0.77	1.0	1.08	0.72	0.61
Test for interaction			**0.005**	0.96			0.88	0.32			0.21	0.18			**0.01**	0.57
	6 weeks of age (1 day post-EPI1) : 5 days				**6 weeks of age (5 days post-EPI1 +/- BCG) : 5 days**				**10 weeks of age (1 day post-EPI2) : 5 days**							
	BCG at birth	BCG at 6wks	p-value	Adj p-value	BCG at birth	BCG at 6wks	p-value	Adj p-value	BCG at birth	BCG at 6wks	p-value	Adj p-value				
All participants	0.80	0.87	0.69	0.91	0.92	0.71	0.66	0.66	0.55	0.38	0.21	0.34				
Male	0.96	0.66	0.27	0.52	1.08	0.66	0.25	0.45	0.52	0.34	**0.02**	**0.02**				
Female	0.77	0.90	0.22	0.90	0.70	0.98	0.68	0.74	0.68	0.56	0.57	0.72				
Test for			**<0.0001**	0.07			**0.05**	0.65			0.33	0.50				

interaction												
	6 weeks of age (5 days post-EPI1 +/- BCG) : 6 weeks of age (1 day post-EPI1)				10 weeks of age (1 day post-EPI2) : 6 weeks of age (1 day post-EPI1)				10 weeks of age (1 day post-EPI2) : 6 weeks of age (5 days post-EPI1 +/- BCG)			
	BCG at birth	BCG at 6wks	p-value	Adj p-value	BCG at birth	BCG at 6wks	p-value	Adj p-value	BCG at birth	BCG at 6wks	p-value	Adj p-value
All participants	0.98	0.93	0.38	0.46	0.63	0.78	0.27	0.44	0.74	0.54	0.13	0.23
Male	0.96	0.85	0.52	0.80	0.59	0.79	0.10	0.16	0.63	0.54	0.47	0.64
Female	0.98	1.06	0.90	0.67	0.68	0.62	0.94	0.54	0.85	0.56	0.30	0.35
Test for interaction			0.36	0.79			0.47	0.85			0.77	0.86

3.3.7 Haemoglobin

	5 days : Cord Blood				6 weeks of age (1 day post-EPI1) : Cord blood				6 weeks of age (5 days post-EPI1 +/- BCG) : Cord blood				10 weeks of age (1 day post-EPI2) : Cord Blood			
	BCG at birth	BCG at 6wks	p-value	Adj p-value	BCG at birth	BCG at 6wks	p-value	Adj p-value	BCG at birth	BCG at 6wks	p-value	Adj p-value	BCG at birth	BCG at 6wks	p-value	Adj p-value
All participants	1.05	1.08	0.43	0.66	0.70	0.72	0.24	0.24	0.73	0.73	0.93	0.81	0.67	0.68	0.37	0.38
Male	1.04	1.08	0.60	0.86	0.71	0.71	0.90	0.99	0.72	0.69	0.39	0.89	0.66	0.67	0.81	**0.03**
Female	1.06	1.09	0.56	0.63	0.69	0.73	0.15	0.09	0.74	0.76	0.56	0.72	0.67	0.70	0.39	0.64
Test for interaction			0.74	0.98			**0.01**	0.17			**0.02**	0.68			0.41	0.08
	6 weeks of age (1 day post-EPI1) : 5 days				6 weeks of age (5 days post-EPI1 +/- BCG) : 5 days				10 weeks of age (1 day post-EPI2) : 5 days							
	BCG at birth	BCG at 6wks	p-value	Adj p-value	BCG at birth	BCG at 6wks	p-value	Adj p-value	BCG at birth	BCG at 6wks	p-value	Adj p-value				
All participants	0.71	0.66	0.27	0.95	0.68	0.76	0.17	0.73	0.61	0.65	0.24	0.89				
Male	0.70	0.66	0.36	0.97	0.68	0.81	0.08	0.15	0.56	0.66	0.19	0.31				
Female	0.71	0.67	0.58	0.83	0.68	0.72	0.63	0.54	0.65	0.65	0.87	0.99				
Test for			0.79	0.70			0.06	0.20			**0.002**	0.27				

interaction												
	6 weeks of age (5 days post-EPI1 +/- BCG) : 6 weeks of age (1 day post-EPI1)				10 weeks of age (1 day post-EPI2) : 6 weeks of age (1 day post-EPI1)				10 weeks of age (1 day post-EPI2) : 6 weeks of age (5 days post-EPI1 +/- BCG)			
	BCG at birth	BCG at 6wks	p-value	Adj p-value	BCG at birth	BCG at 6wks	p-value	Adj p-value	BCG at birth	BCG at 6wks	p-value	Adj p-value
All participants	1.00	1.03	0.09	**0.05**	0.95	0.97	0.60	0.77	0.91	0.93	0.25	0.39
Male	1.00	1.05	0.07	**0.02**	0.93	0.95	0.62	0.17	0.90	0.93	0.28	0.53
Female	1.00	1.01	0.62	0.64	0.97	0.99	0.81	0.80	0.91	0.93	0.61	0.42
Test for interaction			**0.01**	0.34			0.73	0.46			0.38	0.63

3.3.8 Haematocrit

	5 days : Cord Blood				6 weeks of age (1 day post-EPI1) : Cord blood				6 weeks of age (5 days post-EPI1 +/- BCG) : Cord blood				10 weeks of age (1 day post-EPI2) : Cord Blood			
	BCG at birth	BCG at 6wks	p-value	Adj p-value	BCG at birth	BCG at 6wks	p-value	Adj p-value	BCG at birth	BCG at 6wks	p-value	Adj p-value	BCG at birth	BCG at 6wks	p-value	Adj p-value
All participants	1.04	1.07	0.56	0.88	0.68	0.71	0.16	0.18	0.71	0.71	0.94	0.97	0.66	0.69	0.29	0.37
Male	1.03	10.6	0.68	0.99	0.69	0.70	0.86	0.93	0.70	0.67	0.33	0.57	0.66	0.67	0.54	0.06
Female	1.06	1.07	0.75	0.81	0.67	0.72	0.09	**0.04**	0.73	0.74	0.65	0.74	0.68	0.70	0.47	0.90
Test for interaction			0.65	0.97			**0.002**	0.09			**0.02**	0.51			0.98	0.27

	6 weeks of age (1 day post-EPI1) : 5 days				6 weeks of age (5 days post-EPI1 +/- BCG) : 5 days				10 weeks of age (1 day post-EPI2) : 5 days			
	BCG at birth	BCG at 6wks	p-value	Adj p-value	BCG at birth	BCG at 6wks	p-value	Adj p-value	BCG at birth	BCG at 6wks	p-value	Adj p-value
All participants	0.70	0.66	0.28	0.86	0.68	0.75	0.21	0.90	0.61	0.66	0.22	0.57
Male	0.70	0.66	0.42	0.80	0.67	0.80	0.11	0.34	0.57	0.68	0.12	0.36
Female	0.70	0.66	0.52	0.98	0.68	0.72	0.68	0.58	0.61	0.65	0.83	0.62
Test for			0.92	0.79			0.08	0.36			**,<0.0001**	0.51

interaction												
	6 weeks of age (5 days post-EPI1 +/- BCG) : 6 weeks of age (1 day post-EPI1)				10 weeks of age (1 day post-EPI2) : 6 weeks of age (1 day post-EPI1)				10 weeks of age (1 day post-EPI2) : 6 weeks of age (5 days post-EPI1 +/-BCG)			
	BCG at birth	BCG at 6wks	p-value	Adj p-value	BCG at birth	BCG at 6wks	p-value	Adj p-value	BCG at birth	BCG at 6wks	p-value	Adj p-value
All participants	1.01	1.03	0.30	0.16	0.98	0.99	0.81	0.68	0.93	0.96	0.13	0.19
Male	1.01	1.06	0.14	0.09	0.95	0.98	0.54	0.31	0.92	0.98	0.15	0.52
Female	1.01	1.00	0.79	0.93	1.01	0.99	0.81	0.88	0.93	0.96	0.47	0.30
Test for interaction			**0.001**	0.21			0.16	0.82			0.17	0.75

3.3.9 Mean Cell Volume

	5 days : Cord Blood				6 weeks of age (1 day post-EPI1) : Cord blood				6 weeks of age (5 days post-EPI1 +/- BCG) : Cord blood				10 weeks of age (1 day post-EPI2) : Cord Blood			
	BCG at birth	BCG at 6wks	p-value	Adj p-value	BCG at birth	BCG at 6wks	p-value	Adj p-value	BCG at birth	BCG at 6wks	p-value	Adj p-value	BCG at birth	BCG at 6wks	p-value	Adj p-value
All participants	0.96	0.95	0.24	0.69	0.85	0.85	0.87	0.66	0.84	0.83	**0.03**	0.16	0.81	0.81	0.24	0.27
Male	0.95	0.93	0.25	0.69	0.85	0.85	0.80	0.80	0.84	0.82	**0.008**	0.20	0.81	0.80	0.19	0.14
Female	0.97	0.96	0.40	0.65	0.85	0.85	0.71	0.42	0.84	0.83	0.49	0.43	0.82	0.81	0.57	0.58
Test for interaction			0.39	0.85			0.28	0.85			**0.003**	0.69			0.18	0.48

	6 weeks of age (1 day post-EPI1) : 5 days				6 weeks of age (5 days post-EPI1 +/- BCG) : 5 days				10 weeks of age (1 day post-EPI2) : 5 days			
	BCG at birth	BCG at 6wks	p-value	Adj p-value	BCG at birth	BCG at 6wks	p-value	Adj p-value	BCG at birth	BCG at 6wks	p-value	Adj p-value
All participants	0.90	0.88	0.27	0.95	0.87	0.89	0.28	0.33	0.84	0.84	0.80	0.79
Male	0.90	0.89	0.55	0.89	0.88	0.91	0.35	**0.03**	0.83	0.82	0.65	0.73
Female	0.90	0.87	0.34	0.93	0.87	0.89	0.49	0.97	0.84	0.85	0.89	0.87
Test for			0.37	0.97			0.51	0.16			0.19	0.59

interaction												
	6 weeks of age (5 days post-EPI1 +/- BCG) : 6 weeks of age (1 day post-EPI1)				10 weeks of age (1 day post-EPI2) : 6 weeks of age (1 day post-EPI1)				10 weeks of age (1 day post-EPI2) : 6 weeks of age (5 days post-EPI1 +/- BCG)			
	BCG at birth	BCG at 6wks	p-value	Adj p-value	BCG at birth	BCG at 6wks	p-value	Adj p-value	BCG at birth	BCG at 6wks	p-value	Adj p-value
All participants	0.98	0.97	**0.004**	**0.007**	0.95	0.95	0.93	0.89	0.97	0.95	0.13	0.16
Male	0.99	0.97	**0.0004**	**0.001**	0.95	0.96	0.77	0.73	0.97	0.96	0.64	0.71
Female	0.98	0.97	0.32	0.33	0.95	0.94	0.81	0.92	0.98	0.95	0.16	0.21
Test for interaction			**<0.0001**	0.06			0.37	0.87			0.09	0.46

3.3.10 Mean Cell Haemoglobin

	5 days : Cord Blood				6 weeks of age (1 day post-EPI1) : Cord blood				6 weeks of age (5 days post-EPI1 +/- BCG) : Cord blood				10 weeks of age (1 day post-EPI2) : Cord Blood			
	BCG at birth	**BCG at 6wks**	**p-value**	**Adj p-value**	**BCG at birth**	**BCG at 6wks**	**p-value**	**Adj p-value**	**BCG at birth**	**BCG at 6wks**	**p-value**	**Adj p-value**	**BCG at birth**	**BCG at 6wks**	**p-value**	**Adj p-value**
All participants	0.97	0.97	0.52	0.83	0.88	0.87	0.21	0.62	0.86	0.85	0.31	0.71	0.81	0.80	0.10	0.14
Male	0.97	0.96	0.48	0.60	0.87	0.87	0.74	0.77	0.86	0.85	0.13	0.18	0.81	0.79	**0.02**	**0.01**
Female	0.98	0.97	0.69	0.91	0.89	0.87	0.22	0.87	0.86	0.85	0.85	0.70	0.81	0.81	0.72	1.0
Test for interaction			0.63	0.92			**0.01**	0.60			0.08	0.94			**0.001**	0.06

	6 weeks of age (1 day post-EPI1) : 5 days				6 weeks of age (5 days post-EPI1 +/- BCG) : 5 days				10 weeks of age (1 day post-EPI2) : 5 days			
	BCG at birth	**BCG at 6wks**	**p-value**	**Adj p-value**	**BCG at birth**	**BCG at 6wks**	**p-value**	**Adj p-value**	**BCG at birth**	**BCG at 6wks**	**p-value**	**Adj p-value**
All participants	0.91	0.89	0.22	0.95	0.88	0.91	0.14	0.13	0.83	0.83	0.91	0.85
Male	0.91	0.89	0.24	0.68	0.89	0.92	0.16	**0.009**	0.83	0.78	0.12	0.16
Female	0.91	0.90	0.67	0.60	0.87	0.89	0.40	0.69	0.83	0.84	0.67	0.57

Test for interaction			0.33	0.51			0.25	0.16			**<0.0001**	**0.05**
	6 weeks of age (5 days post-EPI1 +/- BCG) : 6 weeks of age (1 day post-EPI1)				**10 weeks of age (1 day post-EPI2) : 6 weeks of age (1 day post-EPI1)**				**10 weeks of age (1 day post-EPI2) : 6 weeks of age (5 days post-EPI1 +/-BCG)**			
	BCG at birth	**BCG at 6wks**	**p-value**	**Adj p-value**	**BCG at birth**	**BCG at 6wks**	**p-value**	**Adj p-value**	**BCG at birth**	**BCG at 6wks**	**p-value**	**Adj p-value**
All participants	0.98	0.98	0.99	0.80	0.93	0.93	0.35	0.45	0.95	0.92	**0.007**	**0.01**
Male	0.98	0.97	0.28	0.37	0.93	0.93	0.96	0.26	0.94	0.91	0.16	0.20
Female	0.97	0.98	0.34	0.40	0.92	0.94	0.17	0.28	0.96	0.92	**0.02**	**0.03**
Test for interaction			**<0.0001**	0.28			**0.03**	0.21			0.51	0.75

3.3.11 Mean Cell Haemoglobin Concentration

	5 days : Cord Blood				6 weeks of age (1 day post-EPI1) : Cord blood				6 weeks of age (5 days post-EPI1 +/- BCG) : Cord blood				10 weeks of age (1 day post-EPI2) : Cord Blood			
	BCG at birth	BCG at 6wks	p-value	Adj p-value	BCG at birth	BCG at 6wks	p-value	Adj p-value	BCG at birth	BCG at 6wks	p-value	Adj p-value	BCG at birth	BCG at 6wks	p-value	Adj p-value
All participants	1.01	1.02	0.16	**0.04**	1.03	1.02	0.23	0.87	1.02	1.03	0.26	0.10	1.0	0.99	0.37	0.41
Male	1.015	1.023	0.25	**0.05**	1.02	1.02	0.95	0.49	1.03	1.03	0.41	**0.02**	1.004	0.99	**0.02**	**0.002**
Female	1.007	1.014	0.26	0.25	1.03	1.02	0.15	0.41	1.02	1.03	0.39	0.63	0.99	1.0	0.67	0.21
Test for interaction			0.65	0.44			**0.004**	0.13			0.87	0.2			**<0.0001**	**0.003**

	6 weeks of age (1 day post-EPI1) : 5 days				6 weeks of age (5 days post-EPI1 +/- BCG) : 5 days				10 weeks of age (1 day post-EPI2) : 5 days			
	BCG at birth	BCG at 6wks	p-value	Adj p-value	BCG at birth	BCG at 6wks	p-value	Adj p-value	BCG at birth	BCG at 6wks	p-value	Adj p-value
All participants	1.01	1.01	0.86	0.23	1.01	1.12	0.44	0.23	0.99	0.99	0.59	0.64
Male	1.01	1.00	0.54	0.44	1.01	1.28	0.37	**0.005**	1.0	0.96	0.09	0.12
Female	1.01	1.03	0.15	0.12	1.02	1.01	0.92	0.78	0.99	0.99	0.80	0.25
Test for interaction			**0.001**	0.22			**0.02**	**0.02**			**<0.0001**	**0.04**

	6 weeks of age (5 days post-EPI1 +/- BCG) : 6 weeks of age (1 day post-EPI1)				10 weeks of age (1 day post-EPI2) : 6 weeks of age (1 day post-EPI1)				10 weeks of age (1 day post-EPI2) : 6 weeks of age (5 days post-EPI1 +/- BCG)			
	BCG at birth	**BCG at 6wks**	**p-value**	**Adj p-value**	**BCG at birth**	**BCG at 6wks**	**p-value**	**Adj p-value**	**BCG at birth**	**BCG at 6wks**	**p-value**	**Adj p-value**
All participants	0.99	1.00	0.15	0.15	0.97	0.98	0.58	0.89	0.98	0.97	0.18	0.17
Male	0.99	0.98	0.74	0.66	0.98	0.97	0.45	0.09	0.98	0.95	0.22	0.18
Female	0.99	1.01	0.09	0.21	0.97	0.99	0.10	0.18	0.98	0.97	0.40	0.42
Test for interaction			**0.02**	0.40			**<0.0001**	**0.04**			0.10	0.45

3.3.12 Red Cell Distribution Width

	5 days : Cord Blood				6 weeks of age (1 day post-EPI1) : Cord blood				6 weeks of age (5 days post-EPI1 +/- BCG) : Cord blood				10 weeks of age (1 day post-EPI2) : Cord Blood			
	BCG at birth	BCG at 6wks	p-value	Adj p-value	BCG at birth	BCG at 6wks	p-value	Adj p-value	BCG at birth	BCG at 6wks	p-value	Adj p-value	BCG at birth	BCG at 6wks	p-value	Adj p-value
All participants	0.99	1.0	0.89	0.90	1.01	0.99	0.21	0.23	1.02	1.01	0.61	0.65	0.93	0.94	0.73	0.68
Male	0.99	0.99	0.86	0.91	1.01	0.99	0.27	0.37	1.04	10.3	0.53	0.63	0.94	0.95	0.67	0.53
Female	1.00	1.00	0.90	0.87	1.01	0.99	0.53	0.44	1.00	1.00	0.88	0.90	0.92	0.93	0.83	0.75
Test for interaction			0.62	0.93			0.52	0.97			0.20	0.64			0.71	0.82

	6 weeks of age (1 day post-EPI1) : 5 days				6 weeks of age (5 days post-EPI1 +/- BCG) : 5 days				10 weeks of age (1 day post-EPI2) : 5 days			
	BCG at birth	BCG at 6wks	p-value	Adj p-value	BCG at birth	BCG at 6wks	p-value	Adj p-value	BCG at birth	BCG at 6wks	p-value	Adj p-value
All participants	1.02	1.00	0.39	0.37	1.02	1.02	0.79	0.79	0.95	0.95	0.95	0.92
Male	1.03	0.99	0.22	0.24	1.02	1.03	0.81	0.82	0.96	1.08	**0.04**	0.08
Female	1.01	1.02	0.76	0.77	1.02	1.01	0.61	0.61	0.94	0.92	0.57	0.68
Test for			**0.01**	0.32			0.21	0.60			**<0.0001**	0.06

interaction												
	6 weeks of age (5 days post-EPI1 +/- BCG) : 6 weeks of age (1 day post-EPI1)				10 weeks of age (1 day post-EPI2) : 6 weeks of age (1 day post-EPI1)				10 weeks of age (1 day post-EPI2) : 6 weeks of age (5 days post-EPI1 +/-BCG)			
	BCG at birth	BCG at 6wks	p-value	Adj p-value	BCG at birth	BCG at 6wks	p-value	Adj p-value	BCG at birth	BCG at 6wks	p-value	Adj p-value
All participants	1.02	1.02	0.94	0.84	0.94	0.92	0.36	0.57	0.93	0.96	0.36	0.31
Male	1.03	1.02	0.82	0.27	0.93	0.95	0.53	0.22	0.94	0.92	0.84	0.82
Female	1.01	1.01	0.94	0.94	0.95	0.88	0.13	0.16	0.93	0.99	0.16	0.12
Test for interaction			0.59	0.71			**<0.0001**	**0.04**			**0.02**	0.24

3.3.13 Red Blood Cells

	5 days : Cord Blood				6 weeks of age (1 day post-EPI1) : Cord blood				6 weeks of age (5 days post-EPI1 +/- BCG) : Cord blood				10 weeks of age (1 day post-EPI2) : Cord Blood			
	BCG at birth	BCG at 6wks	p-value	Adj p-value	BCG at birth	BCG at 6wks	p-value	Adj p-value	BCG at birth	BCG at 6wks	p-value	Adj p-value	BCG at birth	BCG at 6wks	p-value	Adj p-value
All participants	0.40	0.37	0.31	0.47	0.29	0.28	0.71	0.97	0.31	0.28	0.30	0.42	0.30	0.28	0.96	0.55
Male	0.40	0.36	0.31	0.43	0.31	0.28	0.33	0.39	0.31	0.30	0.58	0.57	0.27	0.31	0.33	0.46
Female	0.39	0.38	0.72	0.84	0.26	0.28	0.55	0.31	0.31	0.28	0.40	0.42	0.29	0.26	0.38	0.92
Test for interaction			0.22	0.66			**0.01**	0.21			0.60	0.71			**0.002**	0.26

	6 weeks of age (1 day post-EPI1) : 5 days				6 weeks of age (5 days post-EPI1 +/- BCG) : 5 days				10 weeks of age (1 day post-EPI2) : 5 days			
	BCG at birth	BCG at 6wks	p-value	Adj p-value	BCG at birth	BCG at 6wks	p-value	Adj p-value	BCG at birth	BCG at 6wks	p-value	Adj p-value
All participants	0.78	0.74	0.35	0.39	0.43	0.45	0.63	0.79	0.43	0.42	0.75	0.94
Male	0.78	0.74	0.47	0.51	0.42	0.46	0.46	0.38	0.39	0.41	0.81	0.75
Female	0.78	0.75	0.61	0.66	0.45	0.44	0.88	0.84	0.47	0.42	0.42	0.67
Test for			0.87	0.93			0.10	0.61			0.13	0.29

interaction												
	6 weeks of age (5 days post-EPI1 +/- BCG) : 6 weeks of age (1 day post-EPI1)				10 weeks of age (1 day post-EPI2) : 6 weeks of age (1 day post-EPI1)				10 weeks of age (1 day post-EPI2) : 6 weeks of age (5 days post-EPI1 +/-BCG)			
	BCG at birth	BCG at 6wks	p-value	Adj p-value	BCG at birth	BCG at 6wks	p-value	Adj p-value	BCG at birth	BCG at 6wks	p-value	Adj p-value
All participants	0.35	0.24	0.07	0.07	1.03	1.04	0.82	0.98	0.95	1.02	**0.01**	**0.02**
Male	0.38	0.24	**0.03**	**0.04**	1.00	1.03	0.66	0.76	0.95	1.02	**0.04**	0.13
Female	0.33	0.24	0.44	0.33	1.06	1.05	0.91	0.79	0.95	1.01	0.13	0.17
Test for interaction			0.28	0.84			0.37	0.63			0.70	0.81

3.3.14 White Blood Cells

	5 days : Cord Blood				6 weeks of age (1 day post-EPI1) : Cord blood				6 weeks of age (5 days post-EPI1 +/- BCG) : Cord blood				10 weeks of age (1 day post-EPI2) : Cord Blood			
	BCG at birth	BCG at 6wks	p-value	Adj p-value	BCG at birth	BCG at 6wks	p-value	Adj p-value	BCG at birth	BCG at 6wks	p-value	Adj p-value	BCG at birth	BCG at 6wks	p-value	Adj p-value
All participants	0.69	0.63	0.31	0.58	1.04	1.03	0.91	0.89	0.68	0.63	0.56	0.75	0.99	0.96	0.78	0.54
Male	0.69	0.64	0.37	0.41	1.06	1.05	0.89	0.77	0.68	0.73	0.71	0.64	0.93	1.05	0.44	0.95
Female	0.68	0.62	0.58	0.92	1.01	1.00	0.98	0.78	0.67	0.56	0.38	0.80	1.05	0.91	0.27	0.49
Test for interaction			0.73	0.65			0.89	0.66			**0.04**	0.84			**0.002**	0.68

	6 weeks of age (1 day post-EPI1) : 5 days				6 weeks of age (5 days post-EPI1 +/- BCG) : 5 days				10 weeks of age (1 day post-EPI2) : 5 days			
	BCG at birth	BCG at 6wks	p-value	Adj p-value	BCG at birth	BCG at 6wks	p-value	Adj p-value	BCG at birth	BCG at 6wks	p-value	Adj p-value
All participants	1.45	1.33	0.77	0.73	1.04	1.09	0.63	0.94	1.56	1.45	0.53	0.51
Male	1.53	1.60	0.73	0.37	1.05	1.33	0.09	0.17	1.61	1.62	0.98	0.86
Female	1.36	0.98	0.21	0.27	1.02	0.95	0.86	0.90	1.52	1.41	0.64	0.76
Test for			**0.01**	0.14			**0.01**	0.55			0.61	0.98

interaction												
	6 weeks of age (5 days post-EPI1 +/- BCG) : 6 weeks of age (1 day post-EPI1)				10 weeks of age (1 day post-EPI2) : 6 weeks of age (1 day post-EPI1)				10 weeks of age (1 day post-EPI2) : 6 weeks of age (5 days post-EPI1 +/- BCG)			
	BCG at birth	BCG at 6wks	p-value	Adj p-value	BCG at birth	BCG at 6wks	p-value	Adj p-value	BCG at birth	BCG at 6wks	p-value	Adj p-value
All participants	0.64	0.60	0.63	0.09	1.02	0.98	0.84	0.35	1.41	1.59	0.41	0.44
Male	0.76	0.63	0.30	0.36	1.02	1.19	0.40	0.94	1.33	1.38	0.82	0.87
Female	0.55	0.57	0.95	0.29	1.02	0.79	**0.04**	**0.03**	1.50	1.71	0.53	0.80
Test for interaction			0.1	0.99			**<0.0001**	0.27			0.46	0.76

3.3.15 Neutrophils

	5 days : Cord Blood				6 weeks of age (1 day post-EPI1) : Cord blood				6 weeks of age (5 days post-EPI1 +/- BCG) : Cord blood				10 weeks of age (1 day post-EPI2) : Cord Blood			
	BCG at birth	BCG at 6wks	p-value	Adj p-value	BCG at birth	BCG at 6wks	p-value	Adj p-value	BCG at birth	BCG at 6wks	p-value	Adj p-value	BCG at birth	BCG at 6wks	p-value	Adj p-value
All participants	0.43	0.37	0.11	0.25	0.92	0.95	0.88	0.56	0.29	0.28	0.56	0.80	0.88	0.80	0.44	0.21
Male	0.43	0.39	0.50	0.72	0.95	1.0	0.81	0.48	0.30	0.32	0.74	0.65	0.91	1.0	0.52	0.61
Female	0.42	0.36	0.13	0.25	0.90	0.89	0.98	0.78	0.29	0.24	0.31	0.42	0.85	0.69	0.17	0.27
Test for interaction			0.21	0.54			0.70	0.43			**0.03**	0.32			**<0.001**	0.54

	6 weeks of age (1 day post-EPI1) : 5 days				6 weeks of age (5 days post-EPI1 +/- BCG) : 5 days				10 weeks of age (1 day post-EPI2) : 5 days			
	BCG at birth	BCG at 6wks	p-value	Adj p-value	BCG at birth	BCG at 6wks	p-value	Adj p-value	BCG at birth	BCG at 6wks	p-value	Adj p-value
All participants	2.22	1.84	0.66	0.86	0.73	0.88	0.38	0.82	2.21	1.83	0.35	0.25
Male	2.24	2.41	0.82	0.10	0.69	1.16	0.05	0.13	2.86	1.71	0.29	0.08
Female	2.20	1.20	0.16	0.14	0.78	0.74	0.83	0.20	1.71	1.86	0.88	0.91
Test for			**0.02**	0.08			**0.001**	0.33			**0.01**	0.27

interaction												
	6 weeks of age (5 days post-EPI1 +/- BCG) : 6 weeks of age (1 day post-EPI1)				10 weeks of age (1 day post-EPI2) : 6 weeks of age (1 days post-EPI1)				10 weeks of age (1 day post-EPI2) : 6 weeks of age (5 days post-EPI1 +/- BCG)			
	BCG at birth	BCG at 6wks	p-value	Adj p-value	BCG at birth	BCG at 6wks	p-value	Adj p-value	BCG at birth	BCG at 6wks	p-value	Adj p-value
All participants	0.29	0.26	0.68	0.14	1.07	1.00	0.81	0.15	2.47	2.60	0.80	0.57
Male	0.41	0.28	0.36	0.14	1.05	1.34	0.53	0.31	2.37	2.33	0.96	0.63
Female	0.22	0.25	0.70	0.36	1.09	0.72	0.08	0.07	2.57	2.76	0.84	0.62
Test for interaction			**0.02**	0.78			**0.001**	0.79			0.65	0.95

3.3.16 Lymphocytes

	5 days : Cord Blood				6 weeks of age (1 day post-EPI1) : Cord blood				6 weeks of age (5 days post-EPI1 +/- BCG) : Cord blood				10 weeks of age (1 day post-EPI2) : Cord Blood			
	BCG at birth	BCG at 6wks	p-value	Adj p-value	BCG at birth	BCG at 6wks	p-value	Adj p-value	BCG at birth	BCG at 6wks	p-value	Adj p-value	BCG at birth	BCG at 6wks	p-value	Adj p-value
All participants	0.90	0.80	0.32	0.74	1.13	1.06	0.54	0.53	1.14	1.04	0.49	0.65	1.11	0.80	0.78	0.43
Male	0.91	0.77	0.12	0.13	1.13	1.04	0.44	0.33	1.08	1.21	0.60	0.67	0.97	1.11	0.51	0.46
Female	0.89	0.83	0.96	0.38	1.12	1.09	0.91	0.96	1.22	0.93	0.22	0.84	1.25	1.14	0.63	0.77
Test for interaction			**0.03**	0.16			0.37	0.52			**0.004**	0.97			**0.05**	0.61

	6 weeks of age (1 day post-EPI1) : 5 days				6 weeks of age (5 days post-EPI1 +/- BCG) : 5 days				10 weeks of age (1 day post-EPI2) : 5 days			
	BCG at birth	BCG at 6wks	p-value	Adj p-value	BCG at birth	BCG at 6wks	p-value	Adj p-value	BCG at birth	BCG at 6wks	p-value	Adj p-value
All participants	1.19	1.14	0.90	0.48	1.29	1.25	0.97	0.78	1.38	1.37	0.96	0.74
Male	1.22	2.41	0.56	1.0	1.33	1.50	0.39	0.23	1.29	1.90	0.46	0.52
Female	1.16	0.93	0.35	0.29	1.25	1.11	0.72	0.97	1.48	1.28	0.53	0.89
Test for			**0.008**	0.29			0.06	0.50			**0.007**	0.41

interaction												
	6 weeks of age (5 days post-EPI1 +/- BCG) : 6 weeks of age (1 day post-EPI1)				10 weeks of age (1 day post-EPI2) : 6 weeks of age (1 day post-EPI1)				10 weeks of age (1 day post-EPI2) : 6 weeks of age (5 days post-EPI1 +/- BCG)			
	BCG at birth	BCG at 6wks	p-value	Adj p-value	BCG at birth	BCG at 6wks	p-value	Adj p-value	BCG at birth	BCG at 6wks	p-value	Adj p-value
All participants	1.00	1.02	0.75	0.26	1.00	1.00	0.95	1.0	0.95	1.25	**0.03**	0.06
Male	1.04	1.17	0.23	0.10	0.98	1.11	0.39	0.41	0.94	1.04	0.57	0.60
Female	0.98	0.89	0.70	0.82	1.02	0.89	0.25	0.26	0.97	1.39	**0.05**	0.17
Test for interaction			**0.02**	0.59			**0.001**	0.21			**0.01**	0.50

3.3.17 Monocytes

	5 days : Cord Blood				6 weeks of age (1 day post-EPI1) : Cord blood				6 weeks of age (5 days post-EPI1 +/- BCG) : Cord blood				10 weeks of age (1 day post-EPI2) : Cord Blood			
	BCG at birth	BCG at 6wks	p-value	Adj p-value	BCG at birth	BCG at 6wks	p-value	Adj p-value	BCG at birth	BCG at 6wks	p-value	Adj p-value	BCG at birth	BCG at 6wks	p-value	Adj p-value
All participants	0.93	0.88	0.67	0.91	1.22	1.18	0.70	0.58	0.73	0.69	0.42	0.80	0.98	0.95	0.91	0.65
Male	0.98	0.87	0.38	0.41	1.31	1.17	0.49	0.74	0.81	0.72	0.21	0.16	0.86	1.09	0.25	0.93
Female	0.88	0.89	0.72	0.38	1.11	1.19	0.82	0.52	0.65	0.66	0.84	0.27	1.10	0.87	0.13	0.30
Test for interaction			0.05	0.22			0.14	0.89			**0.02**	0.07			**<0.0001**	0.39

	6 weeks of age (1 day post-EPI1) : 5 days				6 weeks of age (5 days post-EPI1 +/- BCG) : 5 days				10 weeks of age (1 day post-EPI2) : 5 days			
	BCG at birth	BCG at 6wks	p-value	Adj p-value	BCG at birth	BCG at 6wks	p-value	Adj p-value	BCG at birth	BCG at 6wks	p-value	Adj p-value
All participants	1.34	1.0	0.11	0.21	0.81	0.92	0.42	0.79	1.21	0.94	0.11	0.12
Male	1.39	1.06	0.35	0.99	0.86	1.26	0.17	0.95	1.22	1.16	0.81	0.82
Female	1.27	0.90	0.12	0.13	0.75	0.76	0.92	0.68	1.19	0.90	0.18	0.22
Test for			0.44	0.50			**0.02**	0.88			0.20	0.67

interaction												
	6 weeks of age (5 days post-EPI1 +/- BCG) : 6 weeks of age (1 day post-EPI1)				10 weeks of age (1 day post-EPI2) : 6 weeks of age (1 day post-EPI1)				10 weeks of age (1 day post-EPI2) : 6 weeks of age (5 days post-EPI1 +/- BCG)			
	BCG at birth	BCG at 6wks	p-value	Adj p-value	BCG at birth	BCG at 6wks	p-value	Adj p-value	BCG at birth	BCG at 6wks	p-value	Adj p-value
All participants	0.60	0.68	0.93	0.08	0.87	0.95	0.45	0.76	1.32	1.44	0.86	0.59
Male	0.68	0.62	0.91	0.39	0.91	1.22	0.25	0.89	1.16	1.46	0.47	1.0
Female	0.53	0.62	0.86	0.17	0.83	0.72	0.24	0.16	1.51	1.42	0.67	0.92
Test for interaction			0.61	0.77			**0.001**	0.34			**0.05**	0.91

3.3.18 Eosinophils

	5 days : Cord Blood				6 weeks of age (1 day post-EPI1) : Cord blood				6 weeks of age (5 days post-EPI1 +/- BCG) : Cord blood				10 weeks of age (1 day post-EPI2) : Cord Blood			
	BCG at birth	BCG at 6wks	p-value	Adj p-value	BCG at birth	BCG at 6wks	p-value	Adj p-value	BCG at birth	BCG at 6wks	p-value	Adj p-value	BCG at birth	BCG at 6wks	p-value	Adj p-value
All participants	1.37	1.30	0.61	0.13	0.68	0.57	0.35	0.23	0.87	0.75	0.30	0.32	1.13	1.05	0.52	0.09
Male	1.47	1.17	0.21	**0.05**	0.67	0.54	0.49	0.65	0.83	0.82	0.77	0.89	1.20	0.90	0.23	0.15
Female	1.26	1.41	0.55	0.99	0.70	0.61	0.53	0.18	0.91	0.70	0.21	0.12	1.06	1.16	0.86	0.28
Test for interaction			**0.003**	0.14			0.95	0.52			0.29	0.33			**0.02**	0.40

	6 weeks of age (1 day post-EPI1) : 5 days				6 weeks of age (5 days post-EPI1 +/- BCG) : 5 days				10 weeks of age (1 day post-EPI2) : 5 days			
	BCG at birth	BCG at 6wks	p-value	Adj p-value	BCG at birth	BCG at 6wks	p-value	Adj p-value	BCG at birth	BCG at 6wks	p-value	Adj p-value
All participants	0.45	0.54	0.42	0.94	1.02	0.75	0.17	0.16	0.79	0.73	0.60	0.41
Male	0.47	1.06	0.39	0.72	1.22	1.02	0.56	0.53	0.76	0.56	0.42	0.46
Female	1.27	0.44	0.94	0.79	0.82	0.61	0.21	0.21	0.81	0.77	0.65	0.57
Test for			0.21	0.56			0.98	0.91			0.54	0.85

interaction												
	6 weeks of age (5 days post-EPI1 +/- BCG) : 6 weeks of age (1 day post-EPI1)				10 weeks of age (1 day post-EPI2) : 6 weeks of age (1 day post-EPI1)				10 weeks of age (1 day post-EPI2) : 6 weeks of age (5 days post-EPI1 +/-BCG)			
	BCG at birth	BCG at 6wks	p-value	Adj p-value	BCG at birth	BCG at 6wks	p-value	Adj p-value	BCG at birth	BCG at 6wks	p-value	Adj p-value
All participants	1.16	1.64	0.12	0.26	2.18	1.43	0.11	**0.05**	1.38	1.60	0.55	0.73
Male	1.12	1.63	0.34	0.37	2.83	1.30	0.11	0.06	1.56	0.88	0.15	0.60
Female	1.19	1.66	0.24	0.82	1.62	1.59	0.78	0.66	1.23	2.24	0.19	0.81
Test for interaction			0.73	0.53			**0.002**	0.25			**<0.0001**	0.97

3.3.19 Basophils

	5 days : Cord Blood				6 weeks of age (1 day post-EPI1) : Cord blood				6 weeks of age (5 days post-EPI1 +/- BCG) : Cord blood				10 weeks of age (1 day post-EPI2) : Cord Blood			
	BCG at birth	BCG at 6wks	p-value	Adj p-value	BCG at birth	BCG at 6wks	p-value	Adj p-value	BCG at birth	BCG at 6wks	p-value	Adj p-value	BCG at birth	BCG at 6wks	p-value	Adj p-value
All participants	1.03	0.88	0.45	0.42	0.93	0.97	0.97	1.0	0.58	0.56	0.72	0.54	0.77	0.77	0.94	0.67
Male	1.04	0.77	0.19	0.13	0.97	0.92	0.70	0.92	0.59	0.58	0.59	0.71	0.58	0.80	0.36	0.82
Female	1.02	0.97	0.82	0.82	0.88	1.03	0.68	0.92	0.57	0.55	0.99	0.25	1.00	0.75	0.33	0.53
Test for interaction			**0.01**	0.24			0.19	0.96			0.43	0.28			**0.002**	0.67

	6 weeks of age (1 day post-EPI1) : 5 days				6 weeks of age (5 days post-EPI1 +/- BCG) : 5 days				10 weeks of age (1 day post-EPI2) : 5 days			
	BCG at birth	BCG at 6wks	p-value	Adj p-value	BCG at birth	BCG at 6wks	p-value	Adj p-value	BCG at birth	BCG at 6wks	p-value	Adj p-value
All participants	0.97	0.66	0.26	0.50	0.56	0.97	**0.05**	0.19	0.59	0.83	0.50	0.55
Male	1.07	0.91	0.88	0.73	0.69	1.39	**0.03**	0.14	0.34	1.73	**0.02**	0.20
Female	0.86	0.39	0.14	0.15	0.45	0.77	0.27	0.45	1.02	0.72	0.40	0.50
Test for			**0.01**	0.19			0.29	0.80			**<0.0001**	0.43

interaction												
	6 weeks of age (5 days post-EPI1 +/- BCG) : 6 weeks of age (1 day post-EPI1)				10 weeks of age (1 day post-EPI2) : 6 weeks of age (1 day post-EPI1)				10 weeks of age (1 day post-EPI2) : 6 weeks of age (5 days post-EPI1 +/-BCG)			
	BCG at birth	BCG at 6wks	p-value	Adj p-value	BCG at birth	BCG at 6wks	p-value	Adj p-value	BCG at birth	BCG at 6wks	p-value	Adj p-value
All participants	0.59	0.59	0.80	0.21	0.98	0.85	0.75	0.46	1.26	1.65	0.40	0.31
Male	0.75	0.69	0.78	0.43	0.92	1.18	0.28	0.55	1.06	1.46	0.45	0.53
Female	0.49	0.50	0.86	0.48	1.06	0.58	**0.02**	**0.01**	1.49	1.77	0.78	0.59
Test for interaction			0.97	0.66			**<0.0001**	0.08			0.42	0.97