

Evaluation of the non-specific effects of Bacille Calmette-Guérin (BCG) vaccination in healthy UK adults

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Submitted for degree of DPhil in Clinical Medicine

Michaelmas 2017

Word Count: 50,000

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Tuberculosis is the leading single infectious disease killer in the world, and the global burden of LTBI and TB disease, with the addition of antimicrobial drug resistance means there is a desperate need for a safe and effective TB vaccine. BCG is the only licensed vaccine and extremely variable in efficacy. However, BCG potentially exerts a non-specific beneficial effect - protecting infants from other causes of death such as respiratory infections and fever. Data from randomised and observational studies in support have been variable in quality; however the WHO reviewed the data and concluded that the effect was likely real, but that more robust data was needed. The aim of my study was to evaluate the potential non-specific effects of BCG using an *in-vitro* whole blood growth inhibition assay as a surrogate marker of an individual's ability to control growth of bacteria before and after vaccination with BCG. Identification of improved control of bacterial net growth following vaccination with BCG would provide evidence to support large, costly, randomised control trials in TB high burden countries. The assay did not demonstrate improved control in growth of four pathogens in the whole blood GIA following BCG vaccination and was subject to bacterial inoculum batch effect and other variables such as haemoglobin. A PBMC GIA potentially detected a subtle improvement in control of bacteria in the same volunteer cohort, but requires further optimisation. Volunteers who received BCG vaccination had an expected clinical and immunological reaction to vaccination with BCG. Pilot data exploring innate immune 'memory' through secondary antigenic stimulation of PBMCs did not demonstrate a significant increase in cytokine production, but numbers were small and more work is necessary.

Acknowledgements

I would like to start by thanking my supervisor Professor Helen McShane for her unwavering support and belief in my ability to undertake and tackle head on the challenge of this DPhil. It has been a journey with moments of joy and utter exhaustion, but has been made all the better because of her encouragement and friendship. I would also like to thank my other supervisor, Dr David Wyllie, who was always able to provide a valuable and different perspective on the data.

The laboratory work was only possible with the immense help of Rachel Tanner and Danny Wright who spent many, many, long hours slaving over a spiral plater with me well into the dark hours and saved my sanity on many occasions. I would also like to thank Iman Satti, Jenny McNulty, Stephanie Harris, Ashley Jacobs, Ali Hamidi, Julia Beglov, Paulo Bettencourt, Elena Stylianou, Amy Flaxman and Janet Pasricha who all at some point or another helped keep me on track. From the clinical side I would like to thank in particular Sam Vermaak, Ruth Payne, Raquel Lopes Ramon, Mike Riste, Zita Manjaly-Thomas, Linnea Rask, Hannah Johnson, Oliver Griffiths, Navin Venkatraman, Tommy Rampling, Ian Poulson, Rachel Roberts and Alison Lawrie, who all provided excellent support both logistically, but also emotionally on many an occasion.

Some special thanks go to Dr Stuart Schembri, who has through my career taken me under his wing and provided me with some generally excellent career advice and who is in part responsible for where I am today – Grazzi.

To my Oxford family – Alison, Patch, Tamsin, Claire, Ruth, Iain, Charlotte, Olivia and Edward, and my Dundee family - Jacqui, Simon and Beano, Cindy, Ken, Tarryn and Dulcie, I appreciate you friendship more than you will know. Finally I want to thank my family, Struan, Elaine, Cameron and Eliza; Lorne and Eoghainn, but especially my Mum, Dad, and

Lorne, your trips up and down the M6 to visit and help pack, move and unpack me were pure gold, and every moment cherished.

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Abbreviations

~	Approximately
AE	Adverse Event
AG	Arabinogalactan
ALT	Alanine Aminotransferase
ALP	Alkaline Phosphatase
ART	Anti-retroviral therapy
BCC	Basal cell carcinoma
BCG	Bacille Calmette-Guérin
BTS	British thoracic society
C. albicans	*Candida albicans*
CCVTM	Centre for Clinical Vaccinology and Tropical Medicine
CI	Chief Investigator
CIS	Carcinoma in situ
CFP-10	Culture filtrate protein 10kDa
CFU	Colony-forming unit
CNS	Central nervous system
CRF	Case Report Form
DNA	Deoxyribonucleic acid
DT	Diphtheria tetanus vaccine

DTP	Diphtheria tetanus polio vaccine
EC	Ethics Committee
EC-LPS	*E. coli* Lipopolysaccharide
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
ELISPOT	Enzyme-Linked Immunosorbent Spot
ESAT-6	Early-secreted antigenic target 6kDa protein
FBC	Full Blood Count
FCS	Foetal calf serum
F. magna	*Fascioloides magna*
GCP	Good Clinical Practice
GIA	Growth Inhibition Assay
GLP	Good Laboratory Practice
GMP	Good Manufacturing Practice
GM-CSF	Granulocyte macrophage colony stimulating factor
GP	General Practitioner (Family Doctor)
HBsAg	Hepatitis B Surface Antigen
HBV	Hepatitis B Virus
HCG	Human Chorionic Gonadotropin
HCV	Hepatitis C Virus

HIV	Human Immunodeficiency Virus
HKSA	Heat Killed *Staphylococcus aureus*
HKCA	Heat Killed *Candida albicans*
HLA	Human Leukocyte Antigen
ICH	International Committee on Harmonisation
ICAM-1	Intracellular adhesion molecule - 1
ID	Intradermal
IFN-ɣ	Interferon Gamma
IGRA	Interferon Gamma Release Assay
IL	Interleukin
IMP	Investigational medicinal product
IRR	Incidence rate ratio
K. pneumoniae	*Klebsiella pneumoniae*
LAM	Lipoarabinomannan
LB	Lysogeny broth
LFT	Liver Function Test
LM	Lipomannan
LPS	Lipopolysaccharide
LSC	Local Safety Committee
LSM	Local Safety Monitor

MAGP	Mycolylarabinogalactanpeptidoglycan complex
M. bovis	*Mycobacterium bovis*
MDR-TB	Multi drug resistant tuberculosis
MGIA	Mycobacterial growth inhibition assay
MGIT	Mycobacterial growth indicator tube
MOI	Multiplicity of Infection
M. tb	*Mycobacterium tuberculosis*
MHC	Major histocompatibility complex
MRC	Medical Research Council
NHS	National Health Service
NICE	National institute for clinical excellence
NIHR WTCRF	National Institute for Health Research Wellcome Trust Clinical Research Facility
NK	Natural killer cells
ORF	Open reading frame
PBMC	Peripheral Blood Mononuclear Cells
PCR	Polymerase Chain Reaction
PF	Preventative fraction
PG	Peptidoglycan
PHE	Public Health England

PIMS	Phosphatidylinositol
pNPP	4-nitrophenylphosphate
RR	Relative risk
RR-TB	Rifampicin resistant tuberculosis
RSV	Respiratory syncytial virus
SAE	Serious Adverse Event
SAGE	Strategic advisory group of experts
SDG	Sustainable development goals
SEAR	South East Asian Region
Sii	Serum Institute of India
SOP	Standard Operating Procedure
S. agalactiae	*Streptococcus agalactiae*
S. aureus	*Staphylococcus aureus*
SSI	Statens Serum Institut
S. paratyphi	*Salmonella paratyphi*
S. pneumoniae	*Streptococcus pneumoniae*
S. typhi	*Salmonella typhi*
TB	Tuberculosis
TDR-TB	Total drug resistant tuberculosis
TNFα	Tumour necrosis factor alpha

TSB	Tryptone soya broth
T. vaginalis	*Trichomonas vaginalis*
U&Es	Urea and Electrolytes
WHO	World Health Organisation
WTNM	Wild type Newman *Staphylococcus aureus*
XDR-TB	Extensively drug resistant tuberculosis

1 Introduction

1.1 TB and the non-specific effects of BCG – why do we care?

Simply put, tuberculosis (TB) is the leading single infectious disease killer in the world, with an estimated 10.4 million cases globally and 1.7 million TB related deaths in 2016[1]. Despite a 22% reduction in the number of TB related deaths between 2000 and 2015, TB can be found at number 9 of the top 10 causes of death worldwide. The ongoing burden of multidrug (MDR-TB) extensively drug (XDR-TB), and total drug resistant TB (TDR-TB), and the malignant relationship between TB and HIV, means the scientific community faces a significant battle in order to achieve the common aim of both the Sustainable Development Goals (SDGs) and End TB Strategy: to end the global TB epidemic by 2035, with a 95% reduction in TB related deaths and a 90% reduction in TB incidence[2]. Tools required for the successful implementation of these goals include sensitive diagnostic point of care tests to allow quick and easy diagnosis of TB and safe, effective and efficient therapeutic drug regimens for sensitive and drug resistant TB strains[2]. However, arguably the most important tool urgently needed is a safe and effective vaccine which can protect individuals from primary infection, but also prevent those with latent *Mycobacterium tuberculosis* (*M. tb*) infection (LTBI), with or without HIV co-infection, from re-activation.

Bacille Calmette-Guérin (BCG) is a strain of live attenuated *Mycobacterium bovis* (*M. bovis*) which remains the only licensed TB vaccine. BCG, whilst it is good at protecting against TB meningitis and disseminated disease in children, is hampered by its lack of consistent protection against adult pulmonary TB, a particular burden affecting young adults; the group with the highest burden of disease and therefore economic impact[3-5]. Currently there are over 15 candidate vaccines at different stages of development, utilising novel approaches to tackle the problem[6]. They include boosting BCG with a viral vector or

subunit vaccine, and/or replacing BCG altogether with a recombinant BCG or other attenuated whole mycobacterial vaccine[7].

Despite this, there is an important phenomenon which needs not to be forgotten, a phenomenon which not only relates to BCG but other vaccines also – the non-specific or heterologous effects of vaccines. Randomised and observational studies from low income countries with high childhood mortality rates have observed that BCG-vaccinated neonates have lower all-cause mortality rates within the first 6-12 months of life[8-14]. The most consistent effect observed is a reduction in the cases of sepsis, respiratory infection and fever[11, 15]. It is essential that we determine the cogency of this effect, as should it be real, then potential BCG replacement vaccines must be demonstrated to be non-inferior in this regard, in addition to being safe and efficacious against TB.

1.1.1 Mycobacterium tuberculosis and bovis

TB is the disease in humans caused by the *M. tb* complex, a genetically related group of mycobacteria including *M. tb*, *M. bovis*, *Mycobacterium africanum*, and *Mycobacterium canetti*, which can cause TB disease in humans and other animals[16]. It is a slow growing (replication time approximately 20 hours), extremely efficient, acid-fast, rod-shaped bacillus, whose cell wall provides a robust and elaborate armour coating, integral to the complex relationship between the organism and its intracellular survival in its human host. It has a cell wall core, or lower segment, which is made up of a cell membrane followed by a peptidoglycan (PG) layer attached to arabinogalactan (AG) which in turn is attached to a mycolic acid layer. Together this makes up the mycolyl arabinogalactan-peptidoglycan (mAGP) complex. This layer is insoluble and vital for organism viability. The upper segment is comprised of long and short chain fatty acids, and interspersed between both segments are phosphatidylinositol mannosides (PIMs), phthiocerol-containing lipids, lipomannan (LM) and lipoarabinomannan (LAM), which are thought to

have a role in signalling pathways during the disease process[17, 18]. It is this wall structure which provides *M. tb* with the necessary tools to survive the harsh intracellular environment of the human macrophage (Figure 1.1).

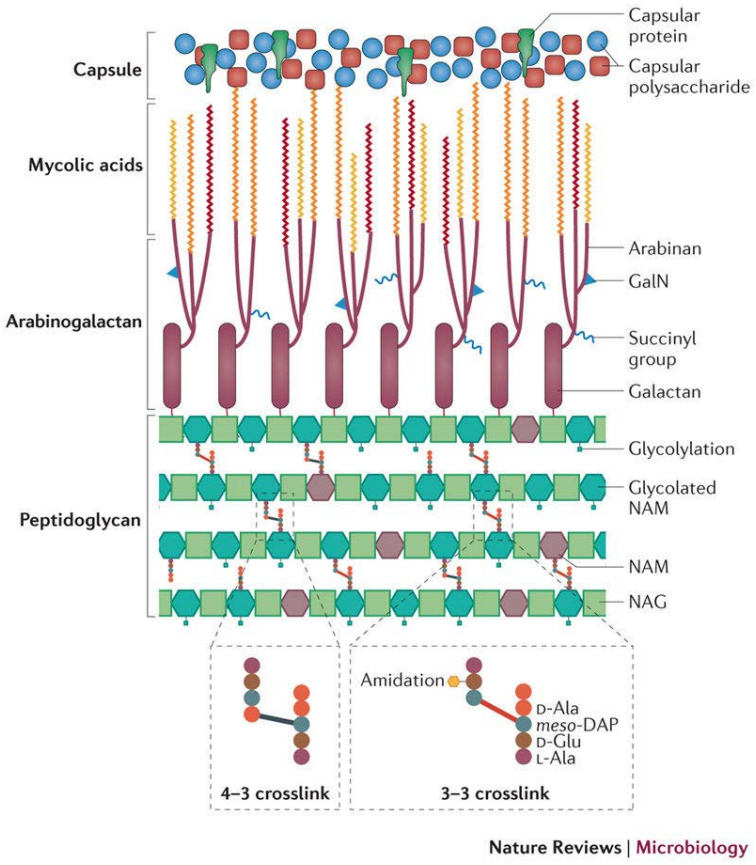


Figure 1.1 Schematic representation of the cell wall structure of mycobacterium.

Taken from http://www.nature.com/nrmicro/journal/v12/n8/box/nrmicro3299_BX1.html [19]

M. tb was initially thought to have been derived from *M. bovis* over 9,000 years ago, however more recent research into the evolution of *M. tb* has suggested that it has a closer affinity to a common parent strain originating from Africa[20], than it has to *M. bovis*; with the implication that both strains developed simultaneously, or that humans may have been responsible for the development of *M. bovis* in cattle, through primary transmission

of *M. tb* between species[21-23]. Development of whole-genome sequencing using DNA microarray has provided a valuable opportunity to explore the underlying differences between *M. tb*, *M. bovis* and daughter BCG strains. A study by Behr et al[24] assembled a DNA microarray comprising nearly all of the open reading frames (ORF) (the section of DNA which is the code for potential protein translation) for *M. tb* strain H37Rv. The H37Rv strain of *M. tb* has been used with success across the globe for research since its first isolation in 1905, with the beneficial features including its retained virulence in animal models, drug sensitivity, and its flexibility to genetic manipulation[25]. This DNA microarray was used to compare the ORFs of H37Rv with *M. bovis* and other BCG strains. They identified 91 ORFs present in H37Rv which were absent in one or more virulent *M. bovis* strains, and an additional 38 ORFs present in *M. bovis*, but absent in some or all strains of BCG. These differences provide evidence for evolutionary change for BCG, but also identify possible candidates for transcription and translation of virulence factors and protective antigens.

1.1.2 Pathogenesis and disease

M. tb is spread when individuals inhale the infected aerosolised droplets, propelled into the air by the cough of people with active pulmonary TB. Only a few bacteria are necessary to cause the spread of the disease. Pulmonary TB disease typically presents with the cardinal triad of fever, night sweats and weight loss, with or without associated cough, sputum production, haemoptysis and anorexia. *M. tb* is able to infect any organ in the body and whilst pulmonary disease is the most common, extra-pulmonary disease (e.g. lymph node, bone, gut, meninges) accounts for 10-42% of patients and depends on other factors such as race, ethnic background, age, co-morbid disease, immune status (immune-compromise resulting from concurrent HIV infection, immunosuppressive therapy or malignancy) and the strain of *M. tb*[26-29]. Symptom onset can be insidious

over many months resulting in inadvertent spread by individuals with active disease, with a diseased individual potentially infecting up to 15 people annually[30].

Once infected, there are one of four possible outcomes[31]:

1. *M. tb* infection elimination either with the innate or acquired immune response
2. Development of LTBI with a bacteria lying dormant within a granuloma
3. Subclinical TB disease
4. Active TB disease

Estimates of LTBI rates have generally been accepted as approximately one third of the world's population. However a more recent study by Houben et al applying mathematical modelling techniques have re-estimated the burden of LTBI to be closer to one quarter[32]. However despite this, at 2.6-3 billion individuals thought to be latently infected, it still remains a very significant potential global disease burden. The risk of developing active TB disease in otherwise healthy individuals solely infected with *M. tb* is quoted as approximately 10% across their lifetime, but in individuals with HIV co-infection this can rise to 10% risk per year[33-36]. The relationship between HIV and TB is undeniable[37, 38] but the question often raised is whether HIV co-infection increases an individual's risk of activation of LTBI or whether it increases risk of primary infection or re-infection[39]. A meta-analysis of TB cluster cases and HIV infection by Houben et al reported that HIV infection was as equally associated with clusters of TB disease as HIV negative cases, and they concluded that HIV infection was likely more often associated with re-infection rather than re-activation in the context of a TB high burden setting[40]. In the age of antiretroviral therapy (ART), ART has been shown to decrease the risk of TB disease in HIV infected individuals by 54-90% and half the recurrence rate[41]. A systematic review and meta-analysis by Suther et al found that ART was strongly associated with a reduction in the incidence of TB regardless of $CD4^+$ count[42]. The meta-analysis was based on studies in developing countries. Gupta et al performed a

study of HIV positive individuals linked to a national TB register in the UK[43]. They found that TB incidence in individuals of black/african background, regardless of HIV status, was unacceptably high, and that in white individuals, there was a significantly higher incidence of TB disease in those with HIV on long term ART, when compared with background non-HIV infected rates. This supported the importance of screening for LTBI in all HIV infected individuals, and screening for HIV in all TB patients.

Treatment considerations are complex and influenced by a multitude of factors including but not limited to; drug susceptibility profile of the *M. tb* isolate, patient co-morbid state, concurrent HIV infection, and central nervous system (CNS) involvement. Current British Thoracic Society (BTS) and National Institute for Clinical Excellence (NICE) Guidelines, based on the World Health Organisation (WHO) recommendations, advocate that for drug-susceptible individuals with no CNS involvement, that they receive six months of rifampicin and isoniazid with pyridoxine, and that during the first 2 months they should also receive ethambutol and pyrazinamide[44]. This obviously varies depending on the influence of other factors listed.

1.1.3 Global disease burden

As previously stated, in 2016 there were 10.4 million cases of TB and 1.7 million deaths, with 374,000 of those deaths in individuals with HIV co-infection[1]. Sixty five percent of cases were in men, 10% were in children and 10% of cases were in HIV positive individuals[1]. The highest burden of HIV and TB co-infection was in southern Africa where levels can exceed 50%[1].

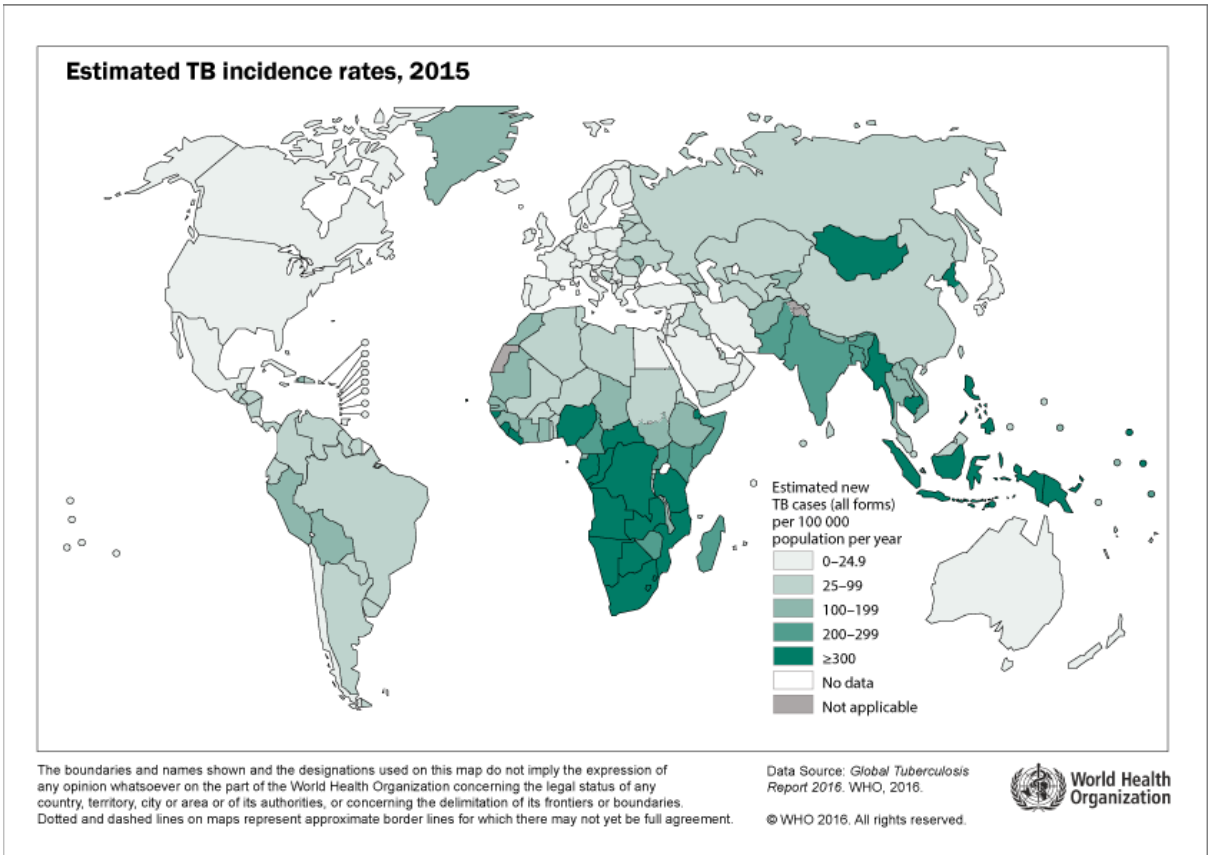


Figure 1.2 Estimated global TB incidence rates.

Taken from WHO Global Health Observatory Map Gallery

(http://gamapserver.who.int/mapLibrary/Files/Maps/Global_TBincidence_2015.png)

There are five countries which accounted for 56% of the total number of cases: India, Indonesia, China, Philippines and Pakistan[1]. India, with 18% of the global population[45], accounts for 25% of all TB cases[46]. Due to an underestimation of rates of TB in the South East Asian Region (SEAR), re-estimations of cases and deaths in 2015 were higher than had previously been thought[46]. The WHO map outlining the estimated TB incidence rates in 2015 is shown in Figure 1.2.

There are 490,000 new cases of MDR-TB with an additional 110,000 rifampicin resistant TB cases (RR-TB) who were eligible for MDR-TB treatment. Forty seven percent of cases of MDR-TB were accounted for by India, China and the Russian Federation[1].

Clearly the burden of disease is enormous, and whilst headway is being made in regards to a reduction in the number of cases and deaths, progress is slow[1] which reinforces the urgent need to find a safe and effective vaccine.

1.2 BCG – A 100 year history

BCG vaccination has been around for over 100 years and is the lasting legacy of the scientific partnership between a clinician, Dr Albert Calmette, and a veterinarian, Camille Guérin. BCG remains the only licensed TB vaccine, and to date has been given to several billion individuals, far outreaching any other single vaccine[47, 48]. A better understanding of the history of BCG can provide understanding of the emergence of the non-specific effects and also why TB vaccine development has been so challenging.

1.2.1 Development of the vaccine

Calmette and Guérin met in the early 1900s and had a common passion for the infectious diseases of their time, working independently on anti-cobra serum, vaccinia virus, tetanus and diphtheria among many[49]. Brought together by their mutual colleague Edmond Nocard[49], the man who was to give them 'lait nocord'[50], the primary strain of *M. bovis* from which BCG would later be derived[51-55], it was Calmette's vision for the successful development of a vaccine for mass immunisation of the population against the rampant disease TB, which was to become their lifetime obsession[49]. Calmette made his first measure to combat the disease by setting up an anti-TB dispensary at the Institute Pasteur, a model based on the TB dispensary founded in Edinburgh in 1887 by Sir Robert

William Phillip[49]. The aim was to provide early diagnosis and treatment for those who were unable to go to the sanatorium, but also provide practical help for patients and their families including information on how to minimise the spread of the disease, a rather modern approach; the key values of which remain vital in the management of TB today[49].

In 1903, the favoured hypothesis of the time was that pulmonary *M. tb* infection was acquired from an intestinal source likely contracted in early life[56]. Therefore the intestinal route became the focus of Calmette and Guérins attention. Their early investigations with cattle, goats and kids showed that kids being fed milk from mother goats whose mammary glands were infected with a virulent strain of *M. bovis* developed small pulmonary miliary tubercles; those who ingested a weakened form of *M. bovis* developed mesenteric lymph node lesions only; and adult cattle and goats given *M. bovis* as a fine emulsion straight into the rumen of the gut (specialised stomach of some plant eating animals designed to help with nutritional absorption by fermentation of plant material by microbial action), showed pulmonary and lymph node lesions[49].

However it was their pursuit of a suitable growth medium to reduce the clumping of the *M. bovis* cultures which led to the discovery of the weakened and less virulent strain of *M. bovis*[52]. The conventional method of culturing *M. bovis* in glycerinated potato medium resulted in a culture containing somewhat difficult to homogenise clumps which hindered the production of a fine suspension needed to ensure ease of transmission across the intestinal wall[49, 52, 56]. They observed that by adding an aliquot of bovine bile, they significantly improved the homogenisation of *M. bovis* and finally adopted the method of culturing on slices of potato soaked in bovine bile and glycerine[52, 57]. What they then discovered was that after a couple of passages of what they now called 'bacille bilie', it was becoming less virulent; a 'dose' of this culture, equal to a 'lethal dose' of the traditionally cultured *M. bovis*, caused only a mild, though slow to resolve, febrile illness[49]. It was

this ‘bacille bilie’ that we would eventually come to call BCG. By 1912, they demonstrated that cattle vaccinated with two intravenous doses of ‘bacille bilie’ were protected from virulent *M. bovis* challenge and remained healthy at 18 months. Autopsy revealed viable *M. bovis* in the pulmonary lymph nodes of the animals but no sign of active lesions[49]. The next stage was long term studies to assess whether vaccination with ‘bacille bilie’ would protect calves from transmission of natural infection from cattle that were actively infected[49]. During this time, the breakout of World War I added an unforeseeable level of complexity[50]. However despite complicated professional and personal conditions, they were able to continue the experiment long enough to show that a single vaccination was able to protect cattle for 18 months and a second dose provided longer protection[49].

By 1921, the ‘bacille bilie’, having now been passaged over 200 times, had been rendered ineffective at causing disease, and when attempts were made to re-culture using the old media (without bile), the bacilli did not regain any of their virulence characteristics. BCG, as it was now called, was taken forward into trials in guinea pigs, rabbits, dog, horse, and monkeys and was found to be unable to cause disease, and subsequent challenge of calves and the young of other species, showed vaccinated animals to be protected from virulent mycobacterial infection[52]. It was following these findings that a baby boy, born to a mother who died shortly after childbirth from TB, was administered BCG orally[49, 50]. Despite living in what was a ‘TB household’, the boy developed well and on the basis of this sentinel case and the work done in the animal models, Calmette authorised a larger study of 217 infants, primarily born to mothers with TB, who were to receive three doses of BCG orally within the first 9 days following birth, to assess if it could prevent transmission[49]. Results of this study reported BCG to be safe and well tolerated. Many more studies were to follow and by 1928, there was an overwhelming recognition that BCG was safe and its use was encouraged[49, 50].

However not everyone was satisfied and there was concern that BCG may revert to its virulent state[58]. In 1930, BCG vaccine development was rocked by the Lubeck disaster; a dark period in history when vaccination of 252 infants with BCG resulted in the death of 72 from TB[59]. Unsurprisingly it decreased confidence in BCG for several years. It was not until two years later that the German medical authorities concluded from their investigation that the cause of the disaster was local contamination of the BCG with a virulent human *M. tb* Kiel strain, which was being studied in the same laboratory, and BCG and the Institute Pasteur were cleared of blame[59].

In the years that followed up until present day, BCG vaccine has been given to several billion people and has yet to be replaced by a better alternative.

1.2.2 BCG strain and variability

Following the successful vaccination of infants in the early studies of 1921, and before the development of lyophilised forms of BCG, live sustained cultures were distributed to various laboratories all over the world for propagation and use in vaccine development[55]. These daughter strains have become morphologically, biochemically and immunologically different, likely due to the inconsistencies in the methods and culture mediums used by the laboratories responsible for maintaining and harvesting the BCG, including the number of passages each strain had been subject to[60]. These differences have been implicated as one of the potential reasons for the wide ranging efficacy seen with BCG (0-94%)[61]. At least 34 countries received BCG from the Institute Pasteur, but only the Gothenburg strain is thought to have been kept as per the conditions recommended by Calmette[55]. Factors contributing to the difficulty in interpreting the results of studies using BCG, both for vaccine research and animal work, was the lack of consistency in the terminology or nomenclature applied to the strain used, and that strains used to make vaccines in one country, came from a strain from another (e.g. BCG-

Russia, is BCG *M. bovis* strain Moscow 361, which is used to produce BCG vaccine at the Serum Institute India). Behr and Small conducted a study to construct the genealogy of 13 BCG strains commonly used in the literature, in particular those used in human trials, using written records and molecular typing techniques to derive a phylogeny. They discovered that DNA fingerprinting was a useful tool to confirm the evolutionary diversity seen in different daughter strains of the original BCG after several hundred passages, and that a better understanding of the differences seen may explain the postulated potential differences in virulence and possible efficacy between strains[24].

As BCG vaccination uptake became more common, interest in vaccine efficacy grew, and by the late 1950s it became apparent that there was significant discordance in vaccine efficacy around the world, particularly against pulmonary TB disease. Landmark studies included the UK Medical Research Council (MRC) trial, which demonstrated 78% protection, compared with studies by the US public health service (Georgia, Alabama and Puerto Rico) which reported less than 30% protection[62-64]. This considerable variability in reported protection continued to be seen in subsequent studies[3, 48, 65].

There are six BCG vaccines which have been given WHO pre-qualification status (https://extranet.who.int/gavi/PQ_Web/). The strains used in these vaccines are BCG strain Danish 1331, strain Moscow 361, strain Tokyo 172, and BCG Bulgaria. Until 2015, BCG SSI (strain Danish 1331) was the licensed vaccine for use in the UK, however a global shortage of the vaccine led to an alternative source being identified by Public Health England (PHE) and measures were put in place to allow the use of BCG Bulgaria (Intervax)[66-68].

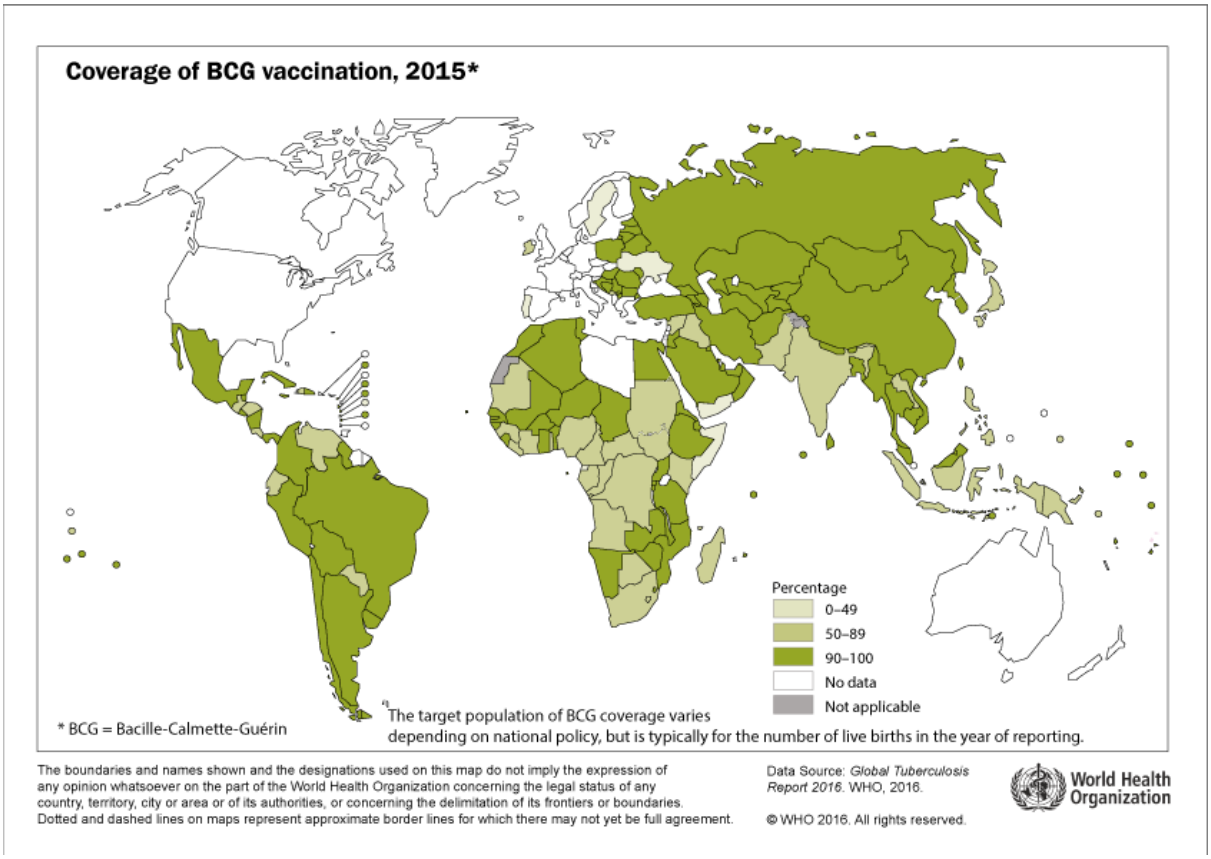


Figure 1.3 Global coverage of BCG vaccination.

Taken from WHO Global Health Observatory Map Gallery

(http://gamapserver.who.int/mapLibrary/Files/Maps/Global_TB_BCG_vaccination_2015.png)

1.2.3 BCG vaccine efficacy

Despite its widespread distribution and uptake, BCG has been plagued by two dominant issues: extremely variable efficacy in protecting against adult pulmonary disease, and reports of strain variability[48]. As mentioned, we know that BCG is good at protecting against disseminated TB disease in the first year of life, but that it is variably efficacious at protecting against adult pulmonary disease with anything between 0 and 80% protection, depending on a variety of factors thought to include geographical location, strain of BCG used, and nutritional status and the possibility of masking of effect caused by exposure to non-tuberculous mycobacteria (NTM)[3, 5, 53, 69-72]. It is possible that different strains

of BCG which have different virulence profiles, may impact on vaccine efficacy, adverse event profile and the nonspecific effects[71], however this has yet to be robustly demonstrated.

1.2.4 BCG and TB vaccine replacement strategies

With the issues surrounding BCG and the ongoing global burden of TB disease, it is easy to understand why an effective and safe vaccine is urgently needed. There are 14 TB vaccine candidates at different stages of clinical development with some phase IIa and IIb studies expected to report imminently, and over 20 in the preclinical stage of development[6]. The vaccine candidates in clinical development (outlined in Table 1.1) include four subunit vaccines pairing together *M. tb* antigens with immunomodulating adjuvants, five viral vectored vaccines, and five whole-cell vaccines using attenuated *M. tb* or other closely related mycobacteria[6].

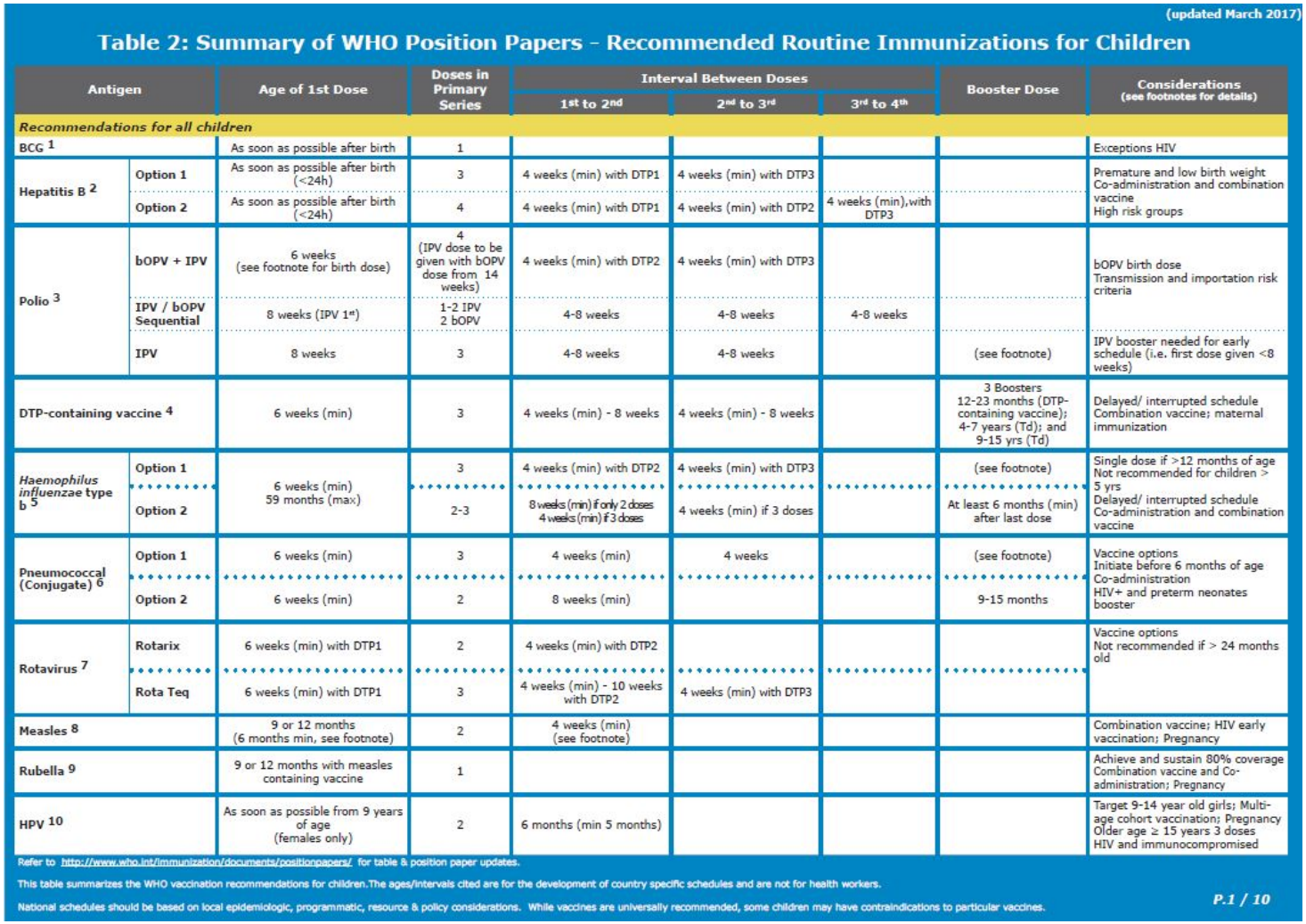
Pre-clinical Development	Phase I	Phase IIa	Phase IIb	Phase III
Recombinant CMV	Ad5Ag85A (aerosol) Viral vector	H4:IC3 Protein/ adjuvant subunit vaccine	M72/AS01E Protein/ adjuvant subunit vaccine	M vacca Whole-cell vaccine
ChAd3 with MVA (aerosol)	ChAdOx1 85A and MVA85A Viral vector	H56:IC31 Protein/ adjuvant	DAR-901 Whole-cell M. obuense	
	MVA85A (aerosol) Viral vector	MTBVAC Live attenuated M tb	VPM1002 Live recombinant BCG	
	MVA85A-IMX313 Viral vector/ adjuvant	ID93/GLA-SE Protein/ adjuvant		
	TB-FLU-04L Viral vector	RUTI Fragmented M tb		

Table 1.1 TB vaccine development pipeline. Adapted from Frick, TAG Pipeline report 2017[6]

1.3 Non-specific effects of vaccines – the debate

Several terms have been used to define the 'non-specific effects' of vaccines. These include 'non-specific effects, 'heterologous effects' and 'non-targeted effects'. For the purpose of this thesis they will be referred to as non-specific effects.

The research relating to the non-specific effects of vaccines is controversial. Randomised controlled studies, in target populations (predominantly infants), are fraught with challenges. The WHO has very clear recommendations relating to vaccination scheduling of infants (Table 1.2), making the opportunity to study the effects of specific vaccinations in isolation using a clean, randomised controlled design, over a medium/ long term follow-up period impossible. The potential non-specific effect of one vaccine may be altered/ masked/ amplified by the administration of another[73]. To break with the recommended vaccination schedule of otherwise healthy infants, we run the risk of exposing them to life threatening infection which could result in death. To deliberately withhold a vaccination which is known to be efficacious would be unethical. Therefore any study looking specifically at the non-specific effects of a vaccine has to be designed with care and where outcome measures are clearly defined.



(updated March 2017)

Table 2: Summary of WHO Position Papers - Recommended Routine Immunizations for Children

Antigen		Age of 1st Dose	Doses in Primary Series	Interval Between Doses			Booster Dose	Considerations (see footnotes for details)
				1st to 2nd	2nd to 3rd	3rd to 4th		
Recommendations for all children								
BCG [1]		As soon as possible after birth	1					Exceptions HIV
Hepatitis B [2]	Option 1	As soon as possible after birth (<24h)	3	4 weeks (min) with DTP1	4 weeks (min) with DTP3			Premature and low birth weight Co-administration and combination vaccine High risk groups
	Option 2	As soon as possible after birth (<24h)	4	4 weeks (min) with DTP1	4 weeks (min) with DTP2	4 weeks (min),with DTP3		
Polio [3]	bOPV + IPV	6 weeks (see footnote for birth dose)	4 (IPV dose to be given with bOPV dose from 14 weeks)	4 weeks (min) with DTP2	4 weeks (min) with DTP3			bOPV birth dose Transmission and importation risk criteria
	IPV / bOPV Sequential	8 weeks (IPV 1st)	1-2 IPV 2 bOPV	4-8 weeks	4-8 weeks	4-8 weeks		
	IPV	8 weeks	3	4-8 weeks	4-8 weeks		(see footnote)	IPV booster needed for early schedule (i.e. first dose given <8 weeks)
DTP-containing vaccine [4]		6 weeks (min)	3	4 weeks (min) - 8 weeks	4 weeks (min) - 8 weeks		3 Boosters 12-23 months (DTP-containing vaccine); 4-7 years (Td); and 9-15 yrs (Td)	Delayed/ interrupted schedule Combination vaccine; maternal immunization
Haemophilus influenzae type b [5]	Option 1	6 weeks (min) 59 months (max)	3	4 weeks (min) with DTP2	4 weeks (min) with DTP3		(see footnote)	Single dose if >12 months of age Not recommended for children > 5 yrs Delayed/ interrupted schedule Co-administration and combination vaccine
	Option 2		2-3	8 weeks (min) if only 2 doses 4 weeks (min) if 3 doses	4 weeks (min) if 3 doses		At least 6 months (min) after last dose	
Pneumococcal (Conjugate) [6]	Option 1	6 weeks (min)	3	4 weeks (min)	4 weeks		(see footnote)	Vaccine options Initiate before 6 months of age Co-administration HIV+ and preterm neonates booster
	Option 2	6 weeks (min)	2	8 weeks (min)			9-15 months	
Rotavirus [7]	Rotarix	6 weeks (min) with DTP1	2	4 weeks (min) with DTP2				Vaccine options Not recommended if > 24 months old
	Rota Teq	6 weeks (min) with DTP1	3	4 weeks (min) - 10 weeks with DTP2	4 weeks (min) with DTP3			
Measles [8]		9 or 12 months (6 months min, see footnote)	2	4 weeks (min) (see footnote)				Combination vaccine; HIV early vaccination; Pregnancy
Rubella [9]		9 or 12 months with measles containing vaccine	1					Achieve and sustain 80% coverage Combination vaccine and Co-administration; Pregnancy
HPV [10]		As soon as possible from 9 years of age (females only)	2	6 months (min 5 months)				Target 9-14 year old girls; Multi-age cohort vaccination; Pregnancy Older age ≥ 15 years 3 doses HIV and immunocompromised

Refer to http://www.who.int/immunization/documents/positionpapers/ for table & position paper updates.

This table summarizes the WHO vaccination recommendations for children.The ages/intervals cited are for the development of country specific schedules and are not for health workers.

National schedules should be based on local epidemiologic, programmatic, resource & policy considerations. While vaccines are universally recommended, some children may have contraindications to particular vaccines.

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Table1.2: WHO vaccination schedule summary. Adapted from WHO website. Provides an overview of the recommended vaccinations for all vaccination programmes globally (http://www.who.int/immunization/policy/Immunization_routine_table2.pdf?ua=1)

The following observations have been made[73]:

- Live vaccines including BCG, polio and measles appear to have a non-specific beneficial effect in some studies which cannot be explained by infection specific targeting[9, 74]
- Inactive or killed bacterial vaccines such as DTP appear to have a non-specific deleterious effect [9, 74]
- The magnitude of the non-specific effect of vaccines is modulated by factors such as sex, vitamin A/iron status, presence/absence of maternal antibodies from maternal BCG, timing of vaccination and in the case of BCG, strain of vaccine delivered[70, 75-79]
- The non-specific effects of one vaccine may be impacted by the administration of another vaccine[80, 81]

Due to the controversial nature of the data, the WHO commissioned two systematic reviews to assess the nature, quality and reproducibility of the data from both an epidemiological and immunological perspective specifically looking at BCG, measles and diphtheria, pertussis and tetanus (DT or DTP) vaccines[82]. Higgins et al conducted a systematic review of randomised and observational studies in order to quantify the effects of these three vaccines on all-cause mortality, including causes other than those which the vaccines are designed to target, and also evaluate the potential bias in the evidence[83]. They identified 68 studies in relation to the three vaccines, reporting on 34 separate birth cohorts. The majority of the birth cohorts came from Africa, and of those, the biggest contribution was from Guinea Bissau. Other contributing countries included North America, South East Asia, Papua New Guinea and Haiti. In regards to assessment of bias, using the 'Cochrane tool for assessing risk of bias in clinical trials', they judged only two of the BCG clinical trials to be at low risk, all others were moderate risk, and for the observational studies, they found them all to be high or very high risk. They concluded that administration of BCG vaccine was associated with a reduction in all-cause mortality (average relative risk (RR) from clinical trials 0.70 (95% CI 0-49-1.01) and 0.47 (95% CI

0.32-0.69)), DTP was associated with increased all-cause mortality (RR 1.38 95% CI 0.92-2.08) which appeared stronger in females. They concluded that these studies were all at high risk of bias and almost always, the DTP was administered with polio vaccine. Measles vaccine was associated with a reduction in all-cause mortality (Clinical trials – RR 0.74 95% CI 0.41-1.07, observational studies – RR 0.51 95% CI 0.42-0.63), again the effect seemed stronger in females, but again, the observational studies were at high risk of bias. In regards to vaccine scheduling, they found seven observational studies where sequence of vaccines were compared, with a suggestion that DTP when given with or after measles vaccine, may have higher all-cause mortality than when given before measles vaccine[83].

Kandasamy et al reported on the immunological outcomes of studies exploring the non-specific effects of vaccines[84]. Using pre-defined eligibility criteria, they identified 77 studies (randomised control trials, cohort studies and case control studies) of which 37 were using BCG as the study intervention. 143 different immunological variables were reported across the studies, with associated differences in measurement units and summary statistics. This high number of variables meant that they were unable to perform a meaningful meta-analysis. What they did report was that studies looking at *in-vitro* interferon gamma (IFN-γ) production following BCG vaccination compared with control, showed a consistent trend towards increased production in vaccinated individuals. There was also a trend towards increased IFN-γ production in vaccinated individuals following *in-vitro* stimulation of cells with *Candida albicans* (*C. albicans*), tetanus toxoid, *Staphylococcus aureus* (*S. aureus*), lipopolysaccharide (LPS) and hepatitis B. Measles vaccination showed an increase in lymphoproliferation to microbial antigens from tetanus toxid and *C. albicans*, and increased humoral immunogenicity to heterologous antigens were noted following DT (HSV and polio antibody titres) and DTP vaccination (increased pneumococcus and polio responses)[84].

On the basis of these independent reviews, the WHO Strategic Advisory Group of Experts (SAGE) on immunisation made the following conclusions[82]:

- The epidemiological review suggested a possible beneficial non-specific effect of BCG on all-cause mortality, and that the current WHO recommended schedule for BCG vaccination was contributory to this effect, therefore this review did not support a change to current policy
- The epidemiological review of the possible non-specific effects of DTP vaccination on all-cause mortality neither confirmed nor refuted the possibility of beneficial or deleterious effects, and therefore could not outweigh the known beneficial specific effects of DTP. Therefore the evidence did not support a change to current policy
- In respect to measles vaccine, the epidemiological review suggested a beneficial non-specific effect on all-cause infant mortality, and like BCG, the evidence was in favour of the current WHO policy on measles vaccination and did not support a change
- The findings of the immunological review neither confirmed nor refuted the possibility of non-specific beneficial or deleterious effects of the three vaccines of interest on all-cause mortality, and there was little confidence in the quality, quantity and kinetics of impact of the non-specific effects of vaccination on infants provided by the reviewed literature
- Recognition was made that non-specific immunological effects following exposure to any antigen, vaccine or natural exposure, are plausible and common but that their biological effects are poorly understood
- Moving forward, the non-specific effects of vaccines on all-cause infant mortality warrants further research, and that the Immunization and Vaccines related Implementation Research Advisory Committee (IVIRAC) be tasked with advising on the priority research questions which need to be answered, so as to impact efficiently on policy decisions, and what study designs needs to be performed in order to reliably answer the questions identified

- Aspects of future trials to be considered include: assessment of feasibility of high quality randomised control trials taking into consideration the substantial ethical and methodological implications, studies need to be sufficiently powered to explore gender difference, studies must prioritise a standardised immunological endpoint capable of answering the specific questions, and emphasis should be made on ensuring research is from a broad range of researchers and a wide range of geographical locations, but using a standardised protocol approach

In 2017 the WHO released a call for public comments on proposed draft protocols to assess the non-specific effects of vaccines[85, 86].

1.3.1 The non-specific effects of BCG – beneficial effect on infant mortality

The non-specific effects of BCG has to date been the most studied, of all the reported non-specific effects, and BCG is the vaccine for which there is the strongest evidence in support of the hypothesis: that BCG improves protection of infants against causes of death other than for which it is targeted. Historically this phenomenon was first documented by a Swedish Physician, Carl Näslund, following the introduction of BCG to Sweden in 1932[87]. He conducted a study of 1323 BCG vaccinated children, and 3350 non-vaccinated children in the Narrbotten region of northern Sweden where there was a high TB burden at the time[87, 88]. He reported that overall mortality was lower in the BCG vaccinated group compared with the non-vaccinated group (6.6% vs 22.2%), and as TB related deaths only accounted for 2% of deaths in this group, this finding could not be explained by specific effects alone[88]. In 1932 the Institut Pasteur summarised animal data, necropsy protocols and child mortality rates of 440,000 children in 46 countries and found similarly that BCG vaccinated infants had a 7.9% mortality compared with 15.3% in non-vaccinated infants[88].

In 1972, the 4th and penultimate report from the 15 year long MRC study was published[89]. They had made some observations and conclusions about BCG and its effect of diseases other than TB. They observed that neither BCG nor vole bacillus vaccination appeared to have an effect on respiratory diseases, death from other causes, or on sarcoidosis cases[89]. However, they did find a possible effect, though not significant, on the number of cases of leukaemia; 2.4 cases per 100,000 in the combined vaccinated group, and 4.1 per 100,000 in the non-vaccinated group, which mirrored data from a Quebec study[90, 91], but both of which were rebutted by data from another study which found no association[92].

Most recently there have been studies, most of which have come from West Africa, to support Näslund's original observation that BCG appears to exert a non-specific effect on all cause child mortality, not simply mortality related to TB disease[9-15, 93]. Studies by Garly et al[14], Nankabirwa et al[94], Aaby et al[11] Kristensen et al[9] and Roth et al[10, 12], have all found a positive correlation between BCG vaccination and infant survival. Beyond Africa, a retrospective epidemiological study of over 400,000 hospitalisations by De Castro et al found that BCG vaccinated infants were much less likely to be admitted with respiratory infection and sepsis than BCG non-vaccinated infants, quoting a preventative fraction (PF) of 41.1% (95% CI 40.3-60.7 $p<0.001$) for respiratory infection and 52.8% (95% CI 43.8-60.7 $p<0.001$)[15].

In opposition to this Haahr et al performed a retrospective cohort study of over 19,000 infants (66% BCG vaccinated) looking at hospitalisation rates at 3 months and 3 years comparing those vaccinated with those not vaccinated in Greenland[95]. They reported an incidence rate ratio (IRR) of 1.07 (95% CI 0.96-1.2) for admissions with infectious diseases, and 1.10 (95% CI 0.98-1.24 for respiratory tract infection at 0-3 months. Between 3-11 months the IRR was 1.00 (95% CI 0.84-1.19) concluding that the results did not support the non-specific beneficial effects of BCG hypothesis. This was also the

conclusion of the Danish Calmette study[96]. They randomised 4262 infants to receive BCG vaccination within 7 days of birth or to a non-vaccinated control group (parental ascent). For all-cause hospitalisations they found no difference between the two groups up to the age of 15 months (BCG vaccinated group 0.49 mean admissions/child compared with 0.47 mean admission/child in the non-vaccinated group)[96]. They also looked at rates of parental reported infant infection, and again found no specific benefit in the BCG vaccinated group (0-3 months IRR 0.87 (95% CI 0.72-1.05) and 3-13 months IRR 1.02 (95% CI 0.97-1.07)). They did however report an interesting finding that there did appear to be a benefit at 0-3 months in children born to mothers who had received BCG (IRR 0.62 (95% CI 0.39-0.98))[76].

One explanation for the polarised results is the geographical location of the studies. As with the disparity seen in the specific BCG efficacy globally, the negative studies were reported from northern hemisphere countries with low background TB rates and lower rates of childhood mortality. This is an important factor to consider when deliberating on the differences. Also, basic access to health care, post-natal support and background exposure to non-tuberculous mycobacteria (NTM) are also possible contributors.

1.3.2 Infant mortality – a global perspective

It is estimated that a staggering 5.9 million children under the age of five die every year, with 2.7 million dying in the neonatal period[97]. There remains a huge disparity in rates between low-middle income countries and high income countries with sub-Saharan Africa reporting the highest levels of childhood mortality: 1 in 12 children will not reach their fifth birthday compared with 1 in 147 in high income countries[97]. Approximately 45% of deaths in children under the age of five is related to malnutrition and 45% will occur in the first 28 days of life (neonatal period)[98]. Neonatal deaths are largely caused by

preterm birth, intrapartum complications and sepsis; after this first month of life, the leading causes of infant mortality are pneumonia, diarrhoeal illness and malaria[98, 99].

The list of pathogens responsible for neonatal sepsis is long. Common causes of neonatal sepsis in the developing world, most often associated with hospital contact, include: S. *aureus*, *Klebsiella pneumoniae* (*K. pnuemoniae*), *Streptococcus agalactiae* (*S. agalactiae*), *Escherichia coli* (*E. coli*), *Pseudomonas aeruginosa* (*P. aeruginosa*), Coagulase negative staphylococci, *Streptococcus pneumoniae* (*S. pneumoniae*), and *Salmonella typhi* and *paratyphi* (*S. typhi, S. paratyphi*)*[100]*. *K. pneumoniae* has been reported as the most common cause of culture positive sepsis in the developing world[101], with *S. agalactiae* infection the most prevalent in the first few days of life[102].

Associated with this, are high levels of antimicrobial resistance amongst the most serious pathogens[103]. Bacterial defences are multiple and include both innate and acquired defences, with the acquired of the greatest concern due to the ease of spread of mutations between bacteria[104, 105]. Bacteria can develop, through genetic mutation, genes encoding enzymes which will destroy antibiotics (e.g. β-lactamases), they can acquire efflux-pumps which expel antibiotics before they can reach the target site, or can develop mutations which cause alteration to their cell wall preventing expression of binding sites which are targeted by antibiotic chemotherapeutic agents[104]. *K. pneumoniae* has long been associated with multi-drug resistance[106]. It produces a variety of enzymes which are efficiently able to inactivate β-Lactam antibiotics, such as extended spectrum β-lactamases, metallo β-lactamases, oxacillinases and carbapenemases[107]. Studies have shown that *K. pneumoniae* has developed resistance factors to the antibacterial agent colistin by development of mutational inactivation of the mgrB gene. This gene is a negative regulator of the PhoPQ signialling system which is involved in the regulation of bacterial envelope remodelling, whilst also contributing to improved bacterial resistance to killing by the innate immune system[108]. By losing regulation of the PhoPQ signalling

system, *K. pneumoniae* has been shown to not only have increased antibiotic resistance, but also to acquire virulence factors[107]. Pathogen resistance is not merely confined to bacteria but has also been demonstrated in parasitic infection such as malaria another major player in infant mortality[109].

Combining the important role infection plays in infant and neonatal mortality and the increasing problems with chemotherapeutic agent resistance, the case for effective vaccines for preventing childhood morbidity and mortality has never been stronger. Additionally, should vaccines provide a non-specific beneficial effect on infant mortality through protection against respiratory infections and fever, the burgeoning weight of multi-drug resistant infection could be lessened. However one of the key gaps in our knowledge is the link between BCG, and the pathogens responsible for the respiratory infection or sepsis for which the vaccine is supposedly protecting the infant from succumbing to. Whilst it is not unreasonable to hypothesise that the non-specific effect of BCG is generalised to all pathogens, it could be more focused, therefore if we knew exactly which infective agent or agents were being non-specifically targeted, then studies could be more targeted in their approach to outcomes measured.

1.3.3 Biological plausibility

The WHO concluded, that for the case of the non-specific effects of BCG, the randomised and observational data was the strongest for BCG over all other vaccines, and there was biological plausibility to the phenomenon[82]. This biological plausibility predominantly stems from the alternative uses of BCG – intravesicle BCG therapy is the mainstay of treatment for non-muscle invasive superficial bladder cancer[110-112], it has been explored in relation to colon cancer treatment[113, 114] melanoma treatment[115, 116] and has been explored as a immunomodulator of Type 1 Diabetes mellitus[117].

1.3.3.1 BCG as a treatment for cancer

BCG has been used with great effect in non-muscle wall invasive, superficial bladder cancer and has been the mainstay of treatment for over 40 years[118]. The original relationship between mycobacteria and cancer dates back as far as 1928[119] and early animals studies demonstrated that BCG primed mice were resistant to transplantation of tumour cells, but also led to the discovery of Tumour Necrosis Factor (TNF)[120, 121]. Yet, much like the relationship between the human host and mycobacteria, the exact mechanism of action of how instillation of a solution containing BCG into the bladder can control tumour growth is unclear[120]. The possible mechanism of action was explored and expressed in a review by Redelman-Sidi et al. in Nature Reviews Urology [120].

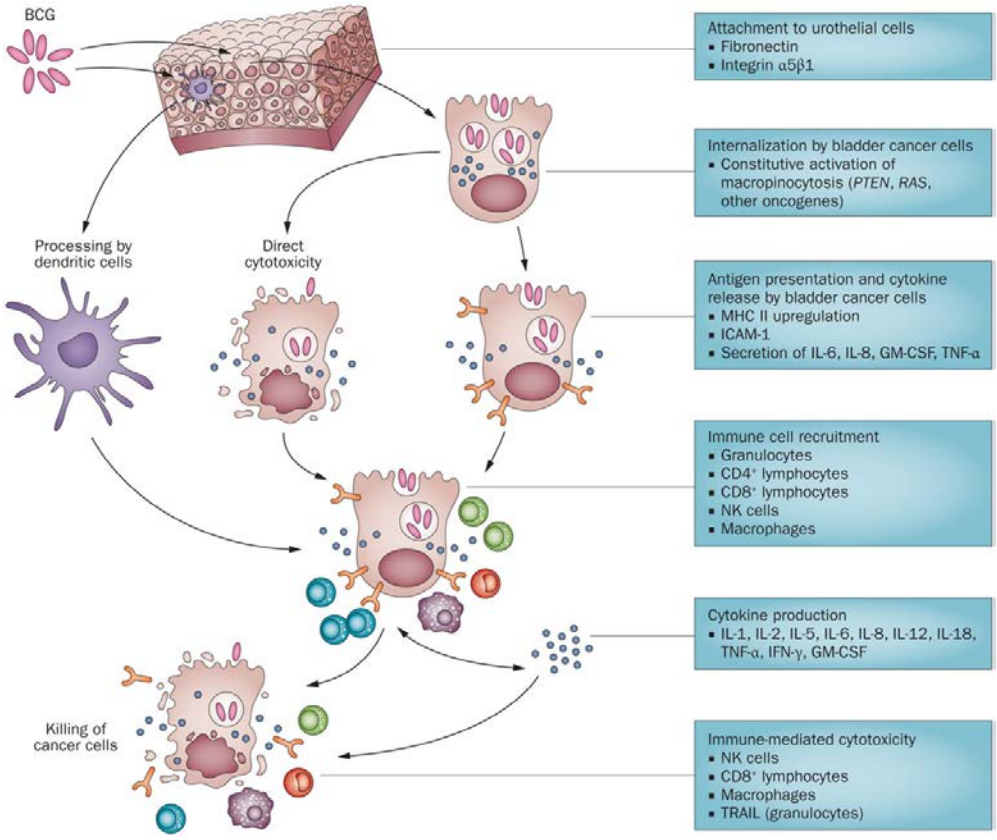


Figure 1.4 Model of the mechanism of action of BCG in bladder cancer. Adapted from Redelman-Sidi et al (2014) The mechanism of action of BCG therapy for bladder cancer – a current perspective Nat Rev Urol. Doi: 10.1038/nrurol.2014.15[120]

They propose the following; instilled live BCG attaches to the bladder wall and become internalised by the bladder cells. The bladder cells then upregulate major histocompatibility complex (MHC) class II and Intracellular adhesion molecule-1 (ICAM-1) to secrete cytokines interleukin-6 (IL-6), interleukin-8 (IL-8), TNF-α and granulocyte macrophage colony-stimulating factor (GM-CSF). With the assistance of dendritic cells they then can begin the process of recruitment of immune cells to the bladder wall such as granulocytes, $CD4^+$ T cells, natural killer (NK) cells and macrophages. This then results in immune mediated cytotoxicity and killing of the cancer cells. The exact mechanism for this is unclear as yet, however they postulate that it could either be secondary to a BCG specific antigen response or somehow, BCG creates a non-specific cross-reactivity with tumour specific antigens leading to targeted killing of the tumour cells (Figure 1.4)[120].

The role of BCG in melanoma has been less straight forward. Original studies from 1976 by Morton et al suggested that there was improved outcome in patients with melanoma who were treated with injection of BCG into the lesion with 90% reduction in the size of the injected lesion and 17% reduction in non-injected lesions and 25% survival at 6 years[116]. Whilst it is used as an immunomodulatory therapy in some cases of stage III melanoma[122], as an anti-cancer vaccine adjuvant, it was of no benefit[123, 124].

The application of BCG has been explored with colon cancer, however, there has to date been little evidence that, as an adjuvant to standard therapy, it has had any effect in contributing additional improvements in outcome[114].

1.3.3.2 BCG as a vaccine for Type 1 Diabetes mellitus

BCG has been tested as a systemic immunomodulatory therapy for early Type 1 Diabetes mellitus. A comprehensive review by Rewers et al evaluated its role and overall there has was no benefit found[117]. However, in June 2017 at the American Diabetic Association Meeting, interim data from a phase I study at Massachusetts General Hospital by Dr

Faustman[125] has reported interesting findings from repeated BCG vaccination therapy in the treatment of established Type 1 Diabetes mellitus[126]. The summary statement reports that BCG may permanently increase the expression of genes which are able to restore the regulatory T cells (Tregs) which can prevent the autoimmune killing of islet cells in the pancreas[126]. The study remains active on Clinicaltrials.gov and is expected to report full findings later in 2017.

1.3.4 Immune mechanisms implicated in the non-specific effects of vaccines

There is compelling emerging evidence for the non-specific effect of vaccines, in particular BCG, at a mechanistic level. Goodridge et al reviewed the literature and proposed two broad types of immunological response – heterologous lymphocyte responses, and innate immune memory or innate 'training'[127]. It is unlikely that a single mechanism in isolation is able to explain the phenomenon which is the non-specific effects of vaccines, but it is reasonable to conclude that, much in the way BCG induces a potent innate immune response which results in the death of bladder cancer cells, it triggers a series of complex pathways within the innate and adaptive immune system which collectively manifests in the clinically significant endpoint of improved infant mortality.

1.3.4.1 Heterologous lymphocyte responses

Under the Goodridge et al proposed heading of heterologous lymphocyte responses, they reported evidence for three possible mechanisms: antigen cross-reactivity, 'bystander activation' of unrelated T/B cells, and stimulation of IFN-γ secreting lymphocytes by primary BCG infection[127].

1.3.4.1.1 Antigen cross-reactivity

The hypothesis is that T cells can in some way develop the ability to react to antigens to which they have not been exposed. Supporting evidence for this comes from a study done by Su et al[128]. They found that human adult derived CD4+ T cells were able to appropriately, and rapidly respond to viral antigens to which they had never been exposed, and that HIV and influenza specific T cells had cross reactive action on exposure to peptides from non-specific organisms isolated from the gut, soil and ocean, including *Fascioloides magna* (*F. magna*) and *Trichomonas vaginalis* (*T. vaginalis*)[128].

1.3.4.1.2 'Bystander activation' of unrelated T/B cells

Goodridge et al next suggested the 'bystander activation' of unrelated T and B cells by infectious or vaccine-induced immune stimulation as a way to boost/sustain already existing antigen-specific immunity[127]. Evidence for this comes from studies which have shown that recent BCG, or co-administration of BCG with other vaccines such as Hepatitis B (Hep B) or influenza is capable of boosting the antibody responses seen to the other vaccines. In a study by Ota et al, they assessed the impact of BCG when given at the time of Hep B and oral polio vaccines (birth), when given with DT vaccine (2 months of age) or at 4.5 months of age when they evaluated responses. They found that there was an increase in Hep B and polio antibody responses, when given at the same time, but no impact on DT responses [129]. A study by Leentjens et al found that when BCG vaccine was administered prior to influenza A (H1N1) vaccine, there was an increase and more rapid production of functional antibody responses[130]. Additionally this study demonstrated a non-specific effect of the influenza vaccine on cytokine responses (IFN-γ, TNF-α, IL-6, IL-1β, and IL-10) to unrelated pathogens: *C. albicans*, *S. aureus* LPS and *M. tb*[130].

In an murine challenge model, Zurli et al found that adjuvanted influenza vaccines provided efficient protection of mice from both viral (targeted) and bacterial (non-

targeted) infection *in-vivo* with *S. aureus* suggestive of a non-specific effect of influenza vaccine[131].

1.3.4.1.3 Stimulation of IFNγ secreting lymphocytes by primary infection

Goodridge et al state that the origin of this hypothesis takes form from studies undertaken during the 1960's[127, 132, 133]. Mackaness et al demonstrated that stimulation of IFN-γ producing lymphocytes following an encounter with a primary pathogen activates a state of heightened immunity against a secondary infection, but that it is not a sustained response, and rapidly drops away on removal of the primary stimulus[132]. However, as Kandesamy et al concluded in their systematic review of the immunological non-specific effects of vaccines, the lack of consistency in approach to assays used and immunological outcomes measured makes our understanding of this possible mechanism incomplete and conflicted[84].

1.3.4.2 Innate immune memory

An emerging concept is that of the 'training' of innate immune cells or 'innate immune memory'. Kleinnijenhuis et al were the first to provide evidence for the adaptive features of the innate immune system in human cells[134]. In a series of elegant experiments, they took peripheral blood mononuclear cells (PBMCs) from otherwise healthy adults pre and 14 and 84 days after BCG vaccination. PBMCs were stimulated *in-vitro* with *M. tb*, heat inactivated *C. albicans*, heat inactivated *S. aureus* and *E. coli* LPS. They demonstrated that following BCG vaccination, there was a 4 to 7 fold increase in the production of IFN-γ, and a 2 fold enhanced production of TNF-α and IL-1β which persisted till day 84. This increased production of pro-inflammatory cytokines was accompanied with increased expression of CD11b and toll-like receptor 4 (TLR4). The mechanism which accounted for this 'training' effect was demonstrated to be through the NOD2 receptor and mediated by increased histone 3 lysine 4 trimethylation. They also demonstrated in an

immunodeficient murine model, that BCG vaccination could protect severe combined immunodeficiency (SCID) mice from overwhelming candidiasis[134].

In a separate study Kleinnijenhuis et al demonstrated increased IL-1β production from PBMCs of a separate cohort of BCG vaccinated subjects, to *in-vitro* stimulation with *M. tb*, which was thought to be due to BCG training of NK cells. This hypothesis was supported by the demonstration that the improved survival in the SCID murine model was in part due to increased activation of NK cells[135].

In a follow-up study, Kleinnijenhuis et al examined the effects of BCG on trained immunity and T helper 1 (Th1) and T helper 17 (Th17) responses up to one year after vaccination[136]. They found that there was increased production of TNF-α and IL-1β following the PBMC stimulation assay following secondary stimulation with *M. tb*, HKSA and HKCA, but that this waned between three months and one year following vaccination. However at one year, the PBMCs had increased expression of CD14, TLR4 and mannose receptor. Production of Th1 (IFN-γ) and Th17 (IL-17 and IL-22) immune responses to non-mycobacterial stimulation remained elevated at one year.

Cheng et al took a slightly different approach by using an *in-vitro* monocyte training model and a genome-wide transcriptome, to demonstrate that induction of aerobic metabolism represents a metabolic basis for trained immunity[137]. They showed that human monocytes, trained with β-glucan, a cell wall constituent of *C. albicans*, displayed high glucose consumption, high lactate production, and a high nicotinamide adenine dinucleotide (NAD+) ratio to NADH, reflecting a shift in cell metabolism, with an increase in glycolysis dependent on the activation of mammalian target of rapamycin (mTOR) through the dectin-1-Akt-HIF-α pathway. They showed that blocking Akt, mTOR or HIF-1α, blocked monocyte induction of trained immunity, and in a murine model, that mice with a defect in HIF-1α, could not mount trained immunity to bacterial sepsis[137].

Bekkering et al have refined an *in-vitro* model of trained innate immunity in primary human monocytes[138]. This model demonstrated that monocytes could be 'trained' *in-vitro* with BCG, and subjected to a secondary *in-vitro* stimulation with *E. coli* -LPS and Pam3Cys, producing a 4 to 5 fold increase in pro-inflammatory cytokines TNF-α and IL-6, and an increase in anti-inflammatory cytokines IL-10 and IL-1ra[138].

Most recently a study by Smith et al has shown in BCG vaccinated infants, a mirrored increase in Epidermal Growth Factor (EGF), IL-6 and platelet derived growth factor (PDGF-AB/BB), following secondary in-vitro stimulation with Pam3Cys, *C. albicans* and *S. aureus* with variable stimulant dependent increase in eight other cytokines and chemokines. There was no TNF-α or IL-1β, but there was an increase in NK cell expression to Pam3Cys[139].

Whilst the evidence for the potential underlying mechanism is beginning to form, it is important to find a way to link these immunological findings to reduced infant mortality from respiratory infection and sepsis. Smith et al have directed the focus from adult and murine models towards an infant study group – the target population – and more work needs to be done to support the progress made.

1.4 A role for a bacterial Growth Inhibition Assay

Taking into consideration what we know immunologically and mechanistically in relation to the non-specific effects of BCG, and the challenges faced in providing evidence to definitively prove or disprove the clinical manifestations of the non-specific effects, the development of an *in-vitro*, or *ex-vivo* functional assay may be of utility in moving this field forward. As a measure of the 'sum of the parts' of the immune system, a whole blood growth inhibition assay (GIA) which could be adapted to a multitude of different pathogens, would provide a valuable tool for the evaluation of the non-specific effects of

BCG, acting as a surrogate marker for an individual's capacity to control growth of bacteria pre and post BCG vaccination. One caveat is that that components of the immune system being measured would include only those present in the peripheral blood at the time of the bleed, therefore providing no information of the immunological mechanism present in the mucosal barrier compartment. This functional approach has been used with success for a variety of different pathogens and more specifically, the use of the MGIA in the TB vaccine community[140]. Developed from the method first described by Wallis et al[141], we have demonstrated that this assay is capable of showing a specific effect of BCG vaccination on reducing mycobacterial growth in humans[140], mice[142] and non-human primates (NHPs) (Tanner et al data unpublished). Work within the group has also shown that it is able to discriminate between humans with active TB, LTBI and controls (O'Shea et al submitted). Additionally the MGIT is able to correlate with *in-vivo* protection in mice[143, 144], *in-vivo* protection from BCG and *M tb* challenge in NHPs, and from epidemiological data from human primary/re-vaccination studies[140].

1.5 Hypothesis

I hypothesised that the ability to control growth of four common bacterial pathogens in a whole blood *in-vitro* GIA would be improved following BCG vaccination and would be dependent on an aspect of innate immunity. Demonstration of an effect of BCG vaccination on the growth of common bacterial pathogens, using an *in-vitro* human model, would provide data to support the funding of large costly clinical studies and provide a model by which BCG replacement candidate vaccines could be evaluated.

1.6 Aim

The aims and objectives of the study are:

1. To determine the non-specific effects of BCG-vaccination in healthy UK adults using the in-vitro GIA as a surrogate marker of the individuals capacity to control growth of *S. aureus*, *E. coli*, S. *agalactiae* and *K. pneumoniae*
2. Identification of potential factors which may confound the functionality of the *in-vitro* GIA as an output measure
3. Identification of immune signatures associated with specific and non-specific effects of BCG which may correlate with bacterial growth inhibition as identified using the GIA

2 Materials and Methods

2.1 Study design

TB038 was a randomised controlled clinical study of healthy, BCG naïve, UK adults, randomised to receive BCG SSI at a standard dose (2-8x10^5pfu) or unvaccinated control. All study documents were reviewed and approved by NRES Committee South Central – Oxford B (REC reference 15/SC/0022) and registered with ClinicalTrials.gov (NCT02380508). It was conducted according to the principles of the Declaration of Helsinki and Good Clinical Practice. Full study protocol and documents are in the appendix.

2.2 Inclusion criteria

Volunteers were required to meet all of the following criteria in order to be eligible to enter the study:

- Healthy adult aged 18-45 years
- BCG naïve
- Resident in or near Oxford (for CCVTM and John Warin Ward) or Birmingham (for NIHR WTCRF) for the duration of the study period
- No relevant findings in medical history or on physical examination
- Allow the investigators to discuss the volunteer's medical history with their GP
- Use effective contraception for the duration of the study period (females only)
- Agreement to refrain from blood donation for the duration of the study and for a period of three months after their last visit
- Give written informed consent

- Allow the Investigator to register volunteer details with a confidential database to prevent concurrent entry into other clinical studies or trials
- Be able and willing (in the Investigators opinion) to comply with all the study requirements

2.3 Exclusion criteria

Volunteers were required meet none of the following criteria in order to be eligible to enter the study:

- Laboratory evidence at screening of LTBI as indicated by a positive interferon gamma release assay (IGRA) response
- Clinical, radiological, or laboratory evidence of current active TB disease
- Previous vaccination with BCG or any candidate TB vaccine
- Within the last year had close household contact with an individual with smear positive pulmonary TB
- Clinically significant history of skin disorder, allergy, immunodeficiency (including HIV), cancer (except BCC or CIS), cardiovascular disease, respiratory disease, gastrointestinal disease, liver disease, renal disease, endocrine disorder, neurological illness, psychiatric disorder, drug or alcohol abuse
- History of serious psychiatric condition
- Concurrent oral or systemic steroid medication or the concurrent use of other immunosuppressive agents
- Concurrent use of long term antibiotic therapy
- History of anaphylaxis to vaccination or any allergy likely to be exacerbated by any component of the BCG vaccine

- Any abnormality of screening blood or urine tests that is deemed to be clinically significant or that may compromise the safety of the volunteer in the study
- Positive hepatitis B surface antigen (HBsAg), HCV or HIV antibodies
- Female confirmed pregnant or intention to become pregnant during the study period, or currently lactating
- Current involvement in another study or trial that involves regular blood tests or an IMP
- Use of an investigational medicinal product (IMP) or non-registered drug, live vaccine, or investigational medicinal device for four weeks prior to dosing with the study vaccine
- Administration of immunoglobulins and/or any blood products within the three months preceding the planned enrolment date
- Any other significant disease, disorder, or finding, which in the opinion of the investigator, may either put the volunteer at risk, or may influence the result of the study, or may affect the volunteer's ability to participate in the study

2.4 Schedule of visits

Schedule of visits including interventions performed are shown below in Table 2.1: Oxford and Table 2.2: Birmingham.

Visit number		1	2	3	4	5	6	7	8	9
Day of visit	Screening	Day 0 (Vaccination)	Day 2	Day 4	Day 7	Day 10	Day 14	Day 21	Day 28	Day 84
Time windows (days)*			+/-1	+/-1	+/-1	+/-2	+/-2	+/-3	+/-3	+/-7
Consent	X									
History & examination	X									
Urinalysis	X									
Blood test	X	X	X	X	X	X	X	X	X	X
ß-HCG urine test	X	X								
Nasal swab^	X^	X^					X^			
Vital signs	X	X								
Faecal sample		X^^			X^^		X^^			
BCG vaccination		X								
Blood for GIA (mL)		5	5	5	5	5	5	5	5	5
Blood for screening (mL)**	10									
Blood for immunology (mL)	10	50	50	50	50	50	50	50	50	50
Blood for Paxgene tube		2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5
Blood for FBC		2	2	2	2	2	2	2	2	2
HLA typing (mL)		4								
S. aureus, E. coli, K. pneumonia and Group B strep antibody titres (mL)		5								
Blood total for visit (mL)	20	68.5	59.5	59.5	59.5	59.5	59.5	59.5	59.5	59.5
Cumulative blood vol (mL)	20	88.5	148	207.5	267	326.5	386	445.5	505	564.5

Table 2.1 Schedule of visit for Oxford volunteers

Visit number		1	2	3	4	5	6	7
Day of visit	Screening	Day 0 (Vaccination)	Day 2	Day 7	Day 14	Day 21	Day 28	Day 84
Time windows (days)*			+/-1	+/-1	+/-2	+/-3	+/-3	+/-7
Consent	X							
History & examination	X							
Urinalysis	X							
Blood test	X	X	X	X	X	X	X	X
ß-HCG urine test	X	X						
Nasal swab^	X^	X^			X^			
Vital signs	X	X						
Faecal sample		X^^		X^^	X^^			
BCG vaccination		X						
Blood for GIA (mL)		5	5	5	5	5	5	5
Blood for screening (mL)**	10							
Blood for immunology (mL)	10	50	50	50	50	50	50	50
Blood for Paxgene tube		2.5	2.5	2.5	2.5	2.5	2.5	2.5
Blood for FBC		2	2	2	2	2	2	2
HLA typing (mL)		4						
S. aureus, E. coli, K. pneumoniae and Group B strep antibody titres (mL)		5						
Blood total for visit (mL)	20	68.5	59.5	59.5	59.5	59.5	59.5	59.5
Cumulative blood vol (mL)	20	88.5	148	207.5	267	326.5	386	445.5

Table 2.2 Schedule of visits for Birmingham volunteers

2.5 Whole blood processing

2.5.1 Reagents

Reagents	Company	Catalogue number
RPMI 1640	Sigma Aldrich	R0883
FCS	Biosera	S1 81 0
L-Glutamine	Gibco	G7513
Penicillin/Streptomycin	Gibco	PO781
Sodium Pyruvate	Gibco	11 360-039
Lymphoprep	Axis Shield	NYC-1114547
Leucosep tubes	Greiner	227290
Casyton tubes	Sedna Scientific	43001
Casyton counting buffer	Sedna Scientific	43003
Cryobabies labels	Wed Scientific Ltd	LCRY-1700
DMSO	Sigma	D2650
2mL screw top tubes	Sarstedt	72.694.006
Benzonase	Novagen	70664-3

Table 2.3 Whole blood processing table of reagents/consumables used

2.5.2 PBMC isolation and freezing

Blood samples are drawn into lithium heparin tubes (Dark Green BD vacutainers™, Becton Dickinson) and processed within 4 hours of collection. Up to 30mL of whole blood is added to each Leucosep tube (Greiner) which was pre-filled with 15mL of Lymphoprep

(Axis Shield). Samples were spun at 1000rpm for 13 minutes at room temperature without brake. Once separation was complete, 4 mL of plasma was aspirated (2 x 2mL) for freezing at -80°C. PBMCs were then collected from the interface layer with a Pasteur pipette and transferred to a new 50mL Falcon tube. Volume was made up to 50mL with R0 media (RPMI (Sigma Aldrich) with 1% Penicillin/Streptomycin (Gibco) and 1% L-glutamine (Gibco)). PBMCs were washed twice with R0 (2 x 1800rpm 5 minutes spins). Cells were re-suspended following second wash in R10 media (RPMI (Sigma Aldrich) with 10% FCS (Biosera), 1% Penicillin/Streptomycin (Gibco), 1% L-glutamine (Gibco) and 1% Sodium pyruvate (Gibco)) and counted using a Casycounter (Sedna Scientific) – 10µL of cell sample suspended into 10mL of Casyton counting buffer, and samples counted in duplicate. The concentration of the cells was then adjusted for ex-vivo ELISpot and the remaining cells frozen in freezing mix (FCS (biosera) with 10% DMOS (Sigma)) in 2mL Sarstedt tubes initially at -80°C for 24 hours and then stored in liquid nitrogen.

2.5.3 PBMC thawing

Samples were removed from storage, thawed in a water bath at 37°C, and rapidly transferred into R10 media in 15mL Falcon tubes. Samples were spun at 1400rpm for 7 minutes and re-suspended at approximately 1 x 10^6cells/mL, in R10 (as above) with Benzonase (Novagen) at 2µL/mL, and rested for 2 hours at 37°C, 5% CO_2. Cells were counted using Casycounter (Sedna Scientific) - 10µL of cell sample suspended into 10mL of Casyton counting buffer (Sedna Scientific). Samples were then re-suspended to desired concentration depending on experiment.

2.5.4 Serum/plasma extraction

Plasma was extracted as detailed above (2.5.2). Blood was collected and coagulated in red BD vacutainers™ (Becton Dickinson). Samples were spun at 1800rpm at room

temperature with the highest brake for 5 minutes. 4mLs of serum was aspirated using a Pasteur pipette and transferred into cryovials (2 x 2mL) and frozen directly at -80°C.

2.6 Microbiology techniques

2.6.1 Reagents

Reagents	Company	Catalogue number
RPMI 1640 + hepes	Gibco	42401018
L-Glutamine	Gibco	G7513
TSB media	Fischer Scientific	12573936
LB media	Sigma Aldrich	L7275-500Tabs
Blood agar Base No. 2 with horse blood	Oxoid, Fischer Scientific	5514940
LB agar	Sigma Aldrich	L7025-500TABS
Staph Brilliance 24 agar plates	Oxoid, Fischer Scientific	P01186
Nasal Swabs	Technical Service consultants Ltd	TS/19-S
PBS	Sigma Aldrich	3813
Water	Life Technologies Ltd	15230188
Glycerol	Sigma Aldrich	G5516-100ml
2mL Sarstedt tubes	Sarstedt	72.694.006
Peptone water	Fischer Scientific	12713586
BD BACTEC plus aerobic media	BD	442023
Human serum (male AB plasma)	Sigma Aldrich	H4522
Mycobacteria stock	AERAS BCG Pasteur	051415MF
BD BBL MGIT tubes	BD	245122

PANTA/enrichment supplement	BD	245124
2mL screw top caps	Sarstedt	72.694.006

2.6.2 Sourcing of bacterial isolates

All four pathogens identified came from clinical isolates. *S. aureus* Wild Type Newman Strain (WTNM) is a well characterised isolate which has been used extensively in the *S. aureus* research group and originated from a clinical isolate. This strain was provided by D. Wyllie, University of Oxford. Wild type *E. coli*, *K. pneumoniae*, and *S. agalactiae* were provided by S. Oakley at the Department of Microbiology, John Radcliffe Hospital, Oxford. Samples were provided on agar slopes (JR microbiology laboratory own) and re-cultured onto LB agar (*E. coli*, *K. pneumoniae*) or Blood Agar Base No. 2 with Horse blood (Oxoid).

2.6.3 Preparation of frozen bacterial stock

In a tissue culture hood, using a 10µL inoculum loop, a sample of wild type bacteria was picked and spread onto LB agar (*E. coli*, *K. pneumoniae*) or Blood Agar Base No. 2 with Horse blood (Oxoid) (*S. agalactiae*). Following overnight culture at 37°C, 3-4 fresh bacterial colonies were picked and inoculated using 10µL inoculum loops, into labelled 50mL Falcon tubes containing 10mL of culture media (TSB (Fischer Scientific) for *S. aureus* and *S. agalactiae*, or LB (Sigma Aldrich) for *E. coli* and *K. pneumoniae*). Bacteria were then cultured overnight at 37°C in an orbital warm air incubator (130rpm for *S. aureus* and 225-250rpm for all other bacteria). The overnight culture was then spun for 10 minutes at 3750rpm and bacteria washed in 10mL PBS. Bacteria were then re-spun for 10 minutes at 3750rpm and re-suspended in 10mL of fresh culture media. Samples were then prepared for freezing in 1mL aliquots in labelled 2mL Sarstedt tubes (Sarstedt) with 10% Glycerol (Sigma Aldrich). Samples were frozen at -80°C.

2.6.4 Preparation of bacterial stock plate from frozen stock

Fresh samples of bacteria were prepared from frozen stocks in a tissue culture hood. Using a 10µL inoculum loop, a scraping of the frozen stock was taken and plated onto Blood Agar Base 2 with Horse Blood (Oxoid). Plates were then incubated overnight (12-24 hours) at 37°C. Stock plates were then ready and stored in the fridge until colonies required for preparation of liquid media culture.

2.6.5 Growth curves

Fresh stock plate of bacteria prepared as per section 2.6.4. Three fresh colonies selected and inoculated into 10mL of fresh sterile media (TSB (Fischer scientific) for *S. aureus* and *S. agalactiae*, or LB media (Sigma Aldrich) for *E. coli* and *K. pneumoniae*). Bacteria cultured overnight (14 hours) at 37°C in an orbital warm air incubator (130rpm for *S. aureus* and 225-250rpm for all other bacteria). Following the overnight culture, 100µL of bacterial culture was inoculated into 10mL of fresh media in a 50mL falcon tube (media dependent on bacteria) and fresh sample put back into orbital incubator at 37°C. Samples of bacterial culture were then taken every 30 minutes and OD_{600} measured using spectrophotometer (1:10 dilution). 100µL of bacterial culture was taken at each time-point and put on ice for quantification of CFU/mL using a microdot technique. CFU/mL was measured by putting a 10µL dot of bacterial culture in duplicate onto LB agar plates (Sigma Aldrich – *E. coli* and *K. pneumoniae*) and Blood Agar Base No 2 with Horse Blood (Oxoid – *S. aureus* and *S. agalactiae*) and incubating for 8 hours at 37°C. Following incubation, micro-colonies were then counted using a microscope and mean CFU/mL measured from duplicates, and growth curves for CFU/mL and OD_{600} could then be plotted using Graphpad Prism.

2.6.6 Growth Inhibition Assay

2.6.6.1 Whole blood GIA

Overnight bacterial cultures were prepared the day before blood collection. Three bacterial colonies were picked using a 10µL loop and inoculated into 10mL of fresh sterile liquid media in a 50mL falcon tube (TSB (Oxoid) *S. aureus* and *S. agalactiae*, LB (Sigma Aldrich) *E. coli* and *K. pneumoniae*) and incubated for 14 hours overnight at 37°C in an orbital warm air incubator (130rpm *S. aureus*, 225-250rpm for all other bacteria). Following overnight incubation, 100µL of bacterial culture was then inoculated into a fresh 10mL of media in fresh 50mL Falcon tube. The second incubation period then started (1.5hrs *K. pneumoniae*, 2hrs *E. coli*, 2.5hrs *S. aureus* and 3hrs for *S. agalactia*e). At the end of the second incubation period, bacteria were removed and centrifuged for 10 minutes at 3750rpm. Supernatant was discarded, and bacterial pellet washed twice in 10mL of PBS (Sigma). Final bacterial pellet was re-suspended in 10mL of MGIT media (RPMI with hepes modification (Gibco) with 1% L-glutamine (Gibco). Serial dilutions of bacterial inoculums were then prepared including 1:10 and 1:100 dilution.

300µL of blood was the transferred into a 2mL Sarstedt tube (Sarstedt) and inoculated with 300µL of bacterial inoculum and mixed gently by inverting tube. Samples were prepared in duplicate for each bacterial inoculum concentration used. Samples were then incubated for 1 hour and 24 hours in an orbital warm air incubator (130rpm *S. aureus*, 225-250rpm for all other bacteria).

Following each incubation period, samples were removed and centrifuged for 5 minutes at 12,000rpm. Supernatants were removed and transferred into fresh 2mL Sarstedt tubes (Sarstedt) and frozen at -80°C. 1mL of sterile water (Sigma Aldrich) was then added to the remaining pellet to lyse cells and release any intracellularised bacteria. Samples were left for 10 minutes with 2 x vortex to disrupt the pellet. Samples were then centrifuged for 10 minutes at 12,000rpm, supernatants discarded and pellet re-suspended in 1mL PBS for

plating on Blood agar Base No 2 with Horse Blood (Oxoid) at suitable dilutions. 100µL of the diluted samples were plated using a spiral plater (Autoplate, Spiral Biotech) at 50 exponentials. Plates were incubated overnight at 37°C and counted using Qcount plate reader and software the following day. 50µL of *S. aureus* and *E. coli* samples were diluted into 5mL of PBS and inoculated into BD BACTEC FX tubes and transported to the JR Hospital for TTP detection. Net growth was calculated using the equation Log10(Sample CFU/mL ÷ Control CFU/mL).

2.6.6.2 PBMC GIA

Overnight bacterial cultures were prepared the day before PBMC sample thawing. 3 bacterial colonies were picked using a 10µL loop and inoculated into 10mL of fresh sterile liquid media in a 50mL falcon tube (TSB (Oxoid) *S. aureus* and *S. agalactiae*, LB (Sigma Aldrich) *E. coli* and *K. pneumoniae*) and incubated for 14 hours overnight at 37°C in an orbital warm air incubator (130rpm *S. aureus*, 225-250rpm for all other bacteria). Following overnight incubation, 100µL of bacterial culture was then inoculated into a fresh 10mL of media in fresh 50mL Falcon tube. The second incubation period then started (1.5hrs *K. pneumoniae*, 2hrs *E. coli*, 2.5hrs *S. aureus* and 3hrs for *S. agalactia*e). At the end of the second incubation period, bacteria were removed and centrifuged for 10 minutes at 3750rpm. Supernatant was discarded, and bacterial pellet washed twice in 10mL of PBS (Sigma). Final bacterial pellet was re-suspended in 10mL of MGIT media (RPMI with hepes modification (Gibco) with 1% L-glutamine (Gibco). Serial dilutions of bacterial inoculums were then prepared including 1:10, 1:100, 1:1000, and 1:10,000 dilutions depending on bacteria.

PBMCs were thawed as per section 2.5.3 and prepared to a concentration of 1 x 10^6 cells per 300µL. 300µL was then added in duplicate to pre-labelled 48 well tissue culture plates. 300µL of bacterial inoculum was then added and mixed gently by pipetting 2-3

times. Samples were prepared in duplicate for each bacterial inoculum concentration used and incubated for 1 hour at 37°C, 5% CO_2.

Following the incubation, samples were removed and transferred into 2mL Sarstedt tubes (Sarstedt) and centrifuged for 10 minutes at 12,000rpm. 500µL of water (Sigma Aldrich) was added to each well. Following completion of spin, supernatants were removed and the water transferred from the wells to the corresponding Sarstedt tude (Sarstedt) and the pellet re-suspended. Samples were then ready for plating onto Blood agar Base No 2 with Horse Blood (Oxoid) at suitable dilutions. 100µL of the diluted samples were plated using a spiral plater (Autoplate, Spiral Biotech) at 50 exponentials. Plates were incubated overnight at 37°C and counted using Qcount plate reader and software the following day. Net growth was calculated using (Log10 CFU/mL sample) ÷ (Log10 CFU/mL of control inoculum).

2.6.7 Nasal swab

Nasal swabs were obtained at screening visit, D0 and D14. Verbal explanation of the procedure was provided to the volunteer. A sterile cotton bud (Technical Service Consultants Ltd) was passed around the rim of both the right and left nostril to collect surface debris (skin cells, commensal bacteria) and then placed directly into a 5mL vial containing 2mL of sterile PBS (Sigma). Samples were stored at +2°C - +8°C and processed within 24 hours. 100µL of the samples were plated at 50 exponentials using the spiral plater (Autoplate, Spiral Biotech) onto *S aureus* selective Staph Brilliance 24 agar plates (Oxoid) and Blood Agar Base No.2 with horse blood (Oxoid). Plates were incubated overnight at 37°C and review the following day. The Staph Brilliance 24 agar plates were used to allow identification of *S. aureus*, and plating onto Blood agar ensured the presence of any commensal bacteria in the sample. Presence of *S. aureus* on the swab was confirmed by the presence of dark blue colonies on the Staph Brilliance 24 agar plates.

2.6.8 Faecal Culture

Volunteers were provided with a safe and hygienic method for stool collection. Samples were deposited within 24 hours of clinic visit and kept in a cool bag with a frozen pack insert in a fridge at +2°C to +8°C and brought with the volunteers to the clinic. Samples were transferred to a -80°C freezer for storage. Samples were then thawed in batches. Once thawed, using a 10µL microbiology loop a sample of stool was suspended in a 3mL bijou of Peptone water (Oxoid) by vortex. Using a 10µL loop, the sample was then streaked onto selective agar plates for identification of relevant bacterial species. Samples of the peptone water suspension were prepared for freezing in 2mL Sarstedt tubes (Sarstedt) with 10% Glycerol.

2.6.9 BCG plating

Remaining BCG vaccine was plated onto 7H11 agar (Sigma Aldrich). Serial dilutions of BCG were prepared in PBS at dilutions of 1:1, 1:10, 1:100, 1:1000, 1:10000, 1:100000 and 1:1000000. Samples were plated in duplicate. Plates were incubated for 3-5 weeks at 37°C and plaques counted.

2.6.10 BCG MGIT

PBMCs were thawed as previously described in section 2.5.3. R10 was substituted with RPMI-MGIT (RPMI with HEPES modification (Gibco), 1% L-glutamine (Gibco) and 10% pooled human serum (PHS)(Sigma)). PHS was heat inactivated for 1 hour at 56°C. PBMCs were re-suspended at $10x10^6$ cells/mL and 300µL of suspension placed into pre-labelled 48 well plates in duplicate for each volunteer. 300µL of BCG Pasteur (100 CFU) was added to each well and to two MGIT control tubes (with added supplement). Plates were

incubated at 37°C for 96 hrs and the control MGIT tubes placed into the BACTEC MGIT machine. MGIT tubes were prepared with 800µL of PANTA enrichment media. Samples from the 48 well plates were transferred into 2mL screw top tubes and centrifuge at 12,00rpm for 10 minutes. 500µL of water was added to each well. Once the spin was complete, the supernatant was removed and discarded and the water from the wells added to the corresponding tube. Samples were then vortexed twice over 10 minutes and the contents added to the MGIT tubes. Tubes were then placed in the BACTEC MGIT machine until positivity reached in TTD. Net growth was then calculated based on the control standard curve.

2.7 Immunology

2.7.1 Reagents

Reagents	Company	Catalogue number
RPMI	Sigma	R0883
L-Glutamine	Gibco	G7513
Penicillin/Streptomycin	Gibco	PO781
Sodium Pyruvate	Gibco	11360-039
FCS	Biosera	S1810
PBS with Tween 20	Sigma	P3563
SEB	Sigma Aldrich	S4881-1 MG
PBS	Sigma Aldrich	3813
PHA-L	Sigma	L4144
PMA	Sigma	P81 39
BCIP/NBT (plus)	Europa Bioproducts	MO711A-1000
Carbonate buffer capsules	Sigma	C-3041
Human IFN-γ ELISPOT kit (ALP)	Mabtech	3420-2A

Tuberculin PPD for *in-vitro*	SSI	2391
Blocker Casein in PBS	Pierce	37528
Ammonium Bicarbonate	Sigma Aldrich	09830
Goat anti-human γ-chain whole IgG alkaline phosphatase conjugate	Sigma	A-3187
5x Diethanolamine	Fischer Scientific	34064
4-nitrophenylphosphate tablets	Sigma	N2765
NUNC immunoplates (442414)	Fischer Scientific	DIA-971-030J
U-bottom 96 well plates	VWR	402030716
ELISpot plates (Multiscreen-IP)	Millipore	MAIPS451 0
16% paraformaldehyde	Lab stock	n/a
Ammonium chloride	Sigma Aldrich	A9434
BSA	Sigma Aldrich	A4918
IgG1κ	Sigma Aldrich	I5154-1MG
Sodium Azide	Sigma Aldrich	769320
$NaHCO_3$ powder	Biochrom	L1703
0.22µm filter 96 well plates	Millipore	MAGVS2210
Mouse monoclonal 4E3 anti-human IgG1 hingh heavy chain	Abcam	Ab99773
PvD012 ClfB pooled fractions	*S. aureus* group	n/a
HOS standard	*S. aureus* group	n/a
Heat killed *C. albicans*	InvivoGen	#tlrl-hkca
Heat killed *S. aureus*	InvivoGen	#tlrl-hksa
E. coli LPS serotype 055:B5	Sigma	Provided by Nijmegen
Human IL-1β Duoset ELISA	R&D	DY201

Human IL-1ra Duoset ELISA	R&D	DY280
Human IL-TNF-α Duoset ELISA	R&D	DY210
Human IL-6 Duoset ELISA	R&D	DY206
Human IL-10 Duoset ELISA	R&D	DY217B
Human IL-INF-γ Duoset ELISA	R&D	DY285B
TMB ELISA substrate (highest sensitivity)	Abcam	Ab171529-1000ml
450nm Stop Solution for TMB substrate	Abcam	Ab171522-10000ml
Gentamicin	Sigma Aldrich	G1272-10ml

2.7.2 IFNγ Enzyme-linked immunosorbent spot (ELISpot) assay

2.7.2.1 PPD ELISpot

ELISpot plates (Millipore) were coated with PPD anti-IFNγ capture antibody and incubated to 1-3 days at 4°C. On the day of the assay, plates were washed with PBS (Sigma) and blocked with R10 media (RPMI (Sigma), 10% FCS, 1% Penicillin/Streptomycin, 1% L-Glutamine, and 1% Pyruvate) for 2.5 hours at 37°C. Cell suspensions were made in R10 media. PBMCs were made to a cell suspension of 1 and 3 x 10^5 PBMC/ 80µL/ well and done in triplicate. Blank wells containing R10 were prepared for subtraction of background levels and SEB was used as positive control. The prepared plates were then incubated for 18-20 hours at 37°C overnight. Following incubation, the plates were washed six times with 0.05% PBS Tween 20 (Sigma). The secondary antibody (Biotin anti-IFNγ antibody) was then added and incubated for 2 hours at room temperature. The secondary antibody was washed off (6x PBS/Tween) and plates coated with Streptavidin conjugated to alkaline phosphatase, and incubated at room temperature for 1 hour. Plates were then washed (6x PBS/Tween) and 50µL of developing buffer containing substrate (Europa Bioproducts). The substrate reaction was terminated at 5-15 minutes by washing with tap water. Plates were then dried overnight and then read

using AID ELISpot reader (Germany). Plates were stored protected from light and humidity.

Each ELISpot plate was calibrated prior to reading. Quality control was performed on each well to eliminate artefact. The results are shown as spot forming cell (SFC) per million PBMC.

2.7.3 Whole Bacteria BCG IgG ELISA

BCG SSI was reconstituted and diluted in PBS to a concentration of 2 x 10^3pfu/mL. 96 well flat bottom NUNC plates (Fischer Scientific) were coated with 50µL and incubated overnight at room temperature. Plates were washed 6 x with 0.05% PBS Tween and plates blocked with 200µL Casein blocking buffer (Pierce) at room temperature. Serum samples including positive and negative controls were diluted 1:10 in Casein blocking buffer and following 1 hour incubation and washing of plates, samples were added at 50µL/well with 50µL of Casein blocking buffer in the blank well for later subtraction of background. Plates were then incubated for 2 hours at room temperature. Plates were washed 6 x with 0.05% PBS tween and 50µL/well of 1:1000 diluted goat antihuman γ-chain whole IgG ALP conjugate (Sigma Aldrich) and incubated for 1 hour at room temperature. Plates were then washed 6 x with 0.05% PBS Tween and 100µL of pNPP (Sigma Aldrich - development buffer) added. Plates were read at 5 minutes intervals until 20 minutes on an ELISA reader (Gen5 ELISA software) at OD405nm. Plate background was subtracted and minute readings used.

2.7.4 Whole bacteria *S. aureus* ELISA

SPA negative *S. aureus* cultures were prepared as per section 2.6.4. Second overnight bacterial culture (orbital warm air incubator (37°C/130rpm) in TSB media (Oxoid)) was

then started. Following culture bacteria were then centrifuged at 3750rpm for 10 minutes and bacteria washed x 2 with PBS. CFU/mL was calculated using spiral plater. Bacteria were then fixed using ice cold 4% Paraformaldehyde for 5 hours. Bacteria were centrifuged and washed x 1 with PBS and re-suspended in 50mM NH_4Cl for 10 minutes. Bacteria were centrifuged and supernatant discarded. Bacterial pellet was then re-suspended in PBS with 1% BSA and 0.1% Azide and stored in the fridge. CFU/mL was calculated and a sample of the fixed bacteria plated onto Blood Agar Base No 2 with Horse blood (Oxoid) to ensure bacterial inactivation.

200µL/well of bacteria suspended at a concentration of $2x10^7$CFU/mL in blocking buffer was added to 96 well filter plates (Milipore) and incubated for 1 hour at room temperature on a plate rocker. Serum dilutions were prepared including standard curve dilutions for positive (HOS) and negative (IgG1κ) controls. Liquid was aspirated from the filter plates using a vacuum manifold and 100µL of serum added to allocated wells and plates stored overnight at 4°C.

Liquid was aspirated and wells washed x 6 with 0.1% PBS/Tween. 100µL of 1:1000 diluted goat antihuman γ-chain whole IgG ALP conjugate (Sigma Aldrich) was added to each well and incubated for 1 hour at room temperature on a plate rocker. Plates were then washed x 6 with 0.1% PBS/Tween and 200µL/well of pNPP added. After 20 minutes, 100µL of the resulting solution was transferred to a NUNC ELISA plate (Fischer Scientific) and read at OD405nm using CLARIOStar software.

2.7.5 *S. aureus* ClfB 4E3 ELISA

NUNC ELISA plates (Fischer Scientific) were coated with 50µL of ClfB 2µg/mL in 0.1M $NaHCO_3$ buffer and incubated overnight at 4°C on a plate rocker. Plates were washed x 6 with 0.1% PBS/Tween and coated with 100µL of blocking buffer (PBS, 1% BSA and 0.1% Tween). Serum dilutions and standard curve of positive control (HOS) and negative

control (IgG1κ) was prepared and 50µL added to each appropriate well following further washing x 6. Plates were incubated overnight at 4°C.

Following incubation plates were washed x 6 with 0.1% PBS/Tween. 50µL of 1:1000 diluted goat antihuman γ-chain whole IgG ALP conjugate (Sigma Aldrich) was added to each well and incubated for 1 hour at room temperature on a plate rocker. Plates were then washed x 6 with 0.1% PBS/Tween and 100µL/well of pNPP added. Plates were read at OD405nm using CLARIOstar software and read every 10 minutes until 1 hour reached.

2.7.6 Cytokine stimulation assay

Protocol was derived from Bekkering et al and Kleinnijenhuis et al[134, 138]. PBMCs were thawed and rested as previously described in section 2.5.3. Cells were prepared to a concentration of 5 x 10^6 cells/mL in RPMI media (with added 10µg/mL Gentamicin, 1% L-glutamine and 1% Pyruvate), and 100µL was added to 96 well round bottom plates (VWR) to a concentration of 5 x 10^5 cells/well. 100uL of secondary antigens were then added to appropriate wells to a final well concentration of 1 x 10^6 microorganisms/mL HKCA, 1 x 10^6 microorganisms/mL HKSA, 1ng/mL EC-LPS and 20µg/mL PPD. Plates were incubated at 37°C for 24 hours. Following incubation, samples were transferred to v-bottom plates (VWR) and centrifuged for 5 minutes at 1800rpm. Supernatants were then removed and transferred to fresh 96 well plates and frozen at -80°C.

2.7.7 Cytokine ELISA: IFNγ, TNFα, IL-1β, IL-1Ra, IL-6, IL-10

Standardised DuoSet ELISA kits (R&D systems) were used for all cytokines. Kits were used as per manufacturer instructions. 96 well plates were coated with 100µL of capture antibody, sealed and incubated overnight at room temperature. Plates were washed three times with wash buffer (0.05% Tween 20 in PBS) and dried. Plates were then blocked

with 300µL of reagent diluent (PBS with 1% BSA) and incubated at room temperature for 1 hour and washed a further 3 times with wash buffer. 100µL of serum and assay standards diluted in reagent diluent were added to appropriate wells, and incubated for 2 hours at room temperature. Plates were then washed x 3 and 100µL of detection antibody, diluted appropriately in reagent diluent was added to each well and incubated for a further 2 hours at room temperature. Plates were then washed again and 100µL of Streptavidin-HRP added to each well and plates were incubated at room temperature out of direct light. Plates were then washed for a final time x 3 and 100µL of substrate solution added and plates incubated for 20 minutes at room temperature. 50µL of stop solution was added to each well and plates read to determine OD at a wavelength of 450nm. Blank wells were used to subtract background for each plate and unstimulated PBMCs were processed to subtract volunteer background.

2.8 Statistical analysis and data management

2.8.1 Statistical analysis for analysis of outcome measure/endpoints

Shapiro-Wilke test for normality was used. Unless both groups passed normality then grouped GIA and immunological data was assumed to be non-parametric and summarised by medians and inter-quartile ranges. One-way analysis of variance (ANOVA) was used to determine significant differences in bacterial net growth over time with post hoc Dunns test for multiple comparisons where appropriate. Between group comparisons were made using Mann-Whitney U test. The Wilcoxon Matched Pairs test was used to make paired comparisons over time for the direct MGIA. Spearman's test of correlation was used to analyse associations between the primary endpoint of GIA net growth and possible volunteer/assay specific confounders and for analysis of correlation between GIA net growth output and BACTEC FX TTP. Additionally, to compare GIA net growth data over time, linear mixed models were used. Fixed effects included in the model were

dummy variables for each visit. In addition, a random intercept for participant ID was fitted to account for clustering of visits within participants. In order to assess the effect of controlling for various potential predictors of outcome, the effect of the following variables were added to an adjusted analysis as fixed effects was assessed: Gender, colonisation status, Hb, WCC, Plts, Hct, Neut, Lymph, Mono, Eosino, Baso, ML ratio, IgG1 (4E3) responses to *S. aureus* ClfB and inoculum concentration. Due to co-linearity, some covariates were dropped in the analysis. This model assumed normality of the residuals assessed by producing qq-plots of the residuals.

3 Clinical results

3.1 Statement of Authorship

All study documents were written and submitted to the relevant ethical review committee by M Wilkie. Volunteer recruitment was performed by N Lella and L Rask (CCVTM Oxford) and the nursing and administrative team in NIHR WTCRF led by A Williams (Birmingham). Assistance with screening was provided by M Riste, R Payne, T Rampling and S Sridhar. Decisions relating to eligibility were taken by M Wilkie and M Riste. Volunteers were confirmed eligible for enrolment and randomisation by M Wilkie, M Riste and Z-R Manjaly-Thomas. Medical supervision of randomisation, administration of BCG vaccination and supervision of the post-vaccination period was provided by M Riste, Z-R Manjaly-Thomas and N Venkatraman in order to ensure M Wilkie remained blind to volunteer randomisation. Nursing support for vaccination, sample collection and follow-up visits were performed by R Lopez Ramon, O Griffiths, I Poulton, P Marriot, M Baker and C Mitton. Nasal swab processing was performed by M Wilkie. Stool sample processing was performed by M Wilkie. Data analysis was performed by M Wilkie.

3.2 Introduction

The observed non-specific effects of BCG have predominantly been reported from studies in infants in low income countries with and without high background levels of TB[11, 12, 145, 146]. It is this group who would be most affected should BCG therefore be replaced, as the WHO recommends that BCG be administered at birth or shortly thereafter, and it is this group, who potentially benefit from the non-specific effects[4]. In order to better understand this phenomenon, the gold standard would be a large, randomised control study of infants in low income countries with high background levels of childhood mortality. However, as discussed in Chapter 1, the logistical and cost considerations of

undertaking such a study, with the associated ethical limitations of withholding lifesaving vaccines, makes the execution of such a study extremely challenging with considerable disagreement on the 'correct methodology' for such a trial[82-84]. There are several different approaches which could be pursued in order to better explore the non-specific effects of BCG vaccination, from the development of an appropriate animal model, robust randomised control trials in the field, and specific cellular and mechanistic studies to compliment the clinical studies. One of the most important considerations however is consensus agreement on well-defined, meaningful endpoints which are measurable and reproducible. There is scope for development of a functional *in-vitro* assay which could measure the 'sum of the parts' of the immune system. If such a study demonstrated a non-specific effect of BCG vaccination, this would provide supporting data for a more targeted approach in infants. TB038 was designed to do exactly this and evaluate an *in-vitro* assay as a way of providing supporting data for the non-specific effects of BCG vaccination. It was a randomised controlled clinical study of healthy, HIV negative, HBV and HCV negative, BCG naïve, UK based adults aged between 18-45 years of age, using a whole blood *in-vitro* growth inhibition assay (GIA) as a surrogate marker of their individual capacity to control growth of bacteria following vaccination with BCG. The successful development of a robust and reliable tool in adults, where the scope to take sufficient volumes of blood in order to perform the necessary optimisation of the assay, would be invaluable in future infant studies, provide an avenue in which to direct more detailed mechanistic work, but also provide a tool for assessing the non-specific effects of future BCG replacement vaccines by.

3.3 Aim

The primary aim of this study was to evaluate the non-specific effects of BCG vaccination using a whole blood *in-vitro* bacterial GIA as a surrogate measure of an individual's

capacity to control growth of *S. aureus*, *E. coli*, *K. pneumoniae* and *S. agalactiae*, before and after vaccination with BCG. Secondary objectives were to identify potential confounders in an adult study population which may impact on the primary outcome of the GIA such as differences in baseline demographics, bacterial colonisation status, and fluctuations in full blood count parameters. As this study was undertaken in adults, administering a licensed vaccine, at a licensed dose, via a licensed route, severe adverse events (SAEs) were not anticipated based on the known side effect profile of the vaccine. Volunteers were monitored for 20 minutes following vaccination as recommended in the SmPC and had a clinical review at each visit following vaccination to ensure safety and report any SAEs which may occur during the study.

3.4 Ethical and regulatory approval

Study protocol and all study documents were submitted to South Central Oxford REC B (REC ref. 15/SC/0022) and approved on 4th February 2015. No approval was required from the Medicines and Healthcare products Regulatory Association (MHRA) as this was a clinical study using a licensed dose, of a licensed vaccine, given via the licensed route (ID BCG SSI 2-8 x 10^5pfu in 100µL).

3.5 Volunteer demographics

Volunteer recruitment is outlined in the Consort diagram (Figure 3.1). In total 79 volunteers were screened for eligibility. Volunteers were recruited from two UK sites: The Centre for Clinical Vaccinology and Tropical Medicine (CCVTM), Oxford, and the NIHR Wellcome Trust Clinical Research Facility (NIHR WTCRF), Birmingham. All volunteers provided written informed consent prior to any study procedures, as outlined in the appendix. Of the 79 volunteers screened (Oxford n=50, Birmingham n=29), 36 were

eligible and 35 were enrolled. 22 were excluded based on medical grounds and 21 withdrew before enrolment could be scheduled. The first volunteer was screened on 12th March 2015, the first schedule of volunteers was enrolled on 5th October 2015 and the final schedule was enrolled on 30th August 2016. The last volunteer last visit was on 22nd November 2016.

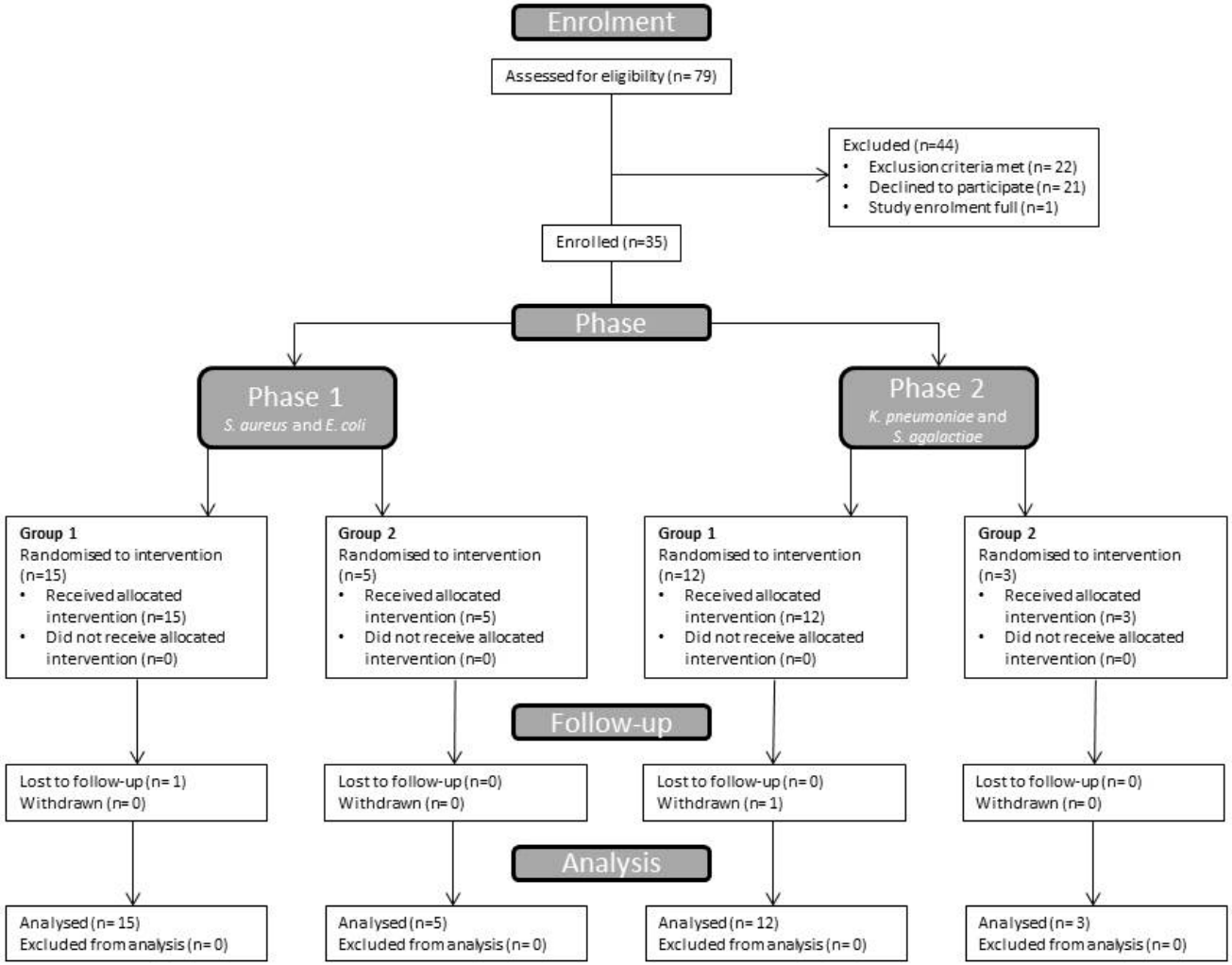


Figure 3.1 Consort Diagram – Schematic representation of volunteer recruitment.

Demographics	Phase 1		Phase 2	
	Group 1 (n=15)	Group 2 (n=5)	Group 1 (n=12)	Group 2 (n=3)
Age				
Mean, years (range)	23 (18-32)	29 (20-45)	31 (23-42)	32 (20-44)
Gender				
Female, n (%)	11 (73)	3 (60)	9 (75)	1 (33)
Place of birth				
UK, n (%)	13 (87)	3 (60)	10 (83)	1 (33)
BMI				
Mean BMI (range)	24 (19-29)	23 (21-25)	29 (20-40)	22 (22-33)
Smoker				
Yes, n (%)	6 (40)	0 (0)	4 (33)	1 (33)
Alcohol				
Yes, n (%)	13 (87)	5 (100)	8 (67)	2 (66)
<14 units/week	11 (73)	5 (100)	5 (42)	1 (33)

Figure 3.2 Volunteer baseline demographics as per phase of enrolment. Group 1 (BCG vaccinated) and group 2 (non-vaccinated controls) shown.

20 volunteers were enrolled into Phase 1 of the study where the bacteria used in the whole blood GIA were *S. aureus* and *E. coli*. They were enrolled over 5 schedules with the first schedule enrolling on 5th October 2015 and the last schedule enrolled on the 29th March 2016, with a total of 15 BCG vaccinated volunteers and 5 non-vaccinated controls. Baseline demographics are outlined in Figure 3.2. Mean age in group 1 was 23 (range 18 - 32 years) and in group 2, 29 (range 20-45). There were more females than males in phase 1, with females accounting for 73% and 60%, of group 1 and group 2 respectively. Of those randomised to group 1, 87% were born in the UK, and in group 2 60%, with the rest of phase 2 coming from Canada (n=2, 10%) and Australia (n=2, 10%), however resident in the UK at the time of enrolment and for the duration of the study. Mean BMI in group 1 was 24 (range 19-29) and group 2, 23 (range 21-25). In group 1, 40% were smokers with no smokers in group 2, 87% of group 1, and 100% of group 2 consumed alcohol. Of those who drank alcohol, 73% of group 1, and 100% of group 2 consumed less than the recommended 14 units per week upper limit (Figure 3.2). Data from this phase was un-

blinded following the final volunteer follow-up visit of schedule 5 on 26th June 2016. Of Phase 1, 19 completed the study, and one volunteer was lost to follow-up (TBT-038033). This volunteer attended D28 but failed to attend their final visit, they were then uncontactable despite all reasonable attempts by the clinical team to get in touch with them. In addition this volunteer (TBT-038033) has missing data from D4 as they were unable to attend the visit. All volunteers randomised to receive BCG vaccination were vaccinated with BCG batch no: 114015A.

15 volunteers were enrolled into Phase 2 of the study where the bacteria used in the whole blood GIA were *K. pneumoniae* and *S. agalactiae*. Volunteers were enrolled over 3 schedules of visits, the first enrolling on 6th June 2016 and the final group enrolling on 30th August 2016 with last volunteer last visit being 22nd November 2016. A total of 12 volunteers were randomised to receive BCG vaccination and three were inoculum controls. Due to BCG expiry and global shortage, it was not possible to enrol a final group. In phase 2, mean age of group 1 volunteers was 31 (range 23-42 years) and in group 2 mean age was 32 (range 20-44 years). The majority of enrolled volunteers were female (67%). In group 1, 83% were born in the UK and 33% of group 2 were born in the UK. The remaining volunteers were born in Germany, Denmark, USA and Canada but resident in the UK at the time of enrolment and for the duration of the study. Mean BMI in group 1 was 29 (range 20 – 40) and in group 2, 22 (range 22-23). 33% of both group 1 and group 2 were smokers, 67% of group 1 and 66% of group 2 consumed alcohol. Overall, 42% of group 1 and 33% of group 2 volunteers drank within the recommended limit of less than 14 units per week (Figure 3.2). 14 volunteers completed all study visits; one volunteer was withdrawn on their final visit due to concurrent antibiotic use. TBT-038039 was commenced on topical antibiotics just prior to their D84 visit. Data was un-blinded when all volunteers had attended their final visit on 22nd November 2016. All volunteers randomised to receive BCG in Phase 2 were vaccinated with BCG batch no. 114022A.

Overall there were 27 volunteers in group 1 (BCG vaccinated), and 8 in group 2 (non-vaccinated control). There was no significant difference between group 1 and 2 for age (p=0.8849 Mann Whitney test Figure 3.3 A), or BMI (p=0.0844 Mann Whitney test Figure 3.3 C). In group 1 (BCG vaccinated) 22% were male and 78% were female volunteers, and in group 2, there were 63% were male and 37% were female (Figure 3.3 B).

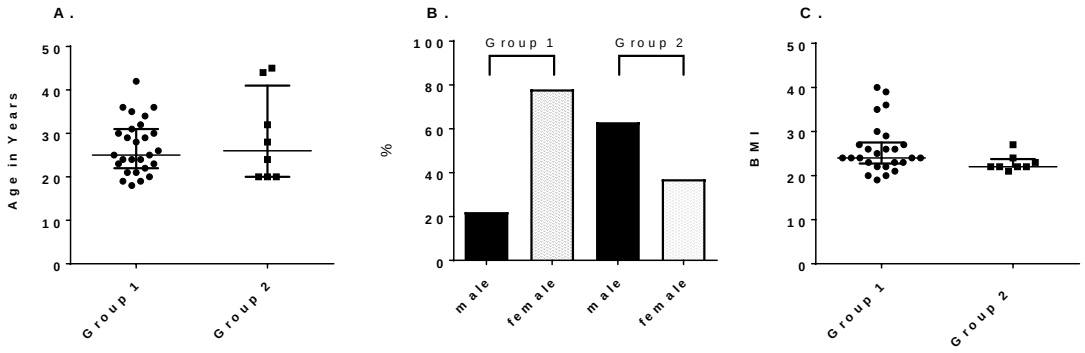


Figure 3.3 Baseline demographics between group 1 and group 2 by age, gender and BMI. A. Age in years, median and interquartile range shown. B. Percentage of male and female volunteers per group. C. BMI, median and interquartile range shown.

The only parameter which demonstrated a significant difference between group 1 and group 2 volunteers at baseline was BMI. Volunteers in group 1 had a higher mean BMI (p=0.03 student T test Figure 3.4).

	Group 1 (n=27)		Group 2 (n=8)		Significance tests		
	Mean	S.D	Mean	S.D	Student's t	Wilcoxon signed rank test	Fisher's exact
Age (years)	26.89	6.21	30.12	9.7	0.4	0.49	
BMI	26.26	5.72	22.49	1.23	0.03	0.04	
Height (m)	1.7	0.08	1.77	0.14	0.23	0.11	
Weight (kg)	75.65	15.4	70.36	9.42	0.25	0.43	
Male	0.26		0.5				0.23
Smoker	0.37		0.12				0.39
Alcohol	0.78		0.88				1
Hb	136.89	10.81	137.5	10.68	0.89	0.72	
Hct	0.41	0.03	0.4	0.02	0.34	0.65	
Plts	282.22	76.72	230.62	51.73	0.04	0.07	
WCC	6.8	1.69	5.71	1.56	0.11	0.13	
Neutrophils	3.96	1.39	3.27	1.14	0.18	0.25	
Lymphocytes	2.11	0.56	1.8	0.39	0.1	0.28	
Monocytes	0.52	0.13	0.44	0.18	0.31	0.16	
M/L ratio	0.26	0.1	0.25	0.11	0.89	0.8	
Basophils	0.05	0.03	0.04	0.03	0.35	0.41	
Eosinophils	0.17	0.09	0.14	0.08	0.27	0.26	

Figure 3.4 Baseline volunteer demographics and FBC analysis at screening for combined phase 1 and phase 2. Mean with standard deviation shown. Significance tests comparing group 1 (BCG vaccinated) with group 2 (non-vaccinated control) showing p values for Student's t test and Wilcoxon signed rank test for mean except when mean was proportionate and Fisher's exact test was used.

3.6 Safety

BCG SSI is a licensed vaccine in the UK, which was administered at the licensed dose and route (ID 2-8x10^5cfu in 100µL) therefore no data on routine vaccine safety was solicited from volunteers during the study. Those who were randomised to receive BCG vaccination had a review of the site of ID vaccination by the clinical team at each follow-up visit. All volunteers had an expected local skin reaction with normal healing during

follow-up. There were no SAEs or Serious Adverse Reactions (SARs) reported during the study period.

3.7 FBC

Volunteers had FBC differential analysis performed at all clinic visits. Volunteer TBT-038033 did not have a FBC taken on D84 as they were lost to follow-up after D28, and D4, as they were unable to attend, and TBT-038038 was withdrawn on D84 due to concurrent antibiotic use therefore no blood was taken. Volunteers from Birmingham did not have FBCs done on D4 or D10 as they did not come to Oxford for those visits. All volunteers had normal blood parameters at baseline (data not shown). Analysis of data from FBC parameters has been performed on the combined group1 and group 2 data from volunteers enrolled into phase 1 and phase 2.

3.7.1 Haemoglobin

Haemoglobin (Hb) in group 1 and group 2 passed normality test (Shapiro Wilk normality test). There was a statistically significant drop in Hb which peaked between D0 and D14 in group 1 (BCG vaccinated) volunteers and was sustained to D28 ($p \leq 0.0001$ One-way ANOVA Holm Sidak's multiple comparisons test; Figure 3.5 A). There was no significance change, between D0 and any other time-point in the non- vaccinated group 2 ($p=0.0885$ One-way ANOVA Figure 3.5 B).

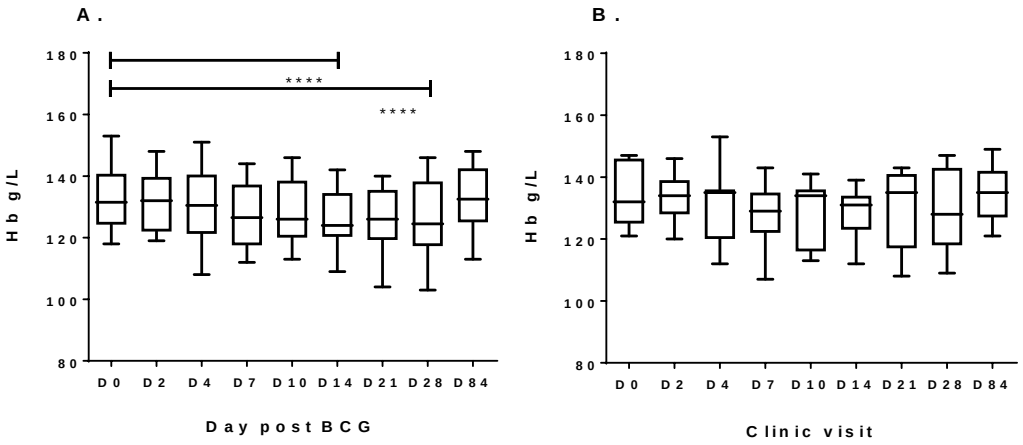


Figure 3.5 Hb concentration trend over time – A. Volunteers in group 1 (BCG vaccinated) B. Volunteers in group 2 (non-vaccinated). Data shown as box-whisker plot with median and maximum range. One-way ANOVA and post-hoc Holm-Sidak's test for multiple comparisons test was performed.

Splitting the groups by male and female volunteers in group 1 (BCG vaccinated) did not alter the finding. There was a decrease in Hb shown in both male and female volunteers (D0 vs D21 male: p=0.0011, female: p=0.0006 one-way ANOVA, Dunn's multiple comparisons test; Figure 3.6 A and B). There was no difference between male and female non-vaccinated volunteers in group 2 (male: p=0.4180 and female: p=0.1251 one-way ANOVA; Figure 3.6 C and D).

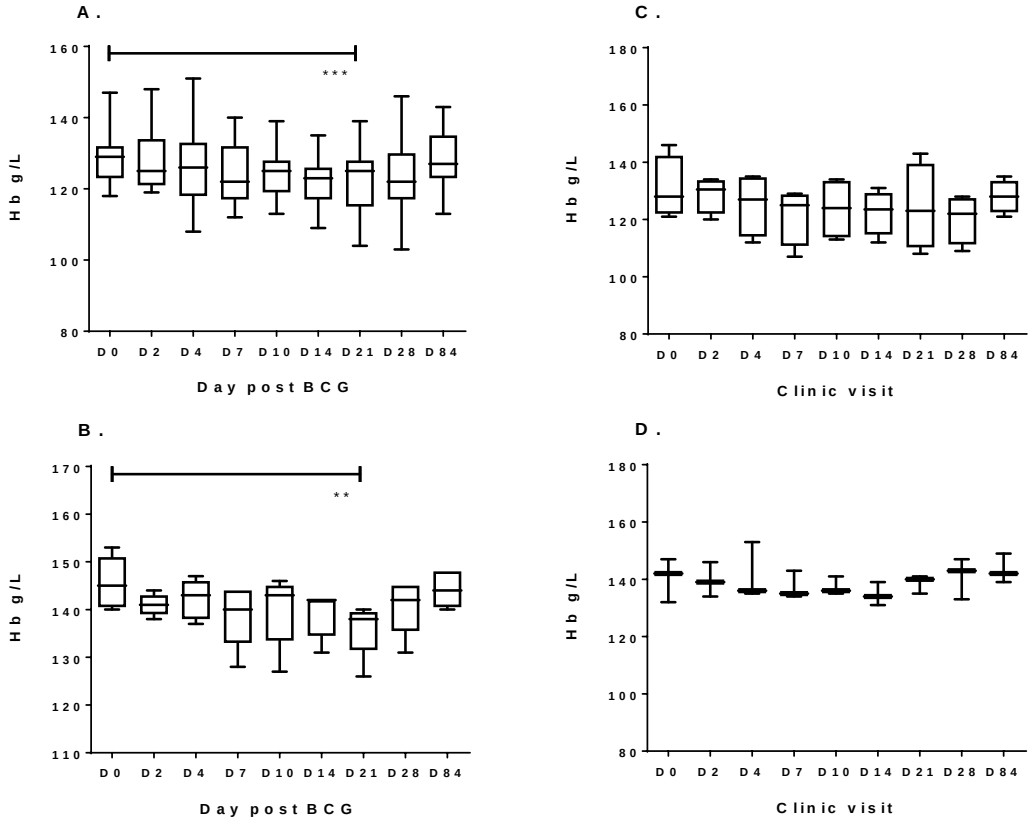


Figure 3.6 Hb concentration trend over time split by gender. A. Female volunteers, group 1 (BCG vaccinated) B. Male volunteers, group 1 (BCG vaccinated) C. Female volunteers, group 2 (non-vaccinated) D. Male volunteers, group 2 (non-vaccinated). Data shown as box-whisker plot with median and maximum range. One-way ANOVA and post-hoc Dunn's multiple comparisons test performed.

3.7.2 Trends in haematocrit, MCV, RBC, MCHb and MCHb concentration

In order to better characterise the change in Hb concentration, analysis of haematocrit (Hct), mean cell volume (MCV) red blood cells (RBC), mean corpuscular Hb (MCHb) and MCHb concentration (MCHb conc.) was performed. Results of Hct, RBC MCHb and MCHb conc. passed normality test (Shapiro Wilk normality test), but MCV did not.

In group 1 (BCG vaccinated) there was a significant decrease in Hct following D0 which peaked at D21 ($p \leq 0.0001$ one-way ANOVA, Holm Sidak's multiple comparisons test;Figure

3.7 A). In group 2 (non-vaccinated) there was no significant decrease in Hct, (p=0.260 one-way ANOVA; Figure 3.7 B).

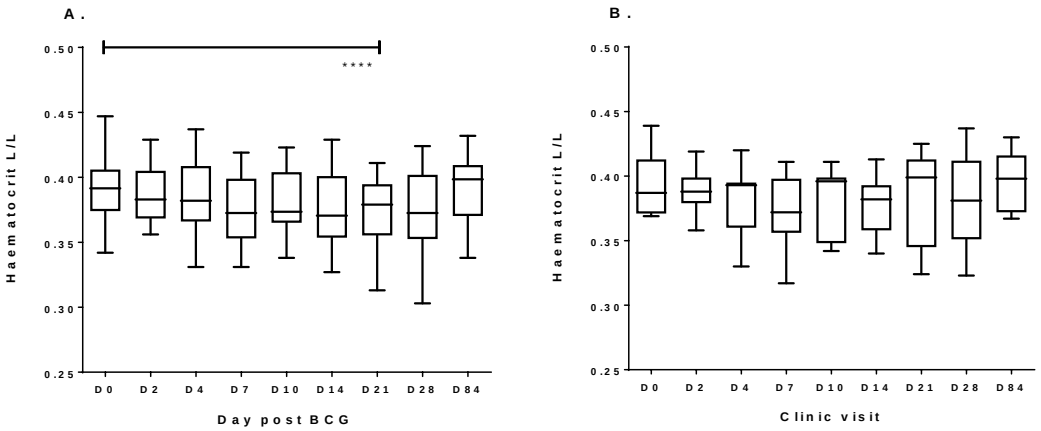


Figure 3.7 Hct trend over time – A. Volunteers in group 1 (BCG vaccinated) B. Volunteers in group 2 (non-vaccinated). Data shown as box-whisker plot with median and maximum range. One-way ANOVA and Holm-Sidak's test for multiple comparisons test performed.

Statistical analysis did not show any significant change in MCV in either group 1 or group 2 (Figure 3.8). In group 1, there was decrease between D0 and D84 however this did not reach statistical significance (D0vs D84 p=0.0533 One-way ANOVA Dunn's multiple comparisons test; Figure 3.8 A).

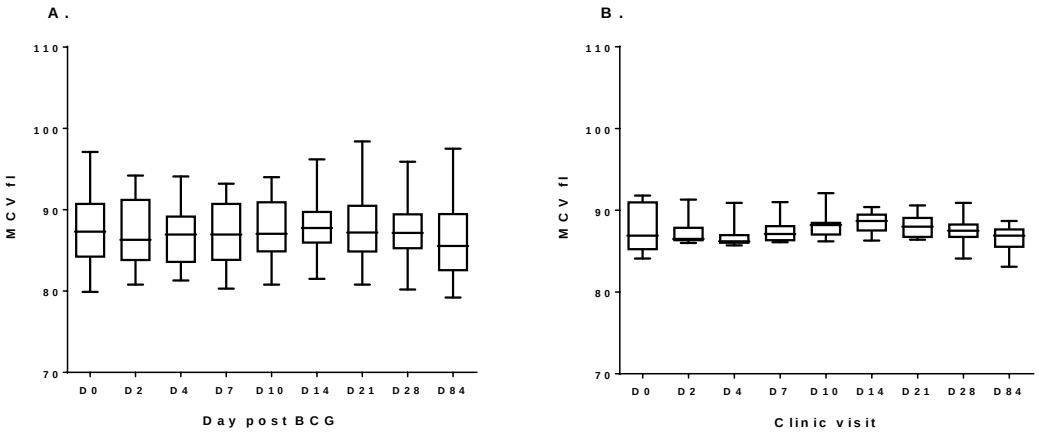


Figure 3.8 MCV trend over time – A. Volunteers in group 1 (BCG vaccinated) B. Volunteers in group 2 (non-vaccinated). Data shown as box-whisker plot with median and maximum range. One-way ANOVA and Dunn's test for multiple comparisons test performed.

As with haematocrit, there was a significant decrease in RBC in group 1 at D7 peaking at D21 (p= 0.0008 and p=0.0001 respectively, One-way ANOVA Holm Sidak's multiple comparisons test; Figure 3.9 A). There was no significant change in group 2 (p=0.2600 One-way ANOVA; Figure 3.9 B).

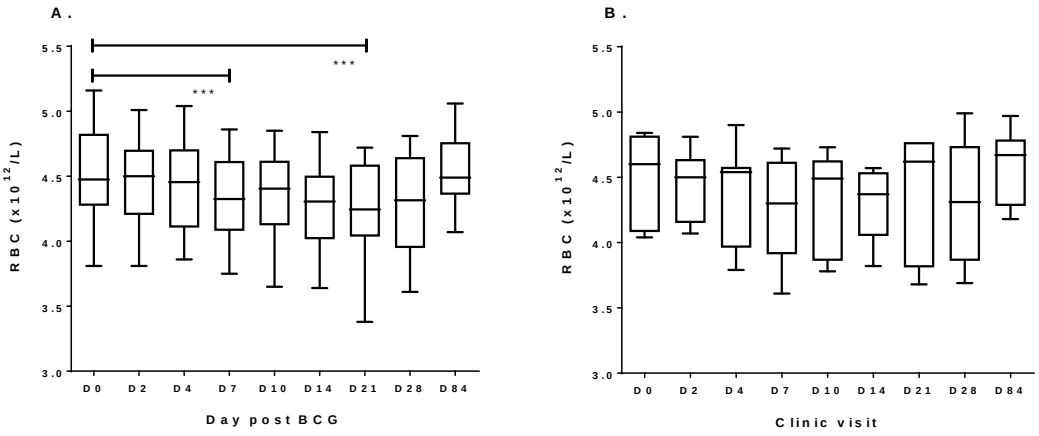


Figure 3.9 RBC trend over time – A. Volunteers in group 1 (BCG vaccinated) B. Volunteers in group 2 (non-vaccinated). Data shown as box-whisker plot with median and maximum range. One-way ANOVA and Holm-Sidak's test for multiple comparisons test performed.

MCHb and MCHb conc. are shown in Figure 3.10. There was no significant difference in either of these parameters over time for either group 1 or group 2 (MCHb - group 1: p=0.0703 One-way ANOVA, group 2: p=0.2307 one-way ANOVA; MCHb conc. – group 1: 0.2719 one-way ANOVA, group 2: p=0.3972 one-way ANOVA).

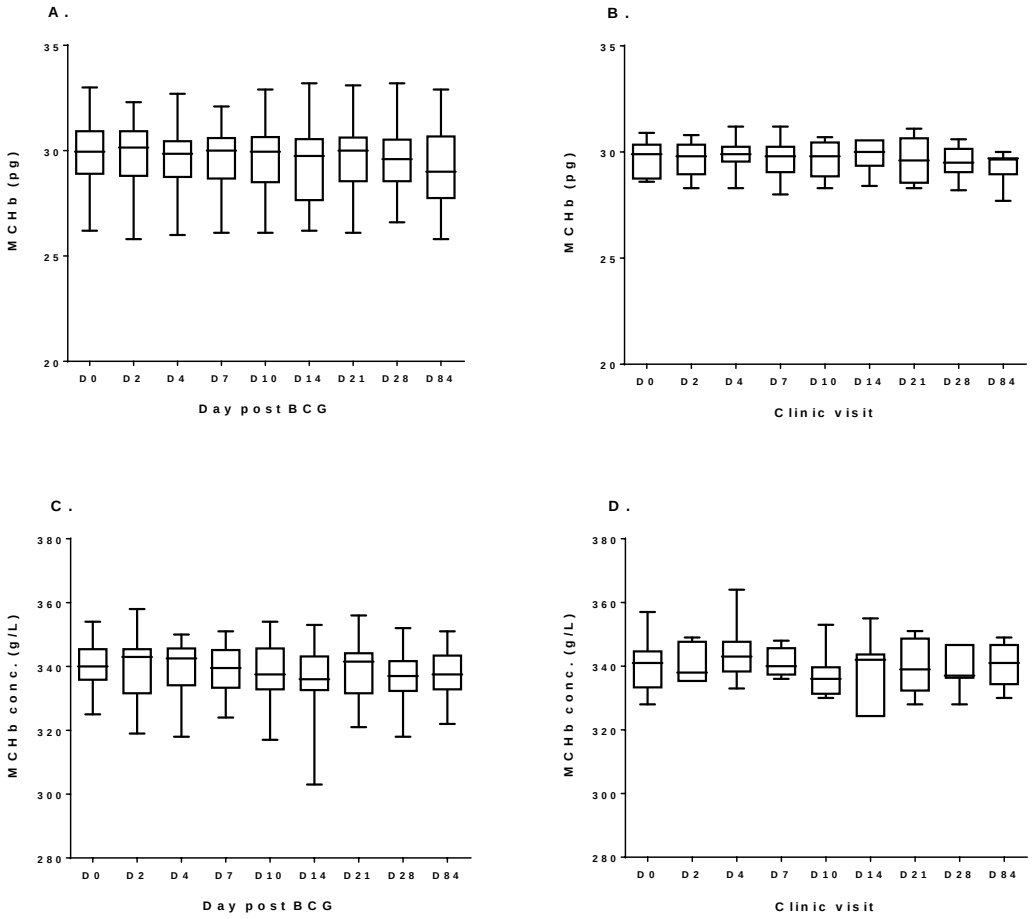


Figure 3.10 Trend in MCHb and MCHb conc. over time. A. MCHb values for volunteers in group 1 (BCG vaccinated) B. MCHb values for volunteers in group 2 (non-vaccinated). C. MCHb conc. values for

volunteers in group 1 (BCG vaccinated) D. MCHb conc. values for volunteers in group 2 (non-vaccinated). Data shown as box-whisker plot with median and maximum range. One-way ANOVA and Holm-Sidak's test for multiple comparisons test performed.

3.7.3 Platelets and white cell differential

There was no significant difference in platelet count or total white cell count (WCC) in group 1 or group 2 over time (platelet count: group 1: p=0.6368 and group 2 p=0.4398 one-way ANOVA, Figure 3.11 A and B; total WCC: group 1 p=0.4984 and group 2 p=0.7906 one-way ANOVA, Figure 3.11 C and D).

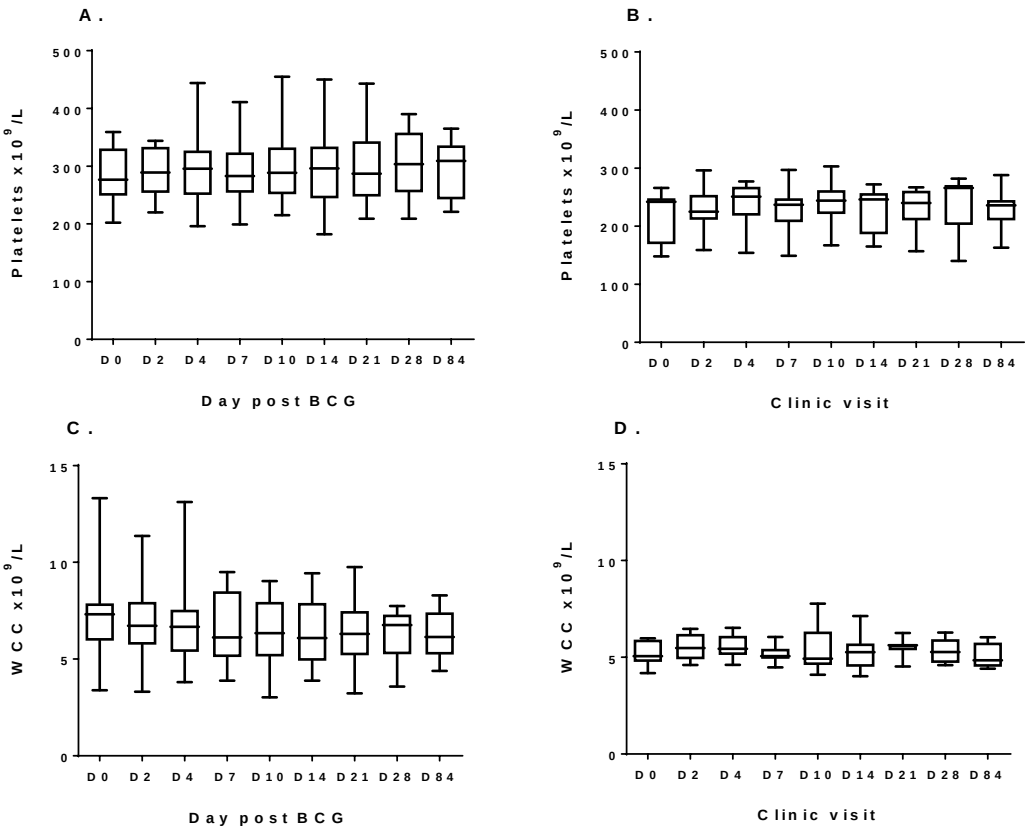


Figure 3.11 Platelet and Total WCC trends over time. A. Platelet counts of volunteer in group 1 (BCG vaccinated) B. Platelet count of volunteers in group 2 (non-vaccinated) C. Total WCC of volunteers in

group 1 D) Total WCC of volunteers in group 2. Data shown as box-whisker plot with median and maximum range. One-way ANOVA and Dunn's test for multiple comparisons test performed.

Neutrophils (group 1 p=0.1013 and group 2 p=0.8558 one-way ANOVA), lymphocytes (group 1 p=0.3128 and group 2 p=0.4787 one-way ANOVA), and eosinophils (group 1 p=0.2778 and group 2 p=0.5292 one-way ANOVA) did not show any change over time in group 1 and group 2 volunteers (Figure 3.12).

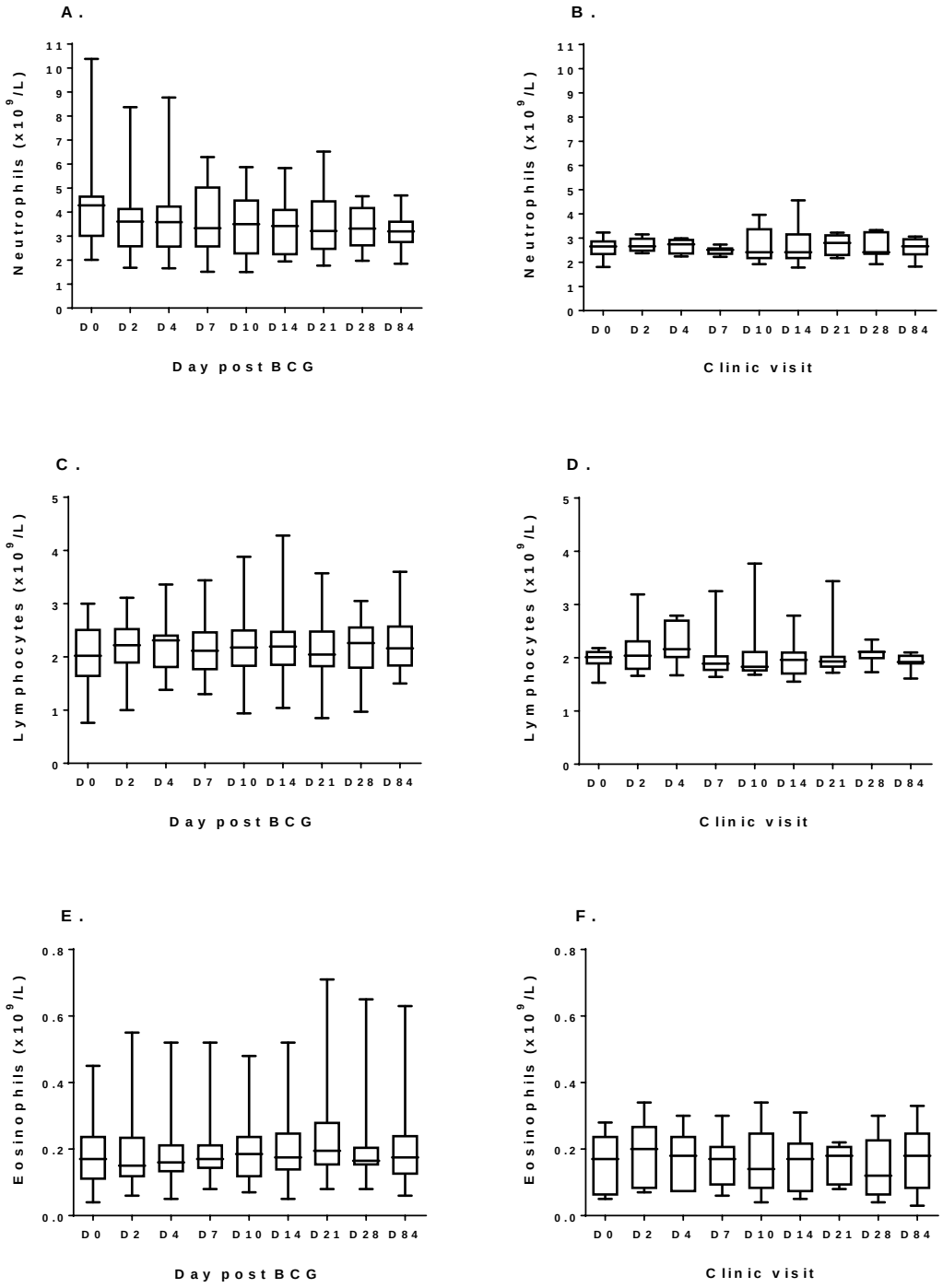


Figure 3.12 Neutrophil, lymphocyte and eosinophil trends over time. A. Neutrophil counts in volunteers in group 1 (BCG vaccinated) B. Neutrophil count in volunteers in group 2 (non-vaccinated) C. Lymphocyte counts in volunteers in group 1 D. Lymphocyte counts in volunteers in group 2. E. Eosinophil counts in volunteers in group 1 F. Eosinophil counts in volunteers in group 2. Data shown as

box-whisker plot with median and maximum range. One-way ANOVA and Dunn's test for multiple comparisons test performed.

Monocyte count decreased over time in group 1 (BCG vaccinated) peaking at D14 (p=0.0002 One-way ANOVA post hoc Dunn's multiple comparisons test; Figure 3.13 A) and was also significant between D0 and D84 (p=0.0088 One-way ANOVA Dunn's multiple comparisons test). This was not seen in group 2 volunteers (p=0.8060 one-way ANOVA; Figure 3.13 B).

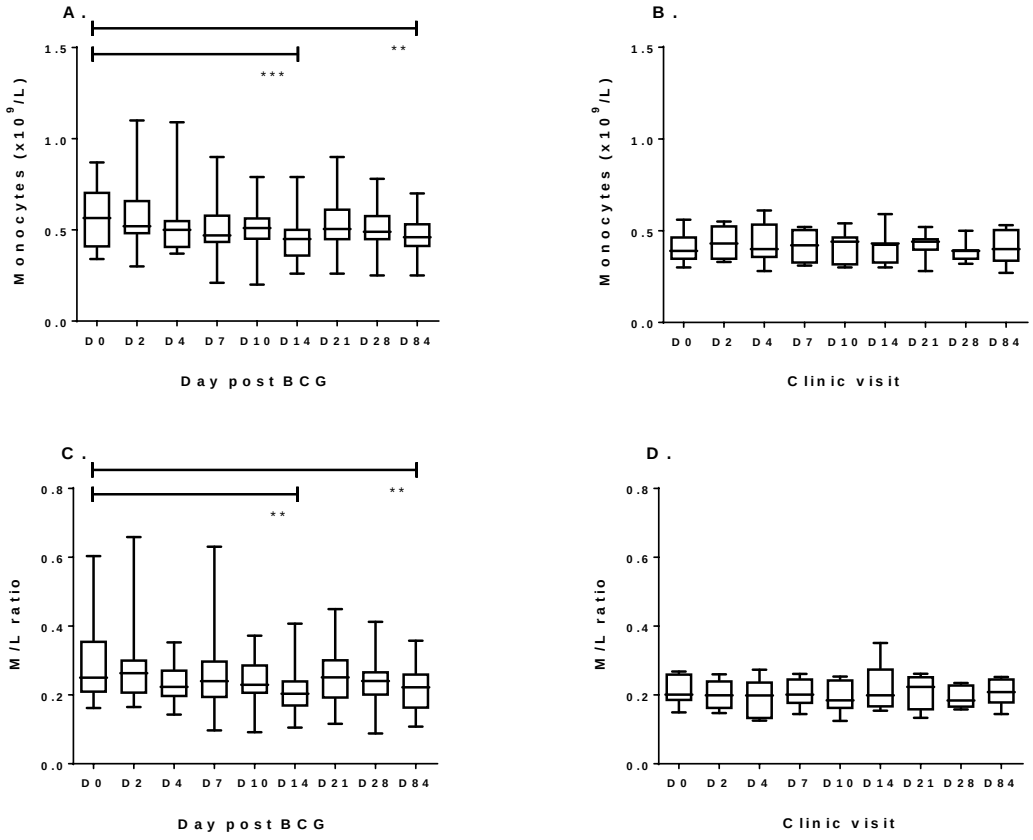


Figure 3.13 Monocyte and M/L ratio trends over time. A. Monocyte counts in volunteers in group 1 (BCG vaccinated) B. Monocyte counts in volunteers in group 2 (non-vaccinated) C. M/L ratio in volunteers in group 1 D. M/L ratio in volunteers in group 2. Data shown as box-whisker plot with median and maximum range. One-way ANOVA and post hoc Dunn's test performed.

Analysis of monocyte/lymphocyte ratio in group 1 (BCG vaccinated volunteers) demonstrated a significant reduction in ratio between D0 and D14 and D0 and D84 ($p=0.0022$ and $p=0.0098$ respectively for One-way ANOVA Dunn's multiple comparisons test; Figure 3.13 C). This was not seen in group 2 ($p=0.1529$ one-way ANOVA; Figure 3.13 D).

Further analysis of monocytes was performed (Figure 3.14). In group 1, 19 (70.4%) volunteers demonstrated a decrease in monocyte concentration and 8 (29.6%) demonstrated an increase. Those who decreased by greater than 30% were 5 (18.5%) and 2 (7.4%) increased.

In group 2, 2 (25%) of volunteers demonstrated a decrease in monocyte concentration, and 6 (75%) demonstrated an increase. 1 (12.5%) had decreased by more than 30%. and 1 (12.5%) increased by greater than 30%.

% change between D0 and D14 monocytes	Group 1 (n=27)		Group 2 (n=8)	
	Decrease	Increase	Decrease	Increase
1-29%	14 (51.8)	6 (22.2)	1 (12.5)	5 (62.5)
>30%	5 (18.5)	2 (7.5)	1 (12.5)	1 (12.5)
Total	19 (70.4)	8 (29.6)	2 (25)	6 (75)

Figure 3.14 Percentage change in monocyte concentration between D0 and D14.

3.8 Bacterial colonisation status

3.8.1 Nasal swabs

Nasal swabs were taken to assess volunteers *S. aureus* colonisation status to better understand the possible confounding it may have on the bacterial GIA. All enrolled volunteers submitted three nasal swab samples. Volunteers were considered negative if all 3 swabs were negative, or positive (carriers), if one or more swab was positive. In total, 16 (45.7%) volunteers in this study were carriers. Of those 16, two were positive on all 3 swabs (5.7% of total). The remaining 19 (54.3%) volunteers were negative on all three swabs (Figure 3.15).

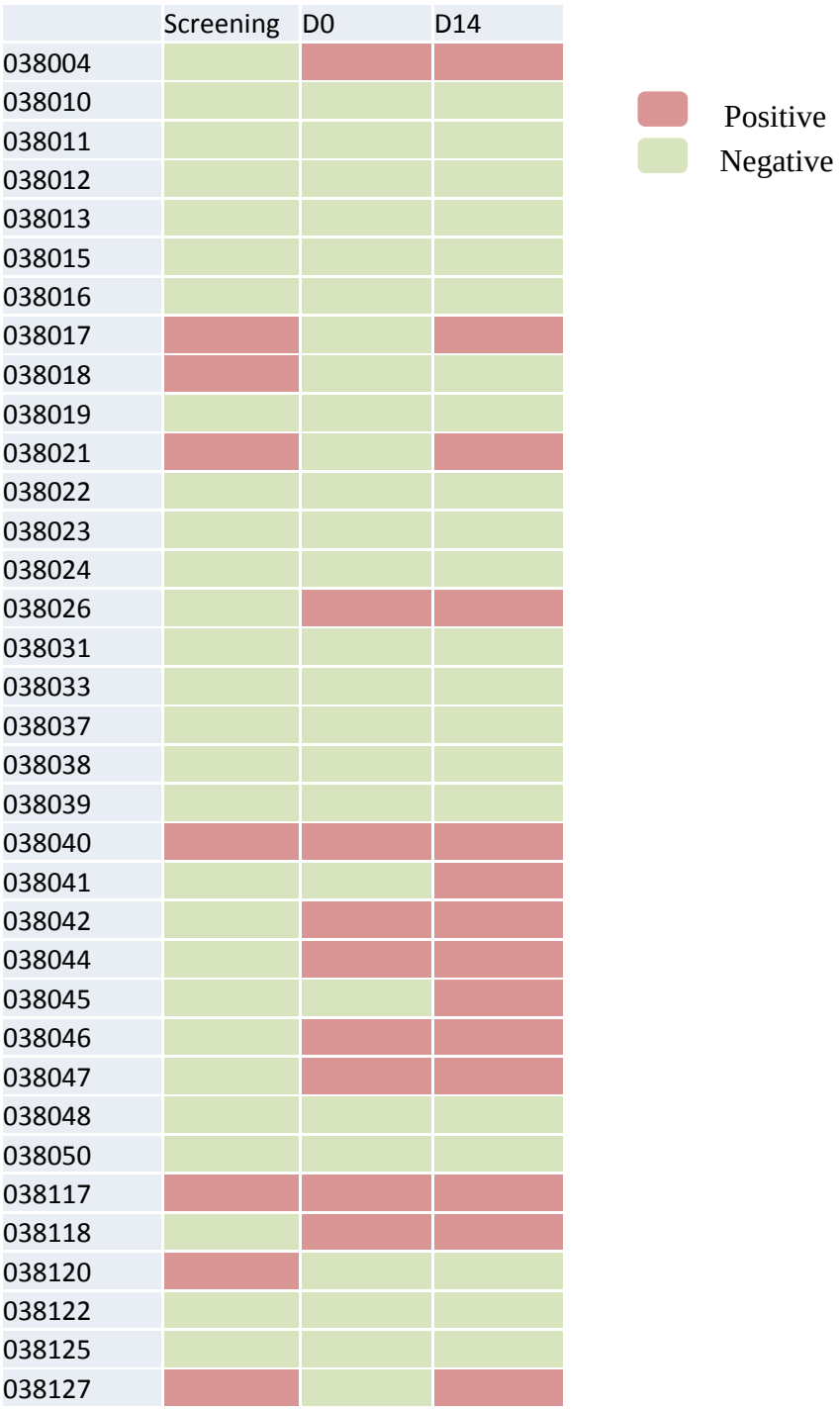


Figure 3.15 Nasal swab results for *S. aureus* carriage status. Results shown per volunteer at Screening, D0 and D14 shown. Results for phase 1 and 2 of enrolment and group1 and 2 shown.

3.8.2 Faecal culture – *E. coli*, *S. agalactiae* and *K. pneumoniae*

Volunteers deposited three faecal specimens during the study at D0, D7 and D14. Samples were processed from frozen and analysed for presence of *E. coli*, *K. pneumoniae* and *S. agalactiae* using chromogenic selective agar. *E. coli* colonies were dark blue, *K. pneumoniae* were magenta and *S. agalactiae* were pink. *E. coli* selective media would also grow other faecal organisms which would grow as salmon pink (e.g. Salmonella species) or red (e.g. Citerobacter species). *K. pneumoniae* was selective only for *Klebsiella* species, but *S. agalactiae* selective agar could grow non-selective species which would appear as a light blue colour.

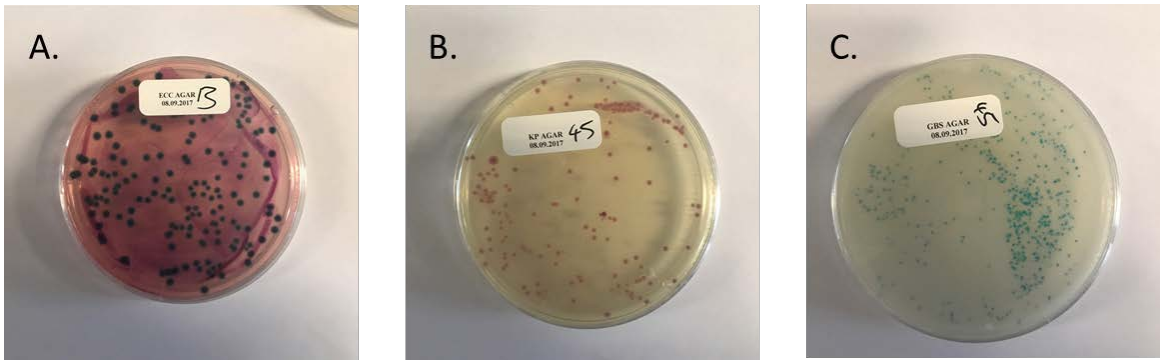


Figure 3.16 Examples of Colonies on selective agar A. *E. coli* selective agar, B. *Klebsiella* selective agar and C. *S. agalactiae* selective agar.

Volunteers were considered to have a positive colonisation status if positive growth for selected bacteria on 1, 2 or 3 of the samples. Only if negative on all three would they be considered to have a negative colonisation status.

Volunteer	D0	D7	D14	Colonisation status
038004		+	+	Colonised
038010	+	+	+	Colonised
038013	+	-	+	Colonised
038016	+	+	+	Colonised
038011	+	+	+	Colonised
038017	+	+	+	Colonised
038018	+	+	+	Colonised
038019	+	+	+	Colonised
038118	+	+	+	Colonised
038024	+	+	+	Colonised
038026	+	+	+	Colonised
038117	+	+	+	Colonised
038015	+	+	+	Colonised
038021	+	+	+	Colonised
038022	+	+	+	Colonised
038023	+	+	+	Colonised
038120	+	+	+	Colonised
038033	+	+	+	Colonised
038122	+	+	+	Colonised
038127	+	-	+	Colonised
038037	+	+	+	Colonised
038038	+	+	+	Colonised
038040	+	+	+	Colonised
038041	+	+	+	Colonised
038045	+	+	+	Colonised
038031	+	-	+	Colonised
038039	+	+	-	Colonised
038042	+	+	+	Colonised
038047	-	+	-	Colonised
038125	+	+	+	Colonised
038012	+	+	+	Colonised
038044	+	+	+	Colonised
038046	+	+	+	Colonised
038048	+	+	+	Colonised
038050	+	+	+	Colonised
			Total	100% colonised (n=35)

Figure 3.17 *E. coli* species colonisation status from faecal culture at D0, D7 and D14.

Volunteer	D0	D7	D14	Colonisation status
038004		+	+	Colonised
038010	+	+	+	Colonised
038013	-	-	-	Not colonised
038016	-	+	+	Colonised
038011	+	+	+	Colonised
038017	+	+	+	Colonised
038018	-	-	-	Not colonised
038019	+	+	+	Colonised
038118	+	-	-	Colonised
038024	+	-	+	Colonised
038026	-	+	-	Colonised
038117	-	-	-	Not colonised
038015	-	+	+	Colonised
038021	-	-	-	Not colonised
038022	+	+	+	Colonised
038023	+	-	+	Colonised
038120	-	-	-	Not colonised
038033	-	-	+	Colonised
038122	+	+	+	Colonised
038127	-	-	-	Not colonised
038037	-	-	-	Not colonised
038038	-	-	-	Not colonised
038040	-	-	-	Not colonised
038041	+	-	+	Colonised
038045	+	+	+	Colonised
038031	-	-	-	Not colonised
038039	-	-	-	Not colonised
038042	-	+	-	Colonised
038047	-	-	-	Not colonised
038125	+	-	+	Colonised
038012	+	+	+	Colonised
038044	-	-	-	Not colonised
038046	+	+	+	Colonised
038048	+	+	+	Colonised
038050	+	+	+	Colonised
			Total	63% colonised (n=22)

Figure 3.18 *Klebsiella* species colonisation species from faecal culture from D0, D7 and D14

All volunteers were positive for *E. coli* species. Twenty two (63%) were positive for *Klebsiella* species, and none were positive for *S. agalactiae*.

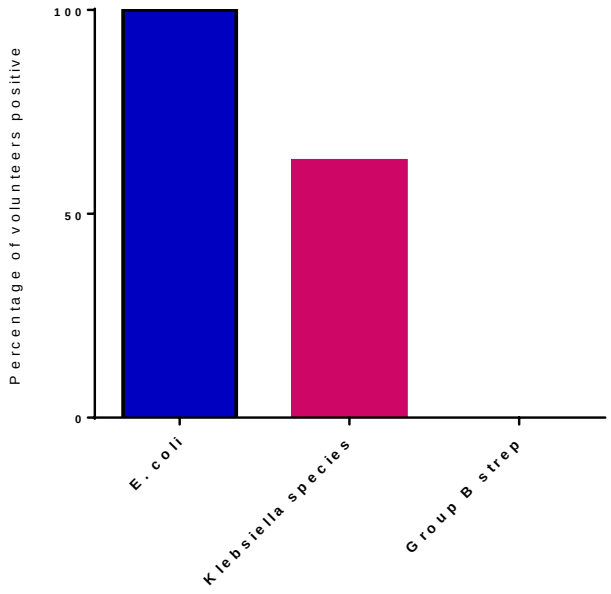


Figure 3.19 Percentage of volunteers positive for faecal carriage of *E. coli*, *Klebsiella* species and *S. agalactiae* on culture.

3.9 Administered BCG dose

BCG was plated on 7H11 agar plates for quantification of dose of BCG administered to volunteers following vaccination. Dose expected as per SmPC was 2-8 x 10^5pfu in an administered volume of 100µL. Volunteers in phase 1 were vaccinated from BCG batch no. 114015A, which expired March 2016 and a total of 5 vials were used. Of the five vials of BCG used, two were within the range of expected dose. Three of the vials used delivered a lower dose than expected (below 2x10^5pfu). Volunteers in phase 2 were vaccinated from BCG batch no. 114022A which had been given a six month extension to the expiry date by PHH due to the global shortage of BCG, a total of 3 vials were used. BCG

from this batch was used in this study during the extended expiry period. Samples plated from each of the three vials used were within the expected range.

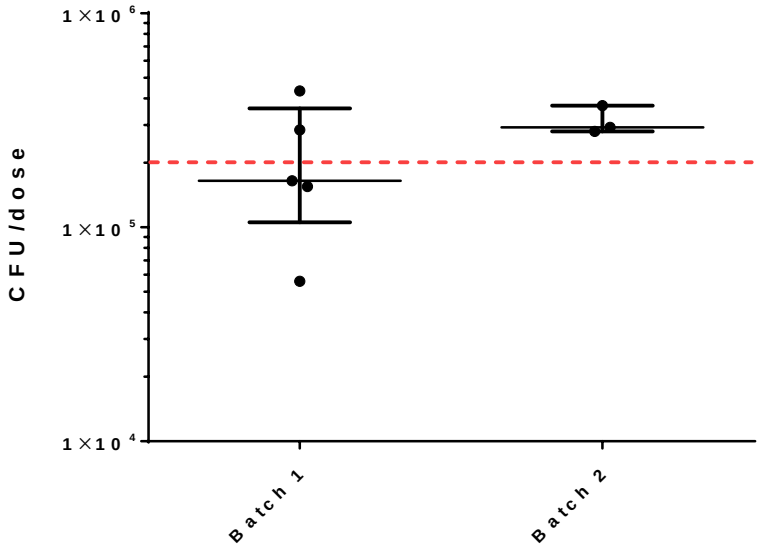


Figure 3.20 BCG CFU/dose administered during phase 1 and phase 2 of study. Each dot represents a single vial from which scheduled volunteers were vaccinated. Median and interquartile range shown. Red dotted line denotes the lower end of the expected range $2x10^5$/100µL of administered dose of vaccine.

3.10 Discussion

Following the successful ethical approval of the study documents on 4th February 2015, the enrolment of volunteers into TB038 was significantly delayed due to the impact of the global shortage of BCG. BCG vaccine demand is in excess of 130 million doses per year and following the inability of two global manufacturers to meet their supply commitment in 2013[147], by 2015 there was a global deficit of approximately 16 million doses, resulting in significant limitations to the supply of BCG for research purposes in the UK[66]. BCG SSI (Danish strain 1331) was the preferred vaccine for use in this study as it was licenced for use in the UK. Alternative sources of BCG vaccine were explored with

identification of BCG Sii (Serum Institute India) as a potential replacement with appropriate substantial amendments made to study documents and ethical approval. However, donation of BCG SSI by Professor Netea in Nijmegen and Professor Pollard at the Oxford Vaccine group enabled us to proceed with BCG SSI for this study. Between 5th October 2015 and 22nd November 2016, 35 healthy, IGRA negative, HIV, HBV and HCV negative, BCG naïve, adult volunteers aged between 18 and 45 years, were enrolled and completed their final visit in the study. The initial sample size aimed for was between 12 and 16 vaccinated volunteers in each phase of enrolment. Calculation of sample size in this study was challenging as the decrease in net growth of the bacteria in the GIA, necessary to see a significant effect was impossible to estimate in this exploratory work. Previous experience in the group with the MGIA had shown that a sample size of 15 was sufficient to see a 50% reduction in mycobacterial growth. Based on this, and our experience with recruitment of healthy volunteers into vaccine studies, in Oxford and Birmingham this sample size was felt to be appropriate and achievable. It was possible to achieve 15 vaccinated individuals in phase 1, but due to expiry of the second batch of BCG, it was only possible to enrol 12 vaccinated volunteers, and the study was terminated early.

Each vaccinated volunteer in group 1 was able to act as their own 'control' as blood drawn on D0 was pre-vaccination. However, concern over bacterial inoculum stability had driven the need to ensure than for every schedule of enrolled volunteers, there would be a non-vaccinated 'inoculum control' – a volunteer whose blood on repeated sampling could control for the daily fluctuations in bacterial inoculum encountered on repeated culture. From our experience with the challenges of volunteer recruitment, it was impractical to have equal numbers of volunteers in group 1 and group 2 as the primary aim was to evaluate the non-specific effects of BCG vaccination, and enrolling 64 volunteers where half were non-vaccinated was not in pursuit of the main endpoint. In an ideal situation, all volunteers would have been enrolled on the same day, thereby minimising the impact of

confounders such as BCG vial dose fluctuation, inoculum variability and blood processing times. However the logistical constraints of the time taken to process the whole blood GIA, meant that a maximum of five volunteers could be enrolled at any one time. Therefore, to accommodate these factors, volunteers were enrolled in schedules of a minimum of three volunteers and a maximum of five, with one 'inoculum control' non-vaccinated volunteer per schedule.

Seventy nine volunteers were screened in total across two recruitment sites in the UK; the CCVTM, Oxford, and the NIHR WTCTF, Birmingham. Of those volunteers 36 were eligible for enrolment, 22 were ineligible on medical grounds but 21 withdrew consent before it was possible to acquire BCG vaccine to permit enrolment. This was a direct result of the delay in procurement of BCG vaccine. Whilst some volunteers were able to wait for the vaccine to become available, many were not able to do so and therefore quite within their rights to withdraw consent. The delay was unavoidable, but once the vaccine was obtained, recruitment was steady and limited thereafter only by laboratory logistics.

Baseline demographics of enrolled volunteers were unremarkable. The age range across both phases of enrolment was wide (18-45 years of age and 20-44 years of age). Considering the natural differences between the immune system of infants and adults, the age range was lower in this study than other vaccine studies in the research group (normal recruitment range 18-55 years of age), but could not be so limited as to cause significant restriction to recruitment. The mean age across both phases of enrolment was significantly different ($p=0.0011$ Mann Whitney test) with the mean age being lower in phase 1 (24 years versus 31 years), however there was no significant difference seen between those randomised to group 1 (BCG vaccinated) or group 2 (non-vaccinated). The majority of volunteers were born in the UK, and of those born outside of the UK but resident here for the duration of the study, none were born in TB high burden countries. Volunteers in group 1 had a slightly higher mean BMI, however this was likely to be due to

the differences in group size and unlikely to have impacted on the outcomes. Smoking of tobacco and consumption of alcohol are both factors for which there is a growing body of evidence of an impact on the innate and adaptive immune system[148, 149]; and are additional key differences between an adult and neonatal study group. Approximately 30% of volunteers enrolled in the study were smokers, and 80% consumed alcohol.

The impact of gender on vaccine responses and in particular the non-specific effects of vaccines is an important consideration for any study exploring the non-specific effects of BCG. Studies have suggested that in the case of the high titre measles vaccine a polarisation towards an increased deleterious non-specific effect of vaccination was seen in females [150], there may be an association with increased deleterious effects of DTP in females[74] and more recently, this has been suggested with malaria vaccine RTS,S/ASO1[151]. In the case of BCG, studies have suggested that the non-specific beneficial effects are strongest in females[77, 146], however the systematic review commissioned by the WHO did not find overwhelming evidence for this[83]. However, there is evidence that females and males respond differently to vaccines, with women often reporting higher levels of side effects[152]. There are several potential mechanisms for this, including differences in male and female innate and adaptive immune responses, impact of sex hormones on immunological cell function and pregnancy[78]. There has been criticism in the way vaccine studies have reported sex differences, with an implication that not enough emphasis is placed on its importance. This study was not powered to assess the impact of sex differences on the primary outcome as numbers were too small and to restrict by gender could impact on recruitment. Overall there were more females than males enrolled with 68% being female. The percentage of females enrolled in each phase was similar. There were more females enrolled in to Group 1 (78%), but in group 2 there were more males (63%). Whilst association between primary outcome and gender will be evaluated, numbers are unlikely to allow for any strong conclusions.

BCG vaccine safety is well documented. It is a safe vaccine which has been given to several billion people. The most common side effect is a local reaction at the vaccination site. Approximately 2 to 6 weeks after ID vaccination with BCG, a small lump may appear which can develop scaling of the skin over the site and redness, can be painful and may discharge a cloudy fluid, and develop into a dry based ulcer. In a small proportion of individuals this can be associated with small tender lymph nodes in the axillae secondary to the skin reaction (1 in 100). Rarely, it can be associated with a mild fever and headache (1 in 100). Between 6 weeks and 6 months, the vaccine site then develops into a small scar. Rare side effects (1 in 1000) include abscess formation in the lymph nodes or BCG-osis, an extremely rare complication of disseminated BCG, mostly commonly seen in immunocompromised people[153]. It is for this reason that BCG is contraindicated in individuals with HIV or who are known to be immunocompromised. Volunteers were HIV, HBV and HCV negative and had a thorough medical history to ensure no undiagnosed condition which may exclude them from participation. The volunteers enrolled in this study had a clinical review of the site of vaccination at every visit and all had a normal skin reaction with a normal healing process. No SAE/SARs were reported during the study.

Iron metabolism is a complex process. Iron is absolutely necessary for complex cellular function and metabolism in humans and is found most abundantly bound to proteins (transferrin, lactoferrin and ferritin) or complexed with haem, as in its free form it is relatively unavailable and toxic[154, 155]. As well as being vital to human cell function, it has been shown to be key in pathogen metabolism and cell function also [156, 157]. This shared dependency is central to the host/pathogen relationship[158] with an intricate balance of mechanisms at work. Early studies demonstrated that host control of iron availability to invading pathogens was able to restrict bacterial growth[159] and studies have shown that pathogenic organisms have developed mechanisms by which they can efficiently obtain iron from their host; such as acquisition from haem and haem containing proteins, mechanisms to bind and uptake iron from transferrin, lactoferrin and ferritin,

gain of ferric iron from siderophores and the uptake of ferrous iron [158]. The intracellular pathogen *M tb* beautifully demonstrates the host/pathogen relationship. Whilst it is not fully clear how Mycobacteria take up iron from host macrophages, blocking cellular uptake of iron by infected macrophages has been demonstrated to inhibit growth of the pathogen [160-162]. Mycobacteria in part block maturation of phagosomes in macrophages and by halting the cell at this early stage of development, allow improved exploitation of the host iron uptake system[157] and studies have shown that mycobacteria are able to utilise and uptake iron from transferrin and intracellular iron stores[163]. *E. coli* has surface transport receptors for iron binding from transferrin[157] and *S. aureus* has been found to preferentially select haem bound iron over transferrin during infections, with identification of a gene encoding a haem-transport system responsible for the transportation of haem into *S. aureus*[164]. As the majority of iron in the host is bound to haem with less than 1% bound to transferrin, this evolutionary advantage therefore maximises the amount of iron available to an organism for utilisation in vital growth functions when invading a host. In a series of elegant experiments by Tanner et al, they demonstrated that there was a positive correlation between Hb and mycobacterial growth in the MGIA, and that Hb gene expression positively correlated with the whole blood MGIA in adults and to a lesser extent in the PBMC MGIA in infants[165]. Therefore, taking this into consideration, it was important to know the impact that fluctuation in volunteer Hb concentration and other cell differentials may have on the outcome of the whole blood bacterial GIA.

Combined analysis of the repeated Hb measure of the volunteers in group 1 compared with group 2 showed a significant drop in Hb concentration at D14 and sustained until D28 in the vaccinated volunteers which was not significant in those not vaccinated. When split by gender, the significant decrease was evident in both male and female volunteers in group 1 and again, not in group 2. Whilst there was not a statistically significant change, there was a visible decrease in mean in group 2, which mirrored group 1 (resolution by

D84), and it is likely that the difference in group size accounts for group 2 not reaching statistical significance. The question raised is whether the decrease seen is a manifestation of repeated blood sampling (60mls over 9 visits over 3 months = 540mLs total), or whether it could be related to vaccination with BCG. The total amount taken during a single blood donation visit is approximately 470mLs and donors are asked not to donate more the 4 times a year for men, and 3 times a year for women. During the three months of this study, volunteers donated between 420mLs (volunteers from Birmingham) and 540mLs (volunteers from Oxford) and were instructed not to donate before enrolment, nor donate blood for three months following completion of the study in order to ensure that sufficient time had been given to allow Hb levels to recover. Despite the decrease, most volunteers remained within the 'normal' range (120-170g/L), and none demonstrated any clinical effects. Almost all returned to baseline by D84. Four volunteers were below the lower limit of normal at the end of the study; however all were at the lower end of the normal range at the start and were recovering by the end of the study. They were given appropriate advice and instruction on dietary measures. Hb and haematocrit as markers of effect of venesection and as an indicator for inclusion in blood donation, has been subject to criticism due to their variability depending on circulating plasma volume, and other markers such as total Hb mass (tHb) have been suggested as more accurate measurements[166]. In the case of these volunteers, whilst haematocrit and RBC concentration mirrored the decrease seen in Hb; MCV, MCHb and MCHb conc. did not. This picture suggests that the drop in Hb was a feature of frequency of blood donation and not on red cell production by the bone marrow, therefore unlikely to be a result of BCG vaccination.

Analysis of other circulating cells did not reveal any significant change in platelets, total WCC, neutrophils, lymphocytes or eosinophils. It would not have been unreasonable in the case of BCG vaccination, that the vaccine, through induction of a limited skin infection with live replicating *M. bovis*, that there might have been some evidence of an

inflammatory response such as a transient increase in neutrophils or platelets. However this was not the case, though it is important to clarify that these measurements were only giving an indication of total circulating number, not an indication of changes in activation status of the cells.

Interestingly there was a significant decrease in levels of circulating monocytes and corresponding M/L ratio in volunteers in group 1. This decrease peaked at D14 but was present at D84 also. Analysis of monocyte fluctuation in both groups demonstrated significant heterogeneity between volunteers, and variability on repeated measures in both groups. In both groups there were volunteers whose level of circulating monocytes increased and well as decreased. A significant number of volunteers varied by up to 29% and taking into consideration technical and laboratory operator errors; this would have to be considered within the scope of 'normality'. In group 1 there was a dominant five volunteers who had a greater than 30% decrease in monocytes who appeared to be driving the significant change seen. It raised the question; is this finding a chance of repeat sampling of a group of adult volunteers, or are the circulating monocytes being activated and recruited to the site of BCG vaccination? The importance of circulating monocytes and ML ratio have been documented in relation to stages of TB disease[167-169], but little is known about effect of BCG on total circulating monocytes. Much like the decrease in Hb, this is most likely an effect of repeated blood sampling and the discordance in group size means that any potential overall decrease in group 2 is masked. Analysis of monocyte activation markers would likely be able to shed light on this but was outwith the scope of the study at this time.

As humans we have a close and personal relationship with bacteria. Complements of bacteria which colonise our skin, nares, and gut are specific to each of us, with the caveat that these 'healthy commensals' have the potential under the right set of conditions to become pathogenic. It is the interplay between the bacteria and our immune system, and

the bacteria with each other at the level of the biofilm, which enables this synergistic environment to, in the majority of cases, remain healthy. Any inequality in the balance, such as factors reducing our immune system or leading to bacteria penetrating the mucosal barrier, can create a state of disease.

S. aureus carriage in the population is determined by many factors including geographical location, age, sex, occupation, presence of skin disease and location of skin swabbed[170]. In the UK, prevalence rates are reported between 20 and 22%[171, 172] but lower in children at approximately 18%[173]. Of the volunteers enrolled in this study 45.7% were considered carriers with positive detection of *S. aureus* colonies on selective agar from nasal swab. Most had 1 or 2 positive swabs and only 5.7% were positive on all three swabs. This demonstrates the importance of performing three swabs, as some individuals have fluctuating colonisation status. Possible reasons for why this cohort had a higher level of carriage that the quoted UK prevalence may have been due to occupation or multiple co-habitation (as in the case of students living in halls of residence).

Determination of *E. coli*, *S. agalactiae* and *K. pneumoniae* colonisation was more challenging. Analysis was made through faecal culture using selective agar. An elegant exploration of the microbiome using genetic sequencing would have been desirable, but was beyond the time constraints of this project. Therefore a binary approach was taken with appropriate storage of samples for potential genetic analysis at a later stage.

E. coli is common. Our gut exposure begins from the moment of birth on passing through the birth canal and exposure to both gram positive and gram negative, aerobic and anaerobic bacteria, with *E. coli* and *Enterococcus* species the first to colonise[174-176]. Most gut *E. coli* are non-pathogenic, however there are several pathogenic strains which include: enterotoxigenic *E. coli*, enteropathogenic *E. coli*, enteroinvasive *E. coli*, enterohaemorrhagic *E. coli*, uropathogenic *E. coli* and verotoxin-producing *E. coli.* Therefore as adults, the expectation would be that we are all carriers of *E. coli* strains.

This was reflected in the faecal culture. ECC selective agar was used to identify the presence of *E. coli* strains. Where *E. coli* colonies grew, they would turn a dark blue with a very dark blue centre. Other species could grow on this agar such as *Salmonella* and *Citerobacter*, however they would grow as light pink and red/purple colonies respectively. Plating of three samples collected on D0, D7 and D14 revealed, as expected, that all volunteers were colonised with *E. coli* species. Five volunteers had at least one negative culture out of the three. This was likely due to poor specimen size and freeze thaw action prior to processing.

Klebsiella species are also common in gut flora. They are opportunistic bacteria which are most commonly associated with hospital acquired infection[101] and have long been associated with multiple drug resistance[177]. Studies have reported gut carriage from faecal culture to be in the region of 5-38% with nasopharynx carriage to be between 1-6%[178-180]. As a pathogen, *Klebsiella* does not survive well on healthy skin and therefore skin carriage levels are negligible and considered transient at most[181]. In developing countries, a study of colonisation of neonates, found gram negative colonisation of which *K. pneumoniae* was the most common, to be associated with increased cases of sepsis[182], and it is commonly found of the list of causes of neonatal sepsis globally[183]. With no definitive reference range for what would be considered the expected level of colonisation of healthy volunteers, it is impossible to comment on whether the 63% of volunteers colonised in this study is high. As with the other bacteria, positivity was variable over the three swabs, and may have been influenced by specimen quality or freeze thaw action.

S. agalactiae has received a great deal of notoriety. It is a common cause of sepsis and death in neonates, passed between mother and infant at birth, and often developing within the first three days of life. Maternal vaginal carriage is variable with approximately 25% of women are thought to be carriers. One study from 1978 found 28% of women

(n=382) to have vaginal colonisation, and as with colonisation with other bacteria, was variable and dependent on race, age, and parity[184]. The gut has been identified as the likely primary source of infection[185]. In this study, using a selective agar, there were no positive faecal cultures. Almost all agar plates grew colonies, but they were pale blue in colour not pink which was indicative of *S. agalactiae*. There are many possible reasons for this: firstly, all volunteers were negative on all three swabs, secondly, the agar was not accurately picking out the correct colonies and therefore there were high false negative rates, or thirdly, due to the less robust nature of *S. agalactiae,* viable bacteria did not survive the freeze thaw process. Further analysis and characterisation of samples would need to be performed to confidently define all volunteers as non-colonised with *S. agalactiae.*

BCG vaccine dose estimated by manufacturers varies depending on strain and manufacturer. Different strains may have different potencies as a vaccine with impact on scar size, specific, and non-specific immunological responses[70, 71]. BCG SSI is reported to deliver 2-8 x 10^5pfu per 100µL dose and has an 18 month shelf life from manufacture and should be administered within six hours of reconstitution. In this study, we used two batches of BCG SSI obtained from two different sources. The first batch (batch no. 114015A) was within the expiry date originally provided by the manufacturer, and overall five vials were used. There was a great deal of variability in dose plated on 5H11 agar. Three of the vials were below the lower end of the dose range. The second batch of BCG SSI (batch no. 114022A) was given a six month extension to its expiry date by PHE due to the already discussed BCG global shortage crisis. Three vials were used all of which were during the expiry extension period, however all were within the expected dose range.

Whilst this finding could be consistent with known issues with variability in dose experienced with BCG vaccine, it is possible that this could have been due to technical error or caused by clumping of the bacteria. All volunteers had an expected skin reaction

therefore the clinical relevance of this is uncertain and will be explored further in Chapter 6.

3.11 Conclusions

- 35 healthy, BCG naïve volunteers were successfully enrolled and completed the study
- Baseline demographics between volunteers in groups 1 and group 2 were unremarkable and similar
- Volunteers in group 1 demonstrated a decrease in Hb following vaccination, however this finding was likely related to frequency of blood sampling rather than a direct response to vaccination
- Decrease in Hb was within the limits of what would be expected from a single blood donation and did not results in any clinical side effects for the volunteers
- There was a decrease in monocyte count following vaccination in group 1, but with significant heterogeneity in volunteer responses across both groups, in a sample of this size meaningful conclusion is impossible and work relating to activation status of monocytes would be valuable in better understanding this finding
- *S. aureus* nasal colonisation in volunteers was 45.7%, higher than expected, but likely influenced by background factors of the study population. As expected, positivity of swabs varied for individuals.
- Faecal colonisation status of *E. coli* in volunteers was as expected at 100% and *Klebsiella* species was 63%. As expected, there were fluctuations in positivity between samples.
- All volunteers were negative for S. agalactiae on faecal culture. Whilst this is possible, though unlikely given that approximately 25% of the population are expected carriers, there are many technical issues with agar and freeze-thaw action of specimen storage which may have reduced viability of any organisms present.

- Dose of BCG administered was variable and could impact on the findings of the whole blood GIA, however this is unlikely.

4 Whole blood GIA results

4.1 Statement of Authorship

S. aureus WTNM strain was made available for use by D Wyllie and the *S. aureus* group, Jenner Institute. Clinical isolates of *E. coli*, *K. pneumoniae* and *S. agalactiae* were identified from blood culture samples at the John Radcliffe Hospital by S Oakely. Frozen stocks of bacteria were prepared by M Wilkie. Initial GIA protocol optimisation for *S. aureus* was performed by R Tanner in collaboration with the *S. aureus* group under the supervision of Dr D Wyllie, the protocol was adapted from the direct mycobacterial growth inhibition assay (MGIA). Further optimisation of *S. aureus* GIA was performed by M Wilkie with the assistance of J Beglov, and A Hamidi. Optimisation of the GIA for the other three pathogens was performed by M Wilkie. Growth curves were performed by M Wilkie using a protocol provided by P Bettencourt. Study GIA samples were processed by M Wilkie with assistance from D Wright and R Tanner for plating of samples onto blood agar. BACTEC FX tubes for time to positivity (TTP) analysis were prepared by M Wilkie and processed by S Oakley and team at the Department of Microbiology, John Radcliffe Hospital, Oxford. Results were provided by S Oakely but all collating of data and analysis was performed by M Wilkie. Data analysis from the GIA was performed by M Wilkie. Statistical support was provided by V Harris (Medical Statistician).

4.2 Introduction

As outlined in chapter 1, the development of a whole blood GIA which could be adapted to a variety of different pathogens, would provide a valuable tool for the evaluation of the non-specific effects of BCG in humans where *in-vivo* challenge is not feasible. As a measure of the 'sum of the parts' of the immune system, such an assay has the potential to provide a surrogate marker for an individual's capacity to control growth of bacteria pre

and post BCG vaccination, with the caveat that the components of the immune system being measured include only those present in the periphery at the time of the bleed, therefore providing no information of the immunological mechanism present in the mucosal barrier compartment. This functional approach has been used with success for a variety of different pathogens and more specifically, the use of the MGIA in the TB vaccine community[140, 186]. Adapted from the method first described by Wallis et al[141], we have demonstrated that the direct MGIA is capable of showing a specific effect of BCG vaccination on reducing mycobacterial growth in humans[140], mice[142] and non-human primates (NHPs) (Tanner et al data unpublished). Work within the group has also shown that it is able to discriminate between humans with active TB, LTBI and controls (O'Shea et al submitted). Additionally the direct MGIA is able to correlate with *in-vivo* protection in mice[143, 144], *in-vivo* protection from BCG and *M tb* challenge in NHPs, and with epidemiological data from human primary/re-vaccination studies[140].

4.3 Aim of Chapter

The aims of the experiments detailed in this chapter were to:

- Assess whether the whole blood direct MGIA method could be adapted for use with *S. aureus*, *E coli*, *K. pneumoniae* and *S. agalactiae*
- Optimise the assay conditions for each pathogen
- Apply the whole blood *in-vitro* GIA method to a study of recent BCG vaccination in healthy BCG-naïve UK adults, to assess whether this assay has the capability to act as a surrogate marker for an individual's capacity to control growth of the four mentioned pathogens
- Explore the correlation between blood lysis plating steps and BACTEC FX TTP output, to assess which would be the most efficient and reliable method

- Determine the impact of fluctuations in bacterial inoculum concentration on GIA outcome measures using a non-vaccinated control volunteer
- Determine whether whole blood in-vitro GIA output is confounded by variables such as FBC differential, gender, or bacterial colonisation status

4.4 Optimisation strategies

4.4.1 *S. aureus* as a test case

Optimised growth conditions for *S. aureus* were provided by the *S. aureus* group. Wild Type Newman strain (WTNM) was used.

4.4.1.1 S. aureus growth in blood – reproducibility and reliability

Blood was taken from six healthy volunteers for use in the *S. aureus* whole blood *in-vitro* GIA. Blood was taken into lithium heparin tubes. Output was measured as CFU/mL and plotted as Log10 CFU/mL. Samples were plated onto horse blood agar and incubated overnight using the spiral plater at 50 exponentials and counted using Qcount plate reading equipment and software. Samples were measured in duplicate. Figure 4.1 shows growth of *S. aureus* in blood incubated for 30 minutes, 1 hour, 2.5 hours and 6 hours. The volunteers differed in their ability to control growth of *S. aureus*, peak killing is at 1 hour and bacteria steadily multiply thereafter. Volunteer C was known to be a carrier of *S. aureus* and have specific antibodies, whereas D and F were not carriers.

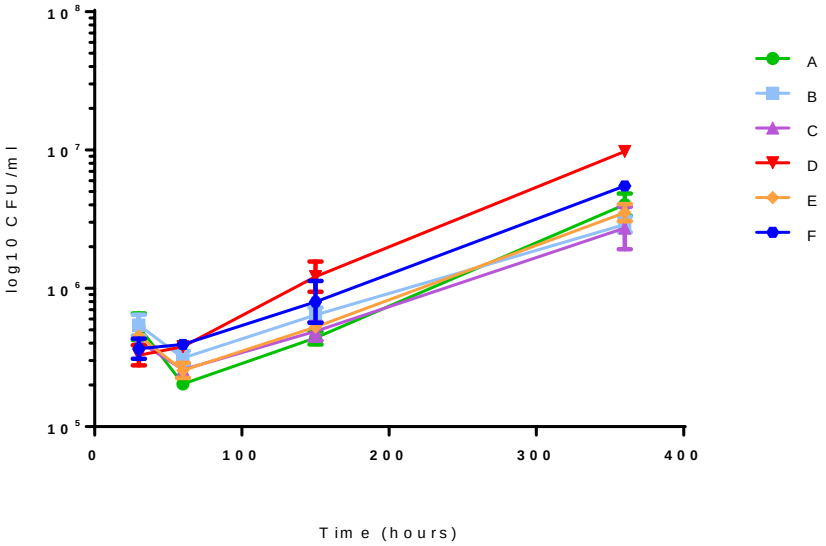


Figure 4.1 *S. aureus in-vitro* GIA from six healthy volunteers following 30 minute, 1 hour, 2.5 hour and 6 hour incubation periods. Each dot represents volunteers with range bars for duplicates shown.

Blood from four volunteers was drawn into lithium heparin tubes, and duplicate samples were incubated with two different inoculum concentrations for *S. aureus* for six hours. Figure 4.2 shows the CFU/mL recovered after incubation with inoculums of 1 x 10^7 CFU/mL and 1 x 10^6 CFU/mL. The same pattern of growth was exhibited by the volunteers at both inoculum concentrations demonstrating reproducibility (Figure 4.2).

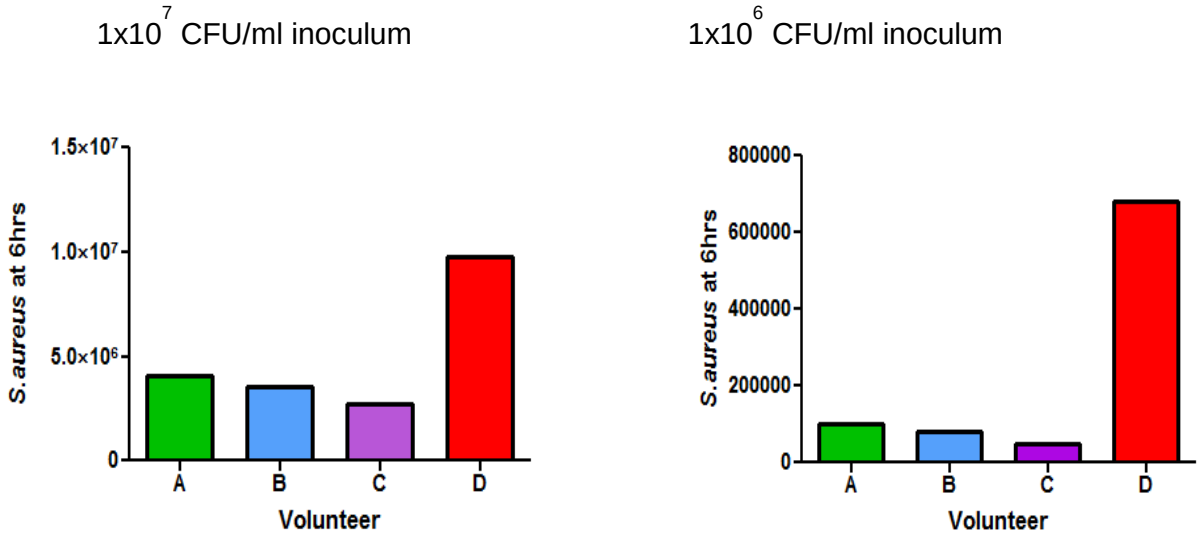


Figure 4.2 CFU/mL recovered from blood of four volunteers incubated for 6 hours with S. aureus. Each column represents a single volunteer and matches the corresponding column in the other graph.

4.4.1.2 S. aureus pilot study

During the initial optimisation of the assay, the opportunity was taken to use whole blood from a small number (n=11) of volunteers enrolled in a separate BCG challenge study (TB031[187]). Appropriate ethical approval was sought. Volunteers were to receive three times the standard dose of BCG SSI (6-24 x 10^5pfu) on day 0 (D0), intradermally (ID), and then have a punch biopsy from the site of challenge at D14. Volunteers were followed up until D28, however no blood draw was planned at that visit. Study time-line is shown in Figure 4.3.

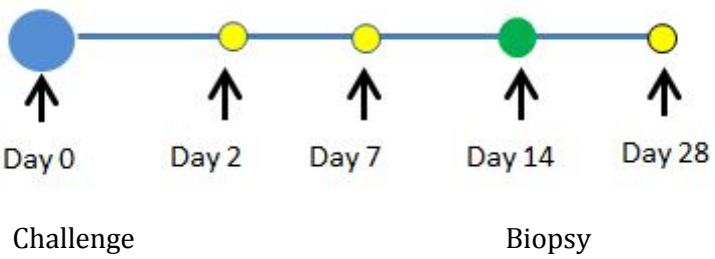


Figure 4.3 Schematic representation of clinic visit for volunteers enrolled in TB031. Day 0 BCG challenge day – volunteers received BCG SSI at 6-24 x 10^5pfu ID. Follow-up visits were performed on D2 and D7. On D14, volunteers attended for a 5mm punch biopsy of the vaccination site and had a final review of biopsy site on D28. Blood was drawn on D0, D2, D7 and D14. No blood was taken on D28.

Results of the net recovery of *S. aureus* from the blood of these 11 volunteers showed a possible trend towards improved control of *S aureus* growth following BCG vaccination but it did not reach statistical significance (1 hour incubation: p= 0.1447 one-way ANOVA Friedmans test; 24 hours incubation p=0.1447 one-way ANOVA Friedmans test; Figure 4.4 A and B respectively). The primary purpose of this pilot study was to ensure that it was logistically possible to process the samples, and this was achieved.

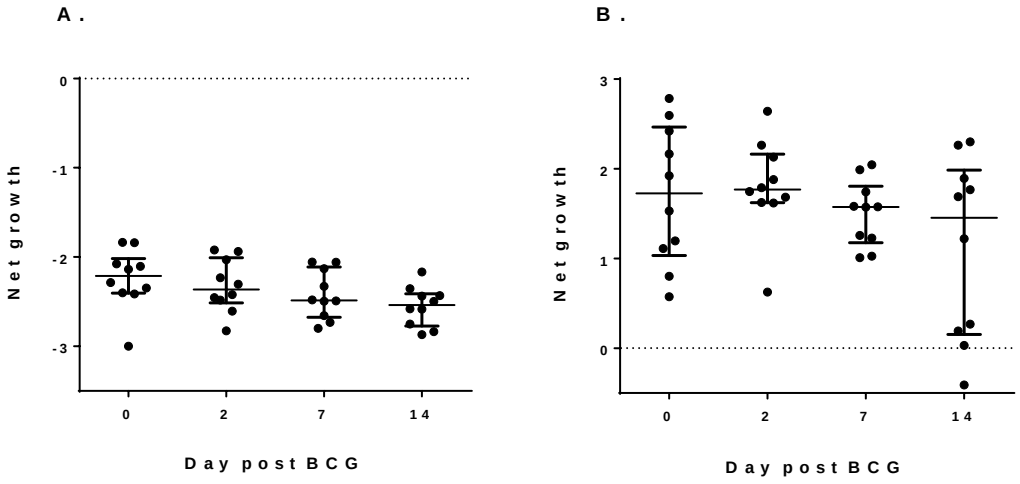


Figure 4.4 Net growth of *S. aureus* from whole blood in-vitro GIA from 11 healthy BCG naïve volunteers who received BCG SSI at 6-12 x 10^5pfu at D0. Blood taken on D0 was pre-vaccination. Blood samples drawn on D0, D2, D7 and D14. Each dot represents the mean of duplicate measurements for each volunteer, median with interquartile range shown. A. net growth following 1 hour incubation, B. net growth following 24 hour incubation.

4.4.1.3 Impact of blood sample delay on net growth following GIA

The impact of a significant time delay from blood draw to laboratory processing was unknown and therefore an experiment was performed to assess whether blood could be drawn from volunteers in Birmingham and processed in Oxford (3-6 hour time delay), or whether volunteers needed to be brought to Oxford for visits and blood draw (volunteers bled and sample processed within 2 hours). Five healthy volunteers provided a blood sample which was taken and processed 2 hours later, with the remaining blood left on the laboratory work bench and then processed at 5 hours post-venesection. One volunteer was unable to participate on the day of collection and could not be replaced. Following the 1 hour incubation, median net growth of samples processed at 2 hours and 5 hours was similar (p=0.4375 Wilcoxon matched-pairs signed rank test; Figure 4.5 A). Following 24 hour incubation, there was no difference between a 2 hour and 5 hour delay to processing (p=0.3125 Wilcoxon matched-pairs signed rank test; Figure 4.5 B).

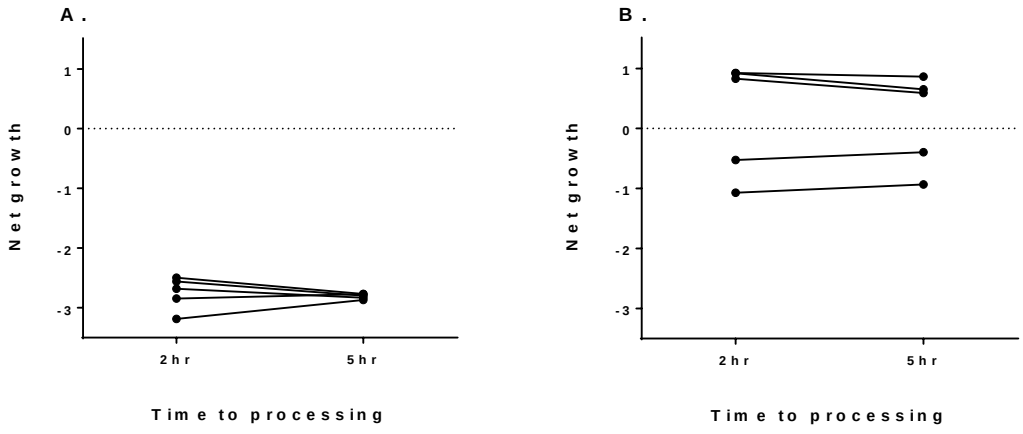


Figure 4.5 Impact of delay in blood processing on *S. aureus* net growth from GIA. A. net growth from samples with 2 and 5 hour delay to blood processing following 1 hour incubation and B. net growth from samples with 2 and 5 hour delay to blood processing following 24 hour incubation. Each dot represents the mean of duplicate samples for each volunteer, line represents paired samples.

4.4.2 Growth curves – reliability of wild type bacteria

Wild type bacteria have a reputation for being unreproducible on repeat culture and different bacteria have different growth kinetics. Assessment of bacterial growth and reproducibility on repeated culture was done by performing growth curve analysis of CFU/mL and cell mass using OD_{600} measurement calculated by spectrophotometry. All four bacteria were assessed.

As *S. aureus* growth kinetics were well understood from previous experience with the *S aureus* group, growth curve analysis was performed from two separate cultures in order to ensure that results were reproducible in our group. As *E. coli* was a wild type strain, and no experience was available with this isolate, growth curves were performed from four separate overnight inoculums. Results for these bacteria are shown in Figure 4.6.

Growth curve analysis of *S aureus* and *E coli* found both bacteria to have highly reproducible growth kinetics.

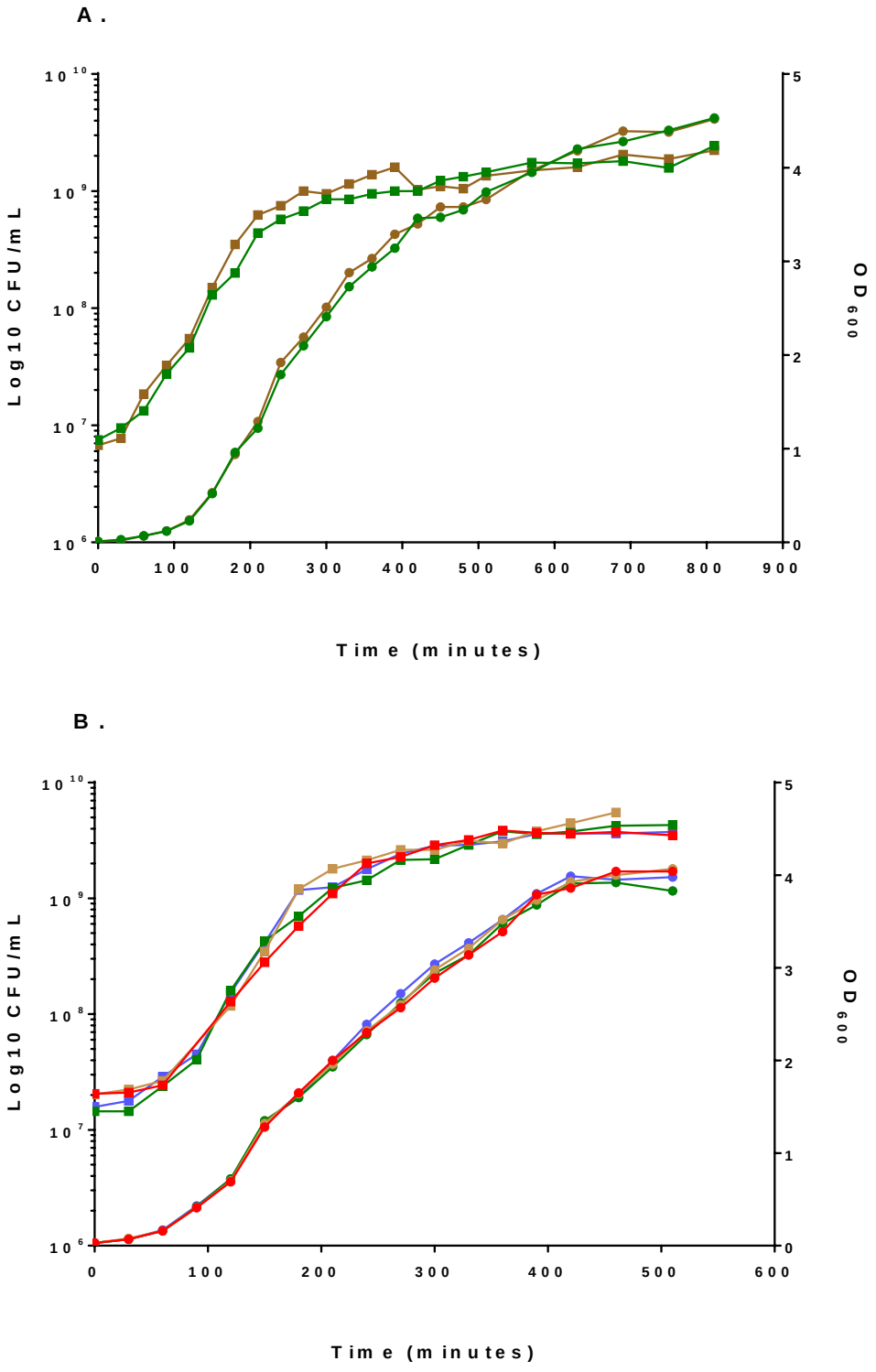


Figure 4.6 Bacterial growth curves measured as CFU/mL and OD_{600}. CFU/mL and OD_{600} are paired by colour of line, CFU/mL is represented by lines with squares, and OD_{600} is represented by lines with circles. A. growth curves for *S. aureus* B. growth curves for *E. coli*.

With the highly reproducible results seen with repeated culture of *S. aureus* and *E.coli*, and due to the time consuming nature of the growth curve analysis, two separate cultures were performed for both *K. pneumoniae* and *S. agalactiae*. Results are shown in Figure 4.7. Again highly reproducible results were obtained from both these wild type bacteria on repeated culture.

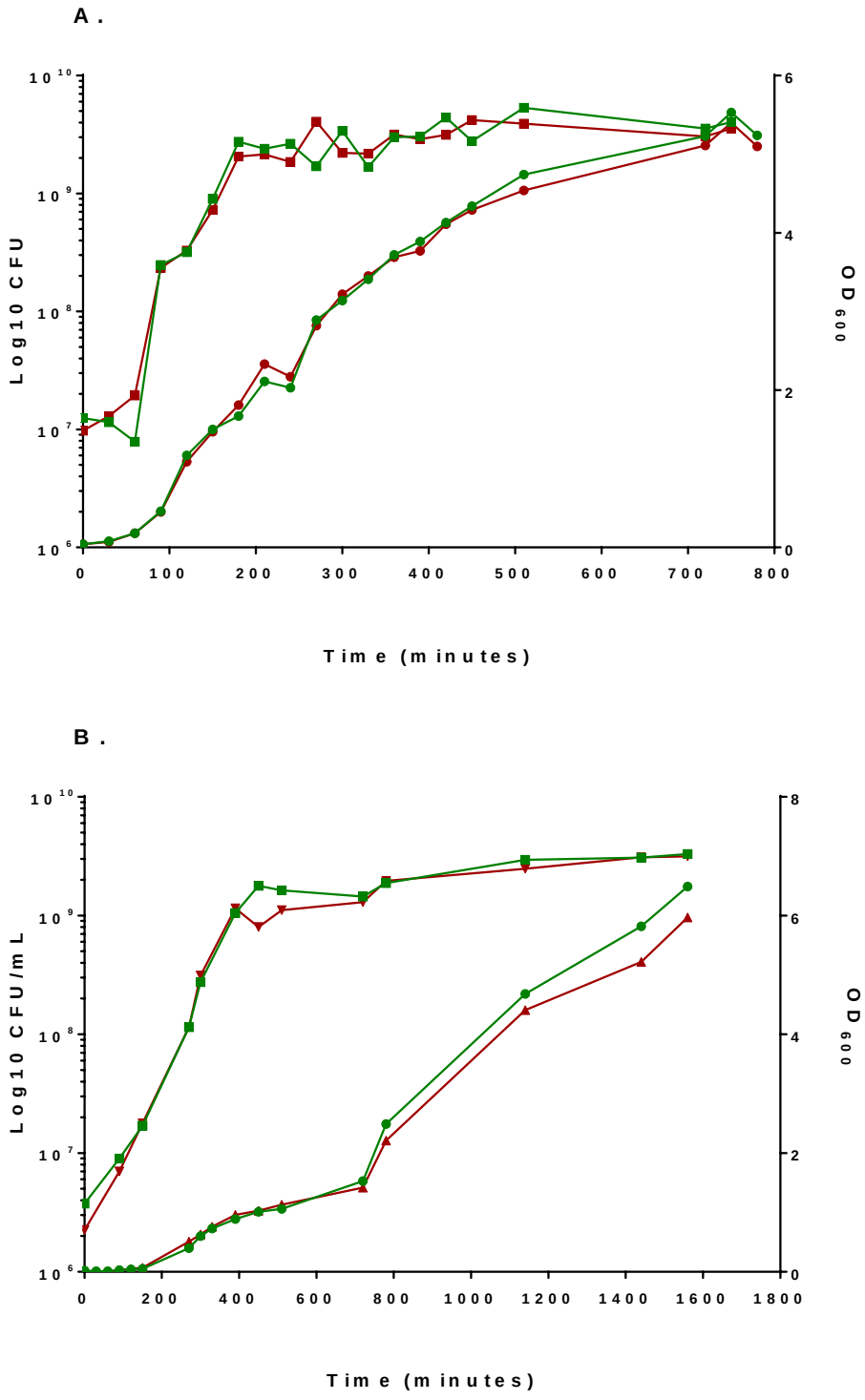


Figure 4.7 Bacterial growth curves measured as CFU/mL and OD_{600}. CFU/mL and OD_{600} are paired by colour of line, CFU/mL is represented by lines with squares, and OD_{600} is represented by lines with circles. A. shows CFU/mL and OD_{600} for *K. pneumoniae*, B. shows the CFU/mL and OD_{600} for *S. agalactiae*

The similarity between CFU/mL curve and OD_{600} curve using a linear regression model. Results are shown in Figure 4.8 for *S. aureus*, Figure 4.9 for *E. coli* and Figure 4.10 for *K. pneumoniae* and *S. agalactiae*.

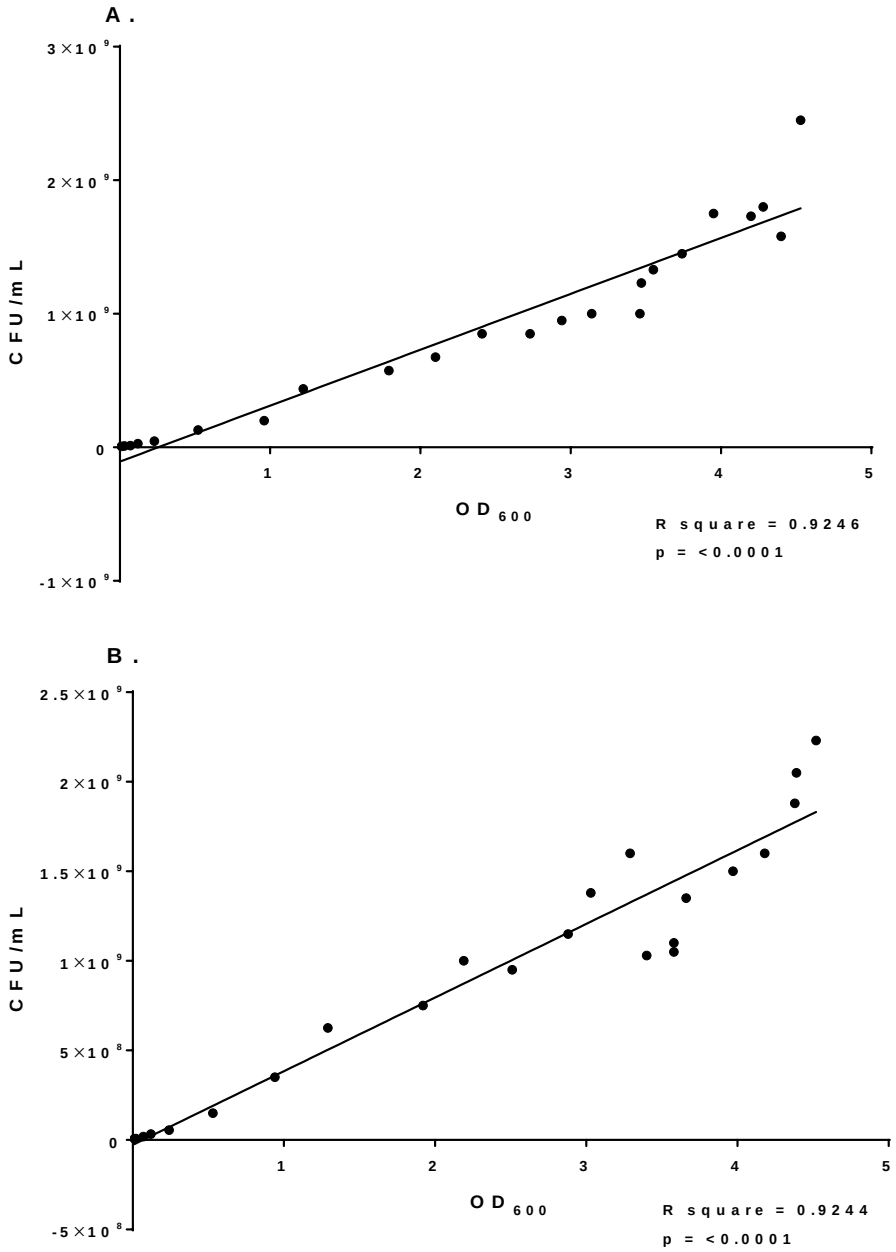


Figure 4.8 Linear regression analysis of CFU/mL and OD curves for *S. aureus* taken from two separate growth curves (A. and B.).

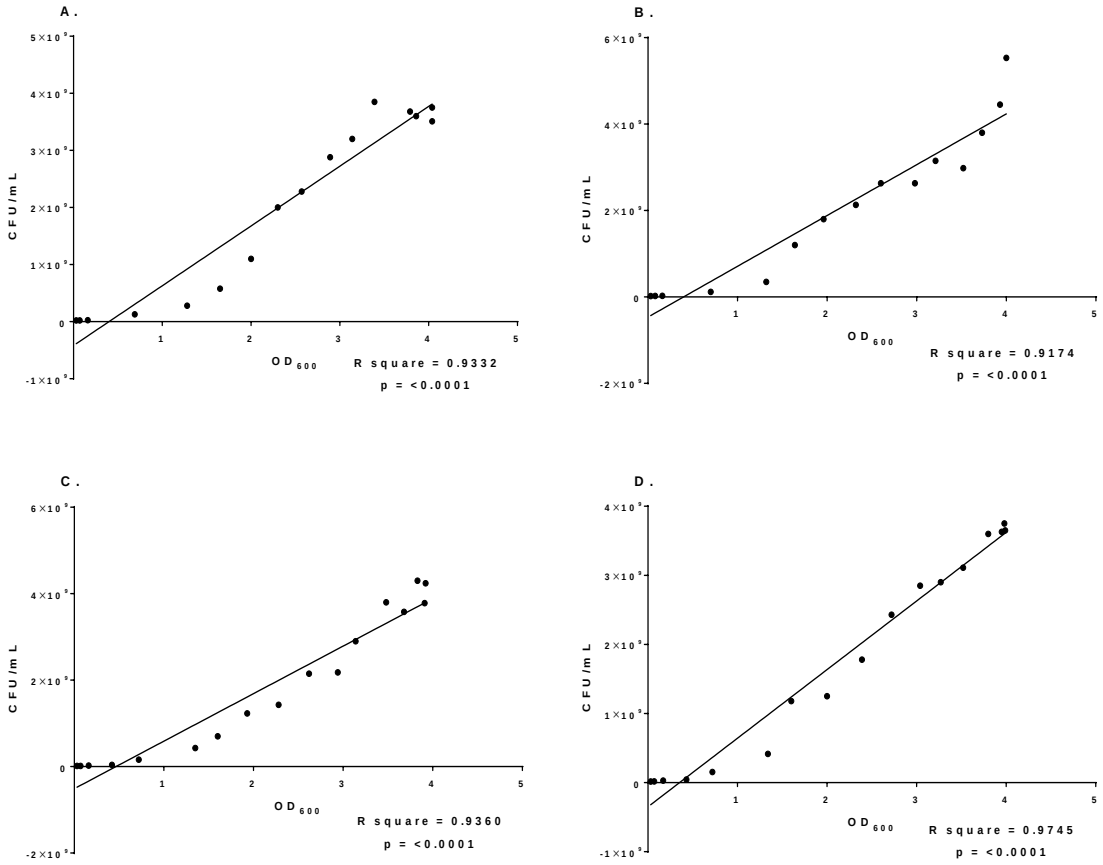


Figure 4.9 Linear regression analysis of CFU/mL and OD curves for *E. coli* taken from four separate growth curves (A. B. C and D.).

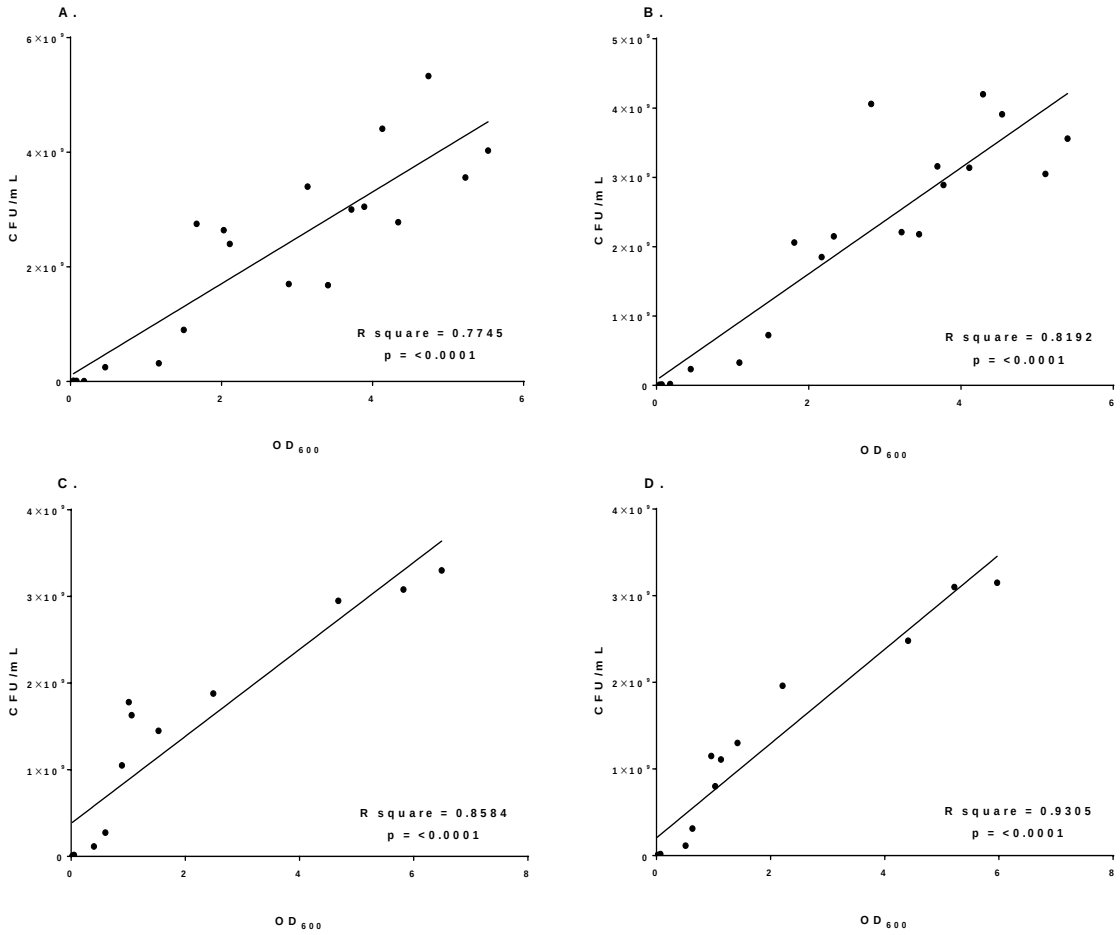


Figure 4.10 Linear regression analysis of CFU/mL and OD curves for *K pneumoniae* taken from two separate growth curves (A and B), and from *S. agalactiae* (C and D).

All four pathogens demonstrated a significant positive correlation between CFU/mL and OD_{600} for all repeated growth curves experiments.

4.4.3 Growth media for bacterial culture

Routine liquid culture of *S. aureus* was in tryptone soya broth (TSB), therefore no change was made to this growth media for this bacterium. I first evaluated whether *E. coli* would grow better in Lysogeny Broth (LB) media (routinely used for culture of *E. coli* laboratory

strains) or whether it would grow better in TSB. Growth curve experiments were set up and performed on both media.

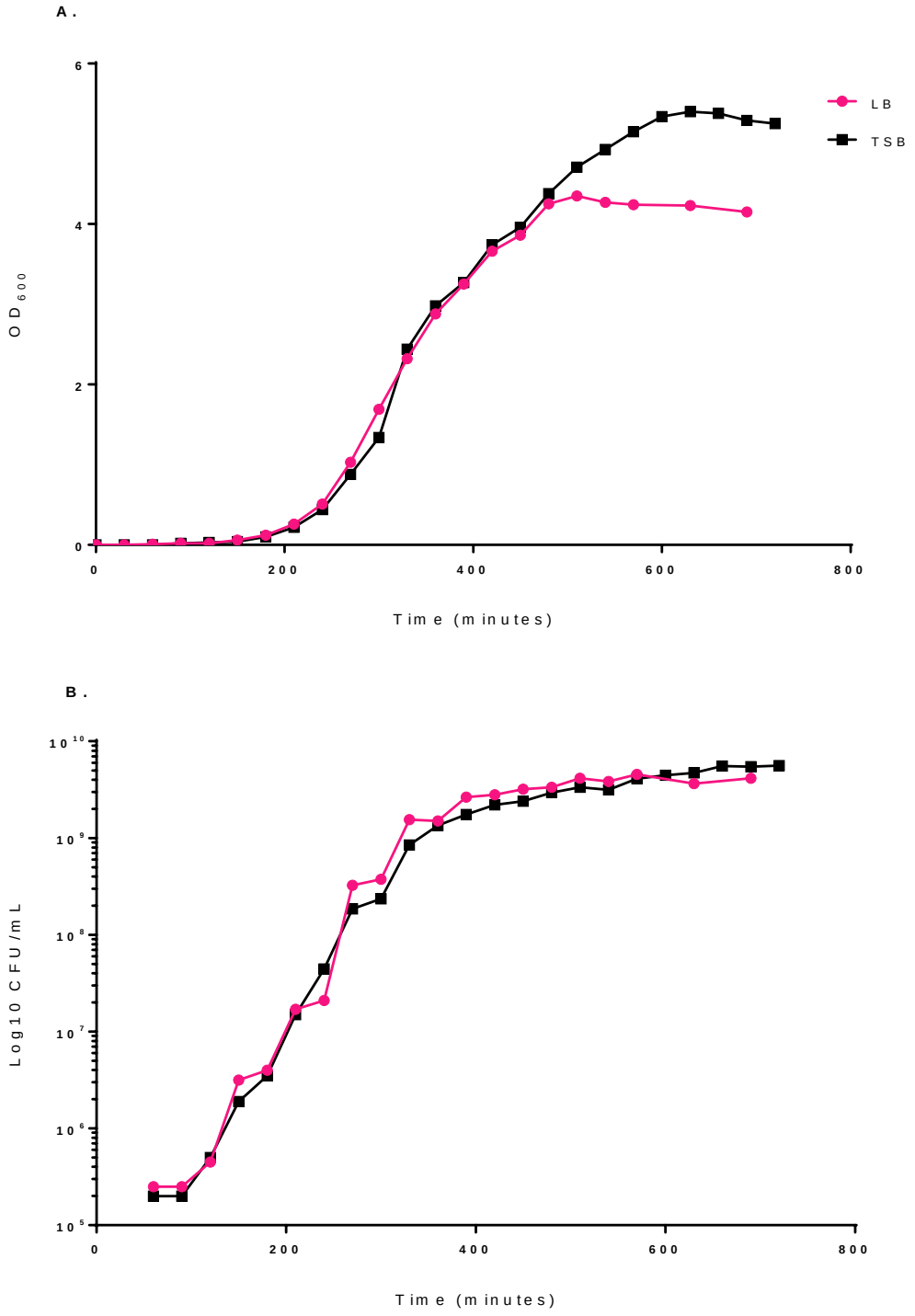


Figure 4.11 Growth curves of *E. coli* grown in LB (blue line) or TSB (pink line) for both OD600 measurements (A) and CFU/mL (B).

Wild type *E. coli* grew equally well in both LB and TSB media (Figure 4.11). Bacterial growth as measured using OD_{600} showed an earlier plateau in bacteria grown in LB, however CFU/mL was the same for both culture media.

K. pneumoniae grew efficiently in LB media and due to the longer growing times of *S. agalactiae*, TSB media was chosen after discussion with the Microbiology Laboratory.

4.4.4 Water bath versus orbital incubator for bacterial culture

Optimal conditions for growth of *S. aureus* as recommended by the *S. aureus* group included culturing the bacteria in an orbital incubator at 37°C and 130rpm. These conditions were maintained throughout the study. However the routine culture of *E. coli* and other bacteria is commonly undertaken in an orbital incubator at 37°C and 225rpm (range 200-250rpm). Due to demand for incubators in the laboratory, almost constant use of two incubators at different settings was logistically challenging, therefore a possible solution was to assess whether *S. aureus* would grow similarly in a water bath environment at 37°C and 130rpm. *S. aureus* was chosen for this analysis as the settings for the other bacteria were more common place in the laboratory, thus accommodating sharing of the equipment more easily.

Growth curves were performed for *S. aureus* comparing its growth kinetics in a water bath and an orbital incubator. Data presented is the mean value for two cultures grown under both conditions with standard deviation. Duplicate cultures were, as before, very similar. There was a suggestion that bacteria cultured in the water bath (Figure 4.12 - represented with black line) grew faster than bacteria in the orbital incubator (Figure 4.12 – represented by pink line).

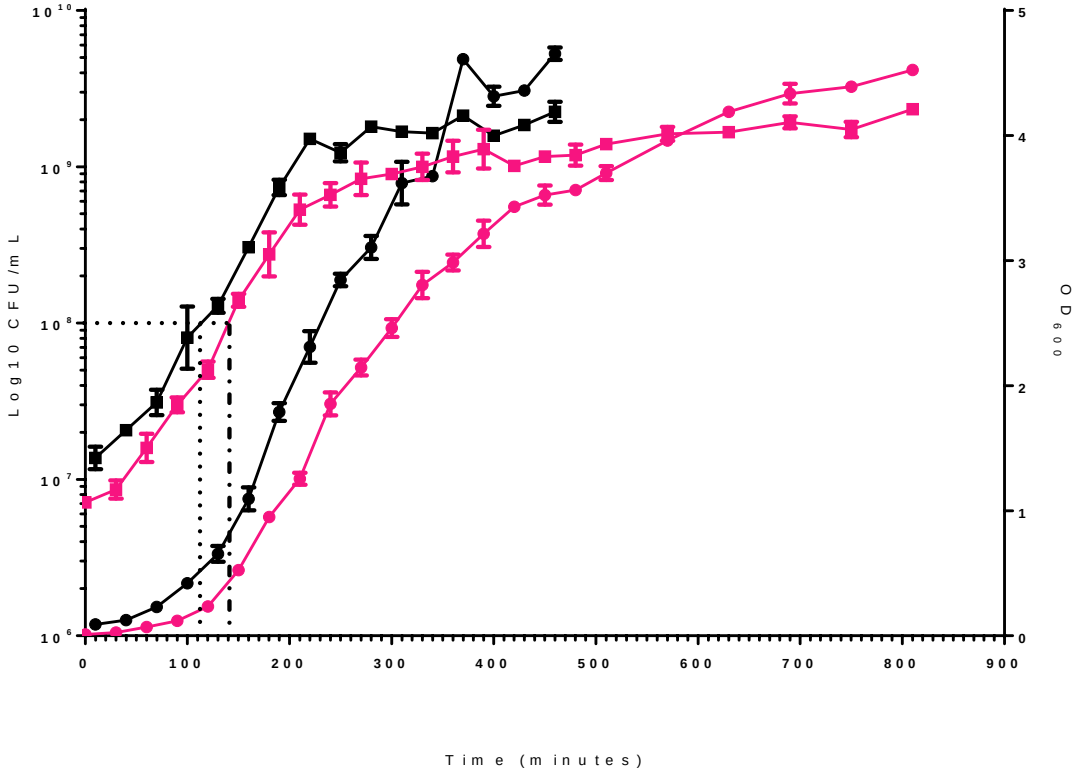


Figure 4.12 Growth curve of *S. aureus* grown in water bath versus orbital incubator. Lines in black represent water bath and lines in pink represent orbital incubator. Lines with squares represent CFU/mL values and lines with circles represent OD_{600} values.

Time taken to reach 1 x 10^8 CFU/mL was 110 minutes for bacteria cultured in the water bath compared with 140 minutes for bacteria cultured in the orbital incubator (Figure 4.12 – lines represented with squares).

4.4.5 Bacterial growth in blood

Based on the result of initial experiments with *S. aureus* (Figure 4.1) as outlined in section 4.4.1.1, the incubation period for the whole blood in-vitro GIA selected for further study were 1 hour and 24 hours. My hypothesis was that peak bacterial killing was detectable

following 1 hour incubation and thereafter, remaining bacteria continued to divide until reaching plateau at 24 hours. This also allowed for navigation of considerable laboratory logistics.

An experiment was performed on *K. pneumoniae* and *S. agalactiae* to ascertain whether these bacteria, like *S. aureus*, demonstrated an apparent peak killing at 1 hour with blood drawn from four healthy volunteers.

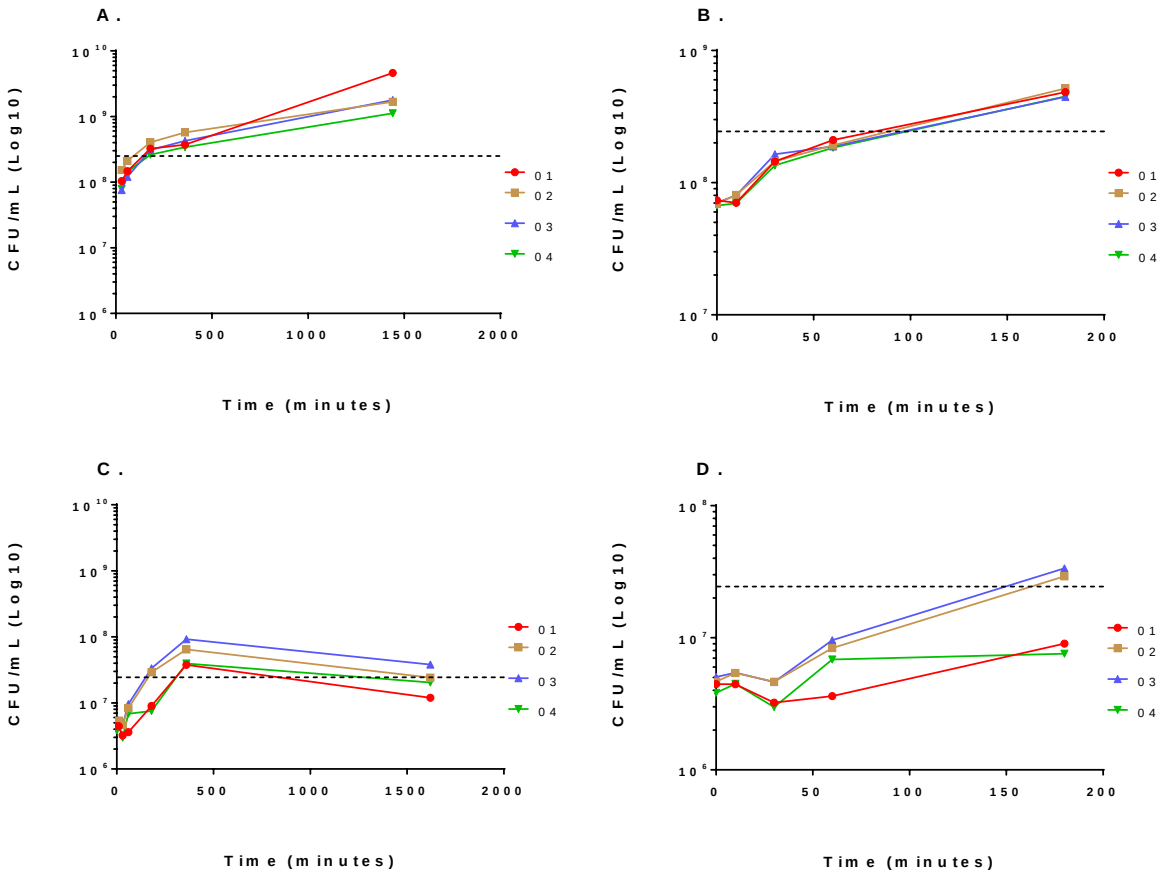


Figure 4.13 Bacterial growth over time in blood. A. *K. pneumoniae* growth in blood drawn from four healthy volunteers, samples processed at 30 minutes, 1 hour, 3 hours, 6 hours and 24 hours, B. *K. pneumoniae* growth in blood processed at 0 minute, 10 minutes, 30 minutes, 1 hour, and 3 hours, C. *S. agalactiae* growth in blood drawn from four healthy volunteers, samples processed at 0 minutes, 10 minutes, 30 minutes, 1 hour, 3 hours, 6 hours and 27, D. *S. agalactiae* growth in blood processed at same time points expanding on early incubations time-points.

Blood was drawn from four healthy volunteers, and samples initially processed at 30 minutes, 1 hour, 3 hours, 6 hours and 24 hours. *K pneumoniae* demonstrated a steady growth rate over time. A follow-up experiment was then performed with blood processed at 0 minute (immediately after mixing blood with bacterial inoculum), 10 minutes, 30 minutes, 1 hour, and 3 hour. *K. pneumoniae* showed and initial lag phase over the first 10 minutes and then steady growth thereafter (Figure 4.13 A. and B.). Blood drawn from four healthy volunteers for processing with *S. agalactiae* were incubated for 0 minutes, 10 minutes, 30 minutes, 1 hour, 3 hours, 6 hours and 27 hours. *S. agalactiae* showed an initial lag phase over the first 10 minutes, with a slight dip in net growth at 30 minutes before multiplying steadily. Evidence of reaching a death phase was demonstrated with net growth dropping at 27 hours (Figure 4.13 C. and D.).

In light of these results and to ensure consistency, incubation periods remained 1 hour and 24 hours for the remainder of the study.

4.4.6 Lithium heparin versus EDTA for blood collection

During the course of the study I observed that the GIA samples for *S. aureus* following incubation at 1 and 24 hours had a macroscopically different appearance to those of *E. coli*. Blood, when combined with the *S aureus* inoculum, developed a difficult to homogenise pellet which was larger after the 24 hour incubation and developed after the first centrifugation step – inversion of the 2mL tube prior to centrifugation did not show a pellet. In comparison, the samples containing *E. coli*, *K. pneumoniae* and *S. agalactiae* inoculum did not form such a pellet. The pellets, which formed after the first centrifugation step for these three bacteria, were easy to disturb and disperse during the water lysis stage of the protocol. Work done using this technique in the *S. aureus* group did not show a pellet, with the key difference being the use of an EDTA tube during blood

collection rather than lithium heparin. In order to explore this, blood samples from five volunteers (Phase 1 Schedule 2) were collected in EDTA and Lithium Heparin tubes for a head to head comparison. Samples were incubated for 1 and 24 hours. There was no difference seen in net growth of bacteria following 1 hour incubation using two of three different inoculum concentrations from samples collected into EDTA tubes (p=0.2188; inoculum concentration ~ 1 x 10^6CFU/mL, and p=>0.999; inoculum concentration 1 x 10^8CFU/mL, Wilcoxon matched-pairs signed rank test: Figure 4.14 columns 1 and 3). At an inoculum concentration of 1 x 10^7CFU/mL there was a significant decrease in net growth between 1 and 24 hr (p=0.0313, Figure 4.14 column 2). Following 24 hour incubation, samples processed from blood collected into lithium heparin tubes showed net growth of bacteria (p=0.0313 for all three inoculums Figure 4.14 columns 4, 5 and 6).

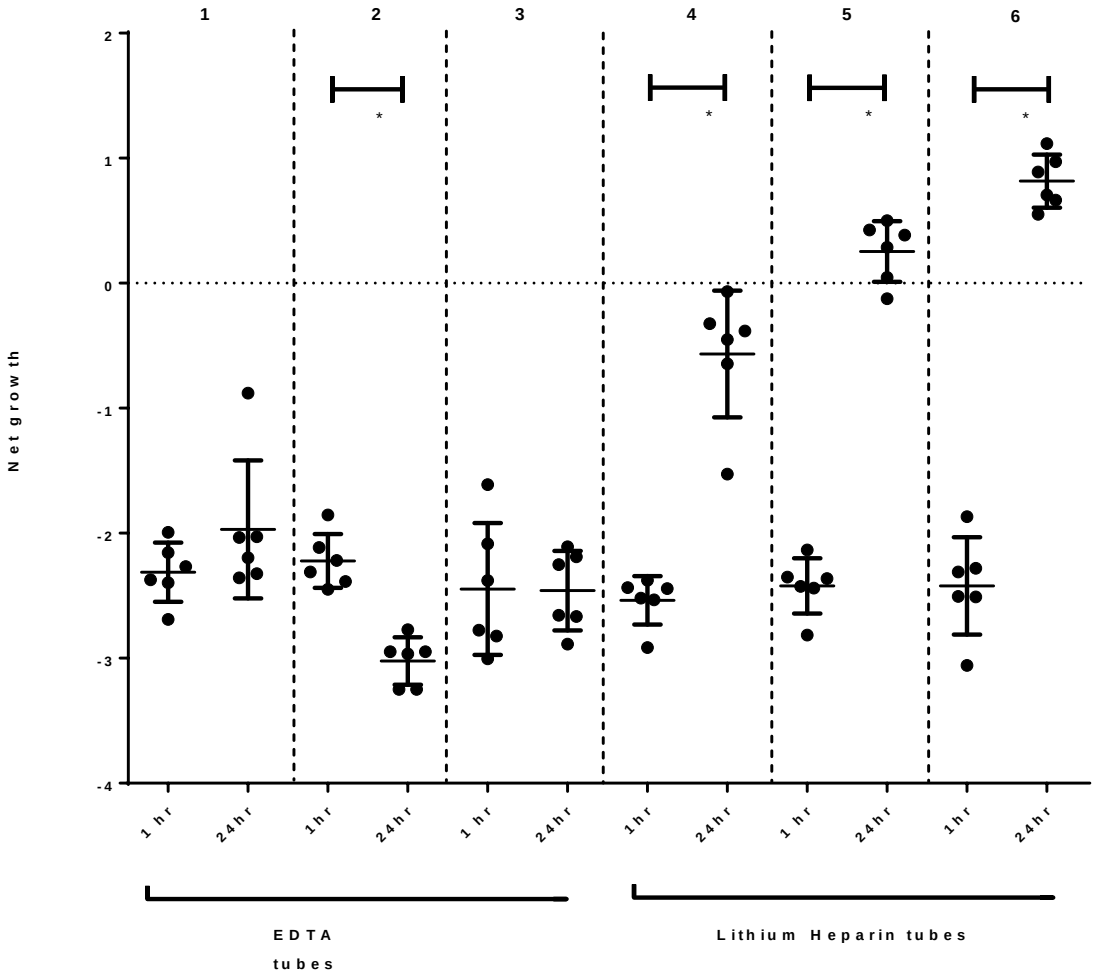


Figure 4.14 EDTA blood collection tubes vs Lithium Heparin: Results shown are for 5 volunteers from Phase 1 Schedule 2. Results show as net growth of *S aureus* following 1 and 24 hour incubation. 1 and 4 - Net growth following 1and 24 hours incubation with inoculum concentration ~ 1 x 10^8 CFU/mL; 2 and 5 - incubation with inoculum concentration of ~ 1 x 10^7 CFU/mL; 3 and 6 -incubation with inoculum concentration of ~ 1 x 10^6 CFU/mL. Wilcoxon matched-pairs signed rank test performed between 1 hour and 24 hour where * represents $p<0.05$

From this schedule, data from one control non-vaccinated volunteer was combined from each of their follow-up visits, providing samples from 9 separate bleeds. The same pattern was seen. There was no statistically significant different in net growth between 1 hour and 24 hour in the GIA processed using blood collected in EDTA tubes at any inoculum concentration (p=0.4961, p=0.2031, p=0.6523; Wilcoxon matched-pairs signed rank test Figure 4.15 column 1, 2 and 3 respectively). There was a significant difference between 1 hour and 24 hour net growth in blood collected using lithium heparin tubes at all inoculum concentrations (1 x 10^6CFU/mL p=0.0039, 1 x 10^7CFU/mL p=0.0039 and 1 x 10^8CFU/mL p=0.0039; Wilcoxon matched-pairs signed rank test, Figure 4.15 columns 4, 5 and 6).

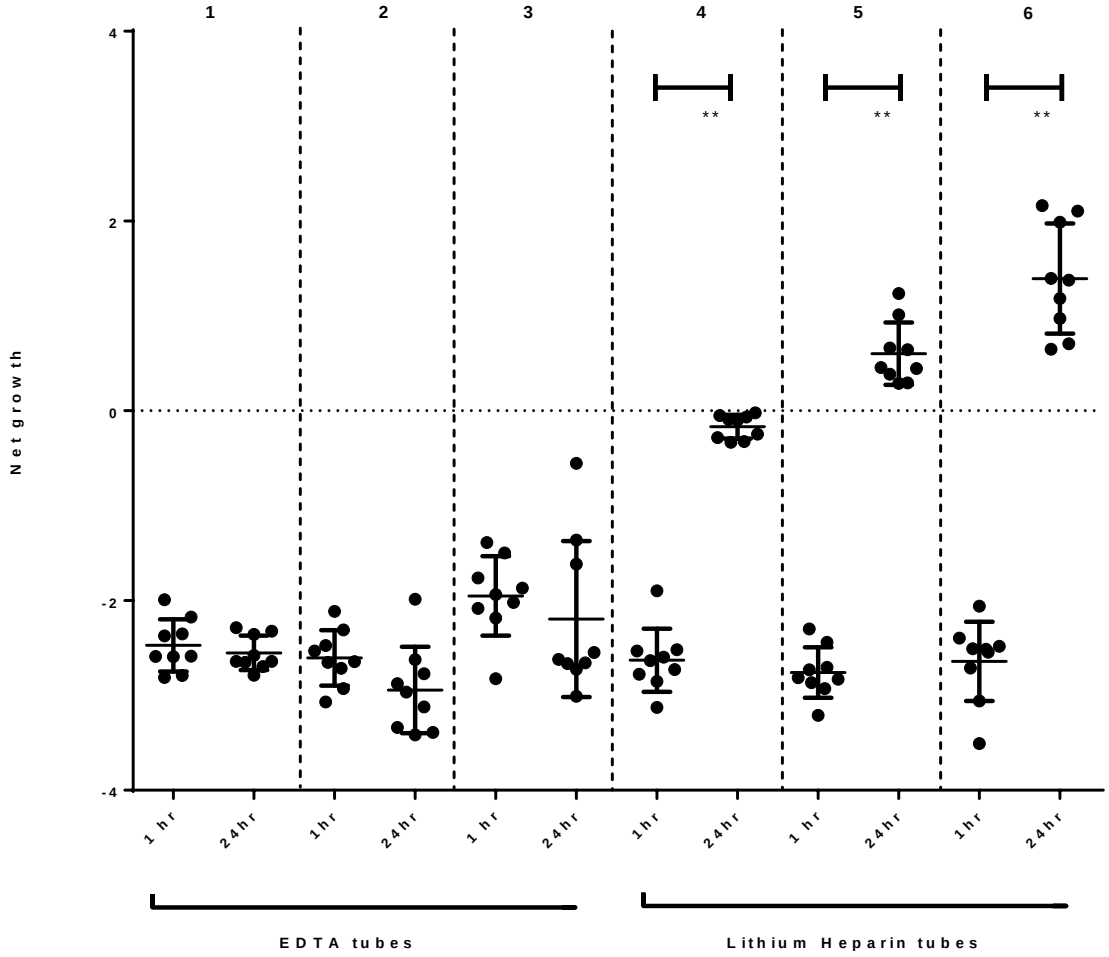


Figure 4.15 EDTA blood collection tubes vs Lithium Heparin: Results shown are for one non-vaccinated volunteer from Phase 1 Schedule 2 with combined results for all time-points. Results show as net growth of *S aureus* following 1 and 24 hour incubation. 1 and 4 - Net growth following 1and 24 hours incubation with inoculum concentration ~ 1 x 10^8 CFU/mL; 2 and 5 - incubation with inoculum concentration of ~ 1 x 10^7 CFU/mL; 3 and 6 -incubation with inoculum concentration of ~ 1 x 10^6 CFU/mL. Wilcoxon matched-pairs signed rank test performed between 1 hour and 24 hour where ** represents $p<0.01$

4.4.7 Inoculum stability and timing of second incubation

One of my concerns in the setting up of this study was the stability of the bacterial inoculums over time on repeated culture. In order to ensure this, timings were kept exact.

All bacteria had a 14 hour primary incubation from fresh colonies picked from agar. Agar stock plates were prepared from frozen stocks, and stock plates replaced every seven days. Following overnight incubation, based on the growth curves previously reported (4.4.2), the optimal duration of second incubation was identified to ensure that bacteria were in the exponential phase of growth, and as far as logistically possible the CFU/mL of each bacterium were the same. The timing of the second incubations evaluated were: 1.5 hours for *K. pneumoniae*, 2 hours for *E. coli*, 2.5 hours for *S. aureus* and 3 hours for *S. agalactiae* (Figure 4.16). *S. agalactiae* had a much longer doubling time than the other three bacteria, and the duration of the second incubation was 6 hours to ensure final inoculum CFU/mL matched the other bacteria. This was not logistically feasible and therefore 3 hours was chosen, allowing for more compatible second incubation times accepting the final inoculum concentration to be approximately a log lower CFU/mL than the other bacteria.

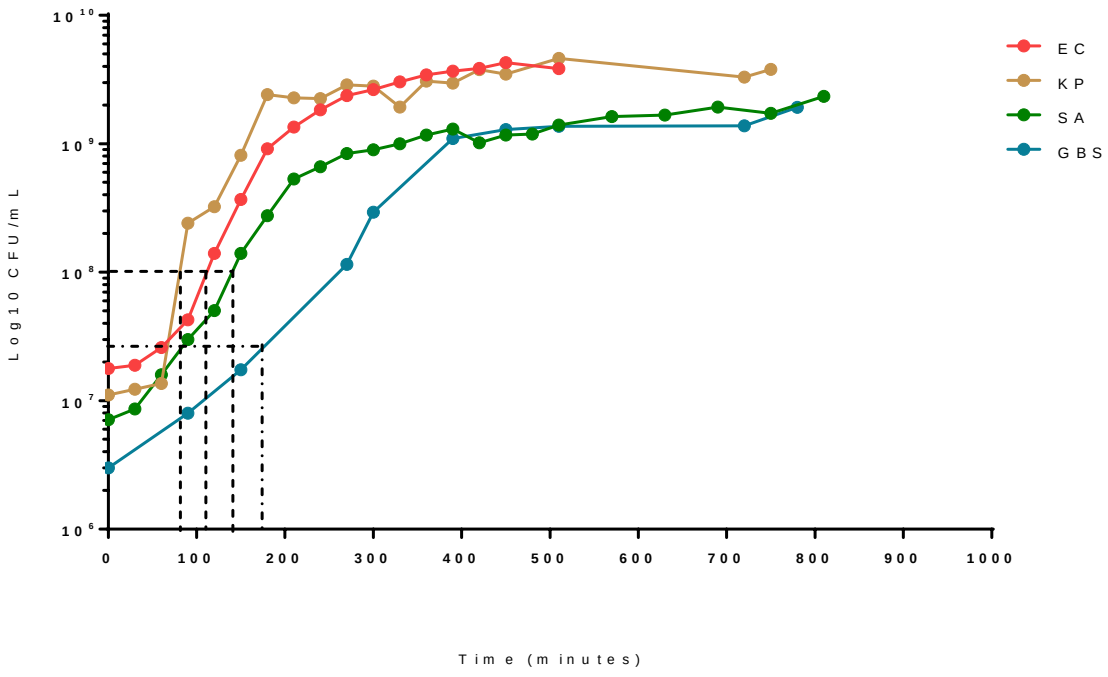


Figure 4.16 Combined growth curves (CFU/mL) for all four bacteria marking the timings for second incubation and approximate CFU/mL. Dotted lines represent optimal point of bacterial exponential

growth phase and therefore timing of second incubation required with approximate CFU/mL to be achieved for each bacterium.

As the optimal concentration of inoculum to potentially see an effect of BCG on bacterial killing was unknown and could not be identified from a single bleed, three inoculum concentrations (~x 10^8, x 10^7 and x 10^6 CFU/mL) were chosen to go forward into the study for *S. aureus*, *E. coli* and *K. pneumoniae*. As *S. agalactiae* was already approximately a log lower in concentration following second incubation, only two concentrations (~x 10^7 and x 10^8 CFU/mL) were chosen in order to maintain concordance across bacteria. Following the second incubation, the desired inoculum concentrations were achieved through a 1 in 10 serial dilution of the original inoculum. All bacteria demonstrated reproducibility and reliability in CFU/mL results across all schedules of the study (Figure 4.17: *S. aureus*, Figure 4.18: *E. coli*, Figure 4.19: *K. pneumoniae*, Figure 4.20: *S. agalactiae*).

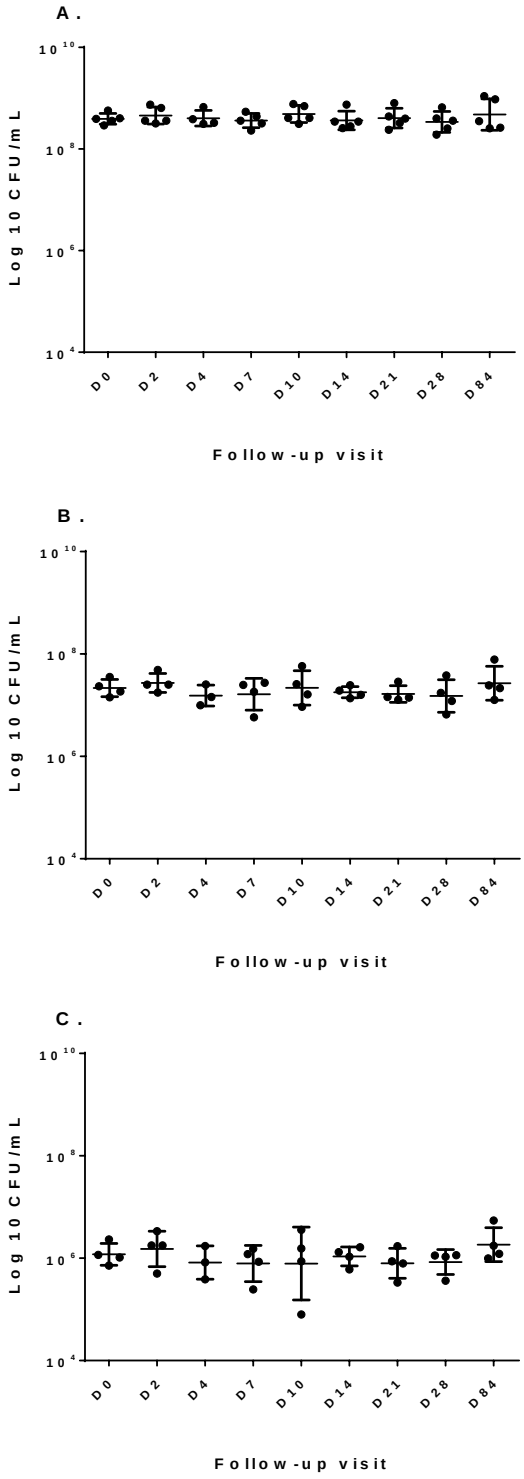


Figure 4.17 *S. aureus* inoculum Log10 CFU/mL following second incubation and subsequent serial dilutions. A. Log10 CFU/mL for 1:1 dilution, B. Log10 CFU/mL for 1:10 dilution and C. Log10 CFU/mL for 1:100 dilution.

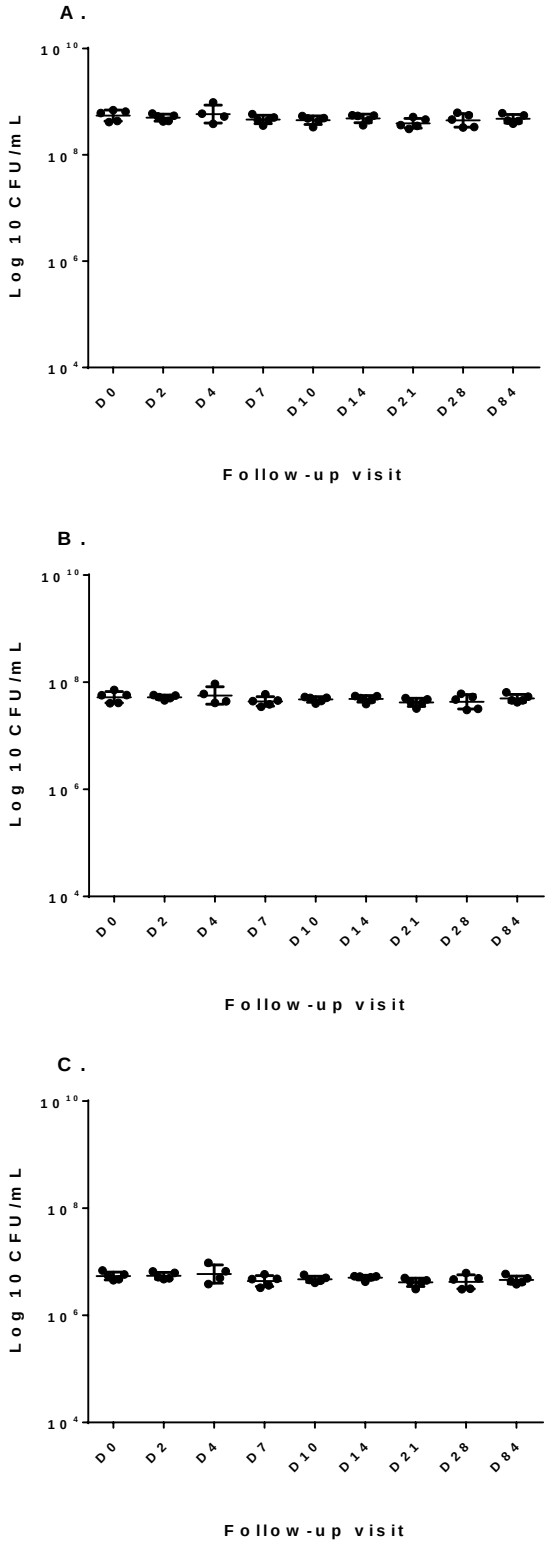


Figure 4.18 *E. coli* inoculum Log10 CFU/mL following second incubation and subsequent serial dilutions. A. Log10 CFU/mL for 1:1 dilution, B. Log10 CFU/mL for 1:10 dilution and C. Log10 CFU/mL for 1:100 dilution.

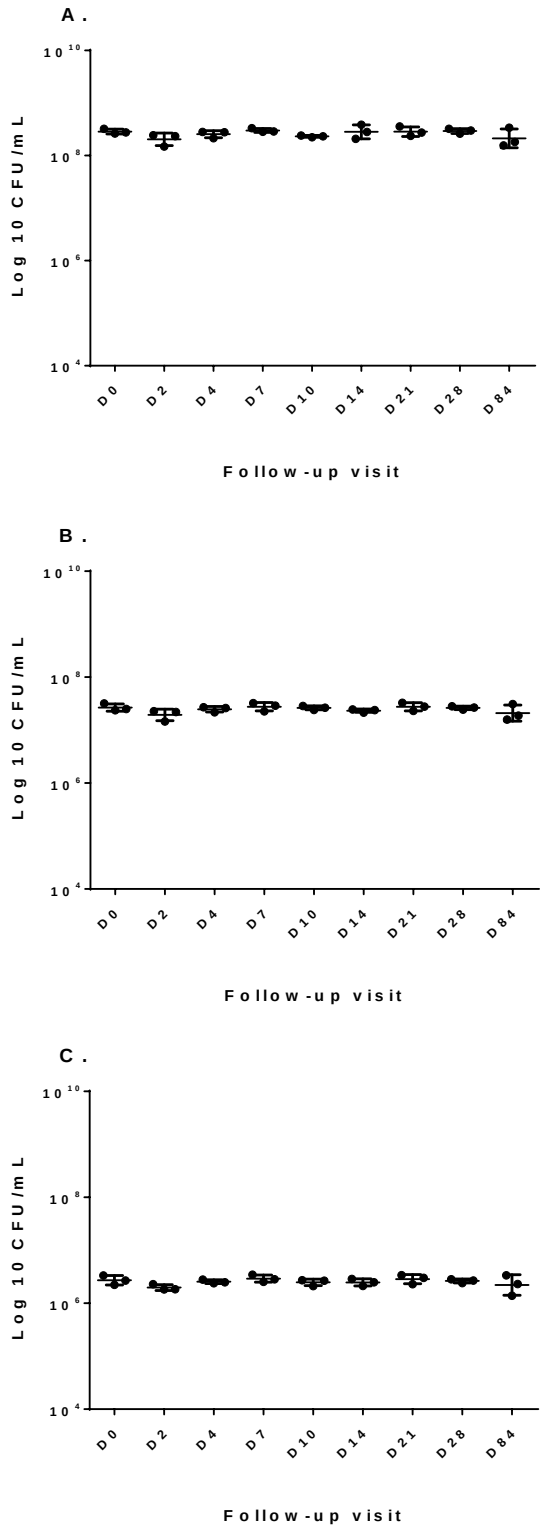


Figure 4.19 *K. pneumoniae* inoculum Log 10 CFU/mL following second incubation and subsequent serial dilutions. A. Log10 CFU/mL for 1:1 dilution, B. Log 10CFU/mL for 1:10 dilution and C. Log10 CFU/mL for 1:100 dilution.

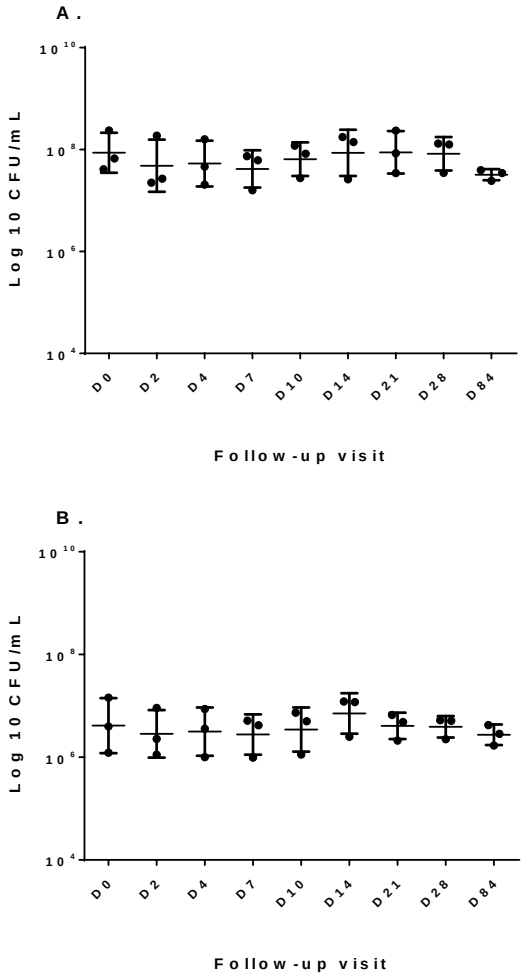


Figure 4.20 *S. agalactiae* inoculum Log 10 CFU/mL following second incubation and subsequent serial dilutions. A. Log10 CFU/mL for 1:1 dilution and B. Log10 CFU/mL for 1:10 dilution.

4.5 Whole blood bacterial growth inhibition assay

As shown in the consort diagram in Chapter 3 (Figure 3.1), for logistical reasons volunteers were enrolled in two phases: Phase 1 – blood drawn from enrolled volunteers were inoculated with *S. aureus* and *E. coli* for the *in-vitro* GIA, and Phase 2 – blood drawn from enrolled volunteers were inoculated with *K. pneumoniae* and *S. agalactiae*. Volunteers enrolled into both phases were randomised to receive BCG SSI at a dose of 2-8 x 10^5pfu or remain a non-vaccinated control. Each volunteer had blood drawn and

processed on Day 0 (D0) pre-vaccination therefore acting their own control, however due to concern over a potential batch effect from the bacterial inoculum, a non-vaccination control was added to each schedule of enrolment to control for this. Data was blinded until the last volunteers form the final schedule of each phase had completed their final follow-up visit. Volunteers enrolled from Oxford site had clinic visits on D0, D2, D4, D7, D10, D14, D21, D28 and D84. Volunteers from Birmingham had clinic visits on D0, D2, D7, D14, D21, D28, and D84; D4 and D10, were omitted from their follow-up as they had to attend Oxford for all of their enrolled study visits and this ensured a more feasible study schedule (Figure 4.21).

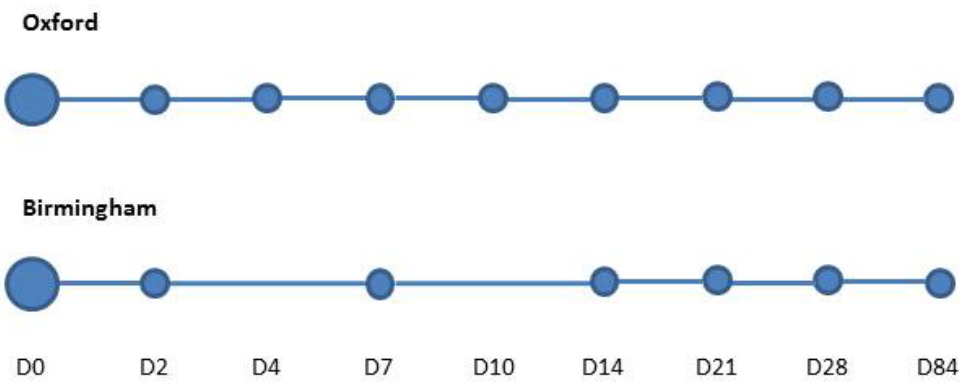


Figure 4.21 Schematic representation of study time-line for blood sample draw.

4.5.1 Phase 1

20 volunteers were enrolled into Phase 1 over five schedules, 15 were randomised to receive BCG SSI vaccination and five were non-vaccination inoculum controls. Five volunteers were enrolled from Birmingham and did not have blood drawn on D4 and D10 for logistical reasons. One volunteer randomised to receive BCG SSI vaccination was lost to follow-up after D28 and was unable to attend their D4 follow-up therefore blood was

not drawn as planned at these time-points. Volunteers attended clinic between 8am and 9am for sample collection and bloods were processed within 2-3 hours after venesection depending on daily laboratory logistics.

Blood was collected into lithium heparin tubes, and was incubated with the bacterial inoculum for 1 and 24 hours.

4.5.1.1 S. aureus: 1 hour and 24 hour incubation

Blood from the volunteers enrolled into schedule 1 were inoculated with only one concentration of *S. aureus*. On review of the blinded data when volunteers in schedule 1 had reached D28, the decision was made to proceed with two lower inoculum concentrations to ensure that the higher concentration was not potentially overcoming any host immune response which might control bacterial growth. Therefore for the higher inoculum concentration, all 20 volunteers were analysed, however for the lower two concentration of inoculum, only 16 were analysed. Mean inoculum CFU/mL for 1:1 dilution was 4.45 x 10^8CFU/mL (range 1.91 x 10^8 – 1.1 x 10^9 CFU/mL), 1:10 dilution was 2.26 x 10^7CFU/mL (range 5.78 x 10^6 – 7.79 x 10^7CFU/mL), and 1:100 dilution 1.33 x 10^6CFU/mL (range 7.93 x 10^4 – 5.46 x 10^6 CFU/mL).

The net growth of *S. aureus* following 1 hour incubation is shown in Figure 4.22. Volunteers are divided by group and by inoculum concentration. Volunteers in Group 1 (BCG vaccinated) showed a statistically significant drop in net growth between D0 and D10 at an inoculum dilution of 1:1 (p=0.0006 Dunn's test of multiple comparisons; Figure 4.22 A). At an inoculum dilution of 1:10, there was significant drop in net growth between D0 and D10 (p=0.0179 Dunn's test of multiple comparisons; Figure 4.22 C). At an inoculum dilution of 1:100, there was a significant drop in net growth between D0 and D4 (p=0.0354 Dunn's test of multiple comparisons; Figure 4.22 E).

Volunteers in Group 2 (non-vaccinated) at an inoculum dilution of 1:1 also showed a statistically significant decrease in net growth between D0 and D10 ($p=0.0014$ Dunn's test of multiple comparisons; Figure 4.22 B). At an inoculum dilution of 1:10 and 1:100, there was no significance difference between D0 and any other time-points ($p = 0.1812$ one way ANOVA Friedmans test, $p = 0.4163$ one way ANOVA Friedmans test respectively; Figure 4.22 D and F).

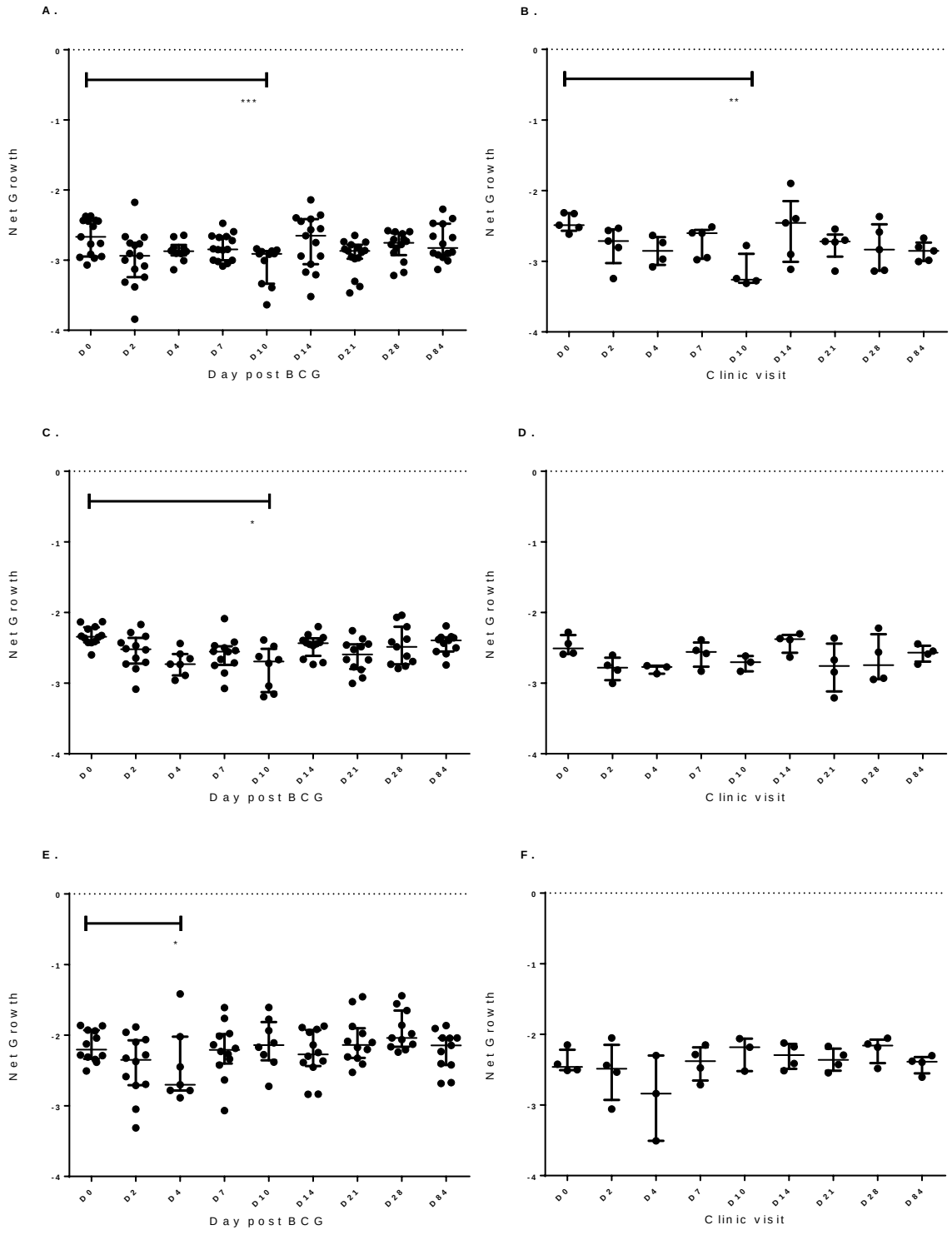


Figure 4.22 *S. aureus* GIA net growth following 1 hour incubation. Each dot represents the mean value of duplicate samples per volunteer; median and interquartile range shown. A. and B. results for GIA with 1:1 dilution (~x10^8CFU/mL) inoculum, C. and D. results for GIA with 1:10 dilution (~x10^7CFU/mL)

inoculum, and E. and F. results for GIA with 1:100 dilution (~x10^6CFU/mL) inoculum. A. Net growth at baseline (pre-vaccination) and following BCG vaccination in Group 1 volunteers, B. Net growth over repeated sampling for non-vaccinated volunteers from Group 2, C. Net growth at baseline and following BCG vaccination in Group 1 volunteers, D. Net growth over repeated sampling for non-vaccinated volunteers from Group 2, E. Net growth at baseline and following BCG vaccination in Group 1 volunteers, F. Net growth over repeated sampling for non-vaccinated volunteers from Group 2. Oneway ANOVA with post hoc Dunn's test of multiple comparisons where appropriate, where * represents $p<0.05$, ** $p<0.01$ and *** $p<0.001$

The net growth of *S. aureus* following 24 hour incubation is shown in Figure 4.23. Again volunteers are divided by group and by inoculum concentration. Volunteers in Group 1 (BCG vaccinated) showed a statistically significant drop in net growth between D0 and D10 at an inoculum dilution of 1:1 ($p=0.0006$ Dunn's test of multiple comparisons; Figure 4.22 A). At an inoculum dilution of 1:10, there was a significant drop in net growth between D0 and D10 ($p=0.0179$ Dunn's test of multiple comparisons; Figure 4.23 C). At an inoculum dilution of 1:100, there was a significant drop in net growth between D0 and D4 ($p=0.0354$ Dunn's test of multiple comparisons; Figure 4.23 E).

Volunteers in Group 2 (non-vaccinated controls) also showed a statistically significant decrease in net growth between D) and D10 with the inoculum dilution of 1:1 ($p=0.0014$ Dunn's test of multiple comparisons; Figure 4.23 B). At an inoculum dilution of 1:10 and 1:100, there was no significance difference between D0 and all other time-points ($p = 0.1812$ one way ANOVA Friedmans test, $p = 0.4163$ one way ANOVA Friedmans test respectively; Figure 4.23 D and F).

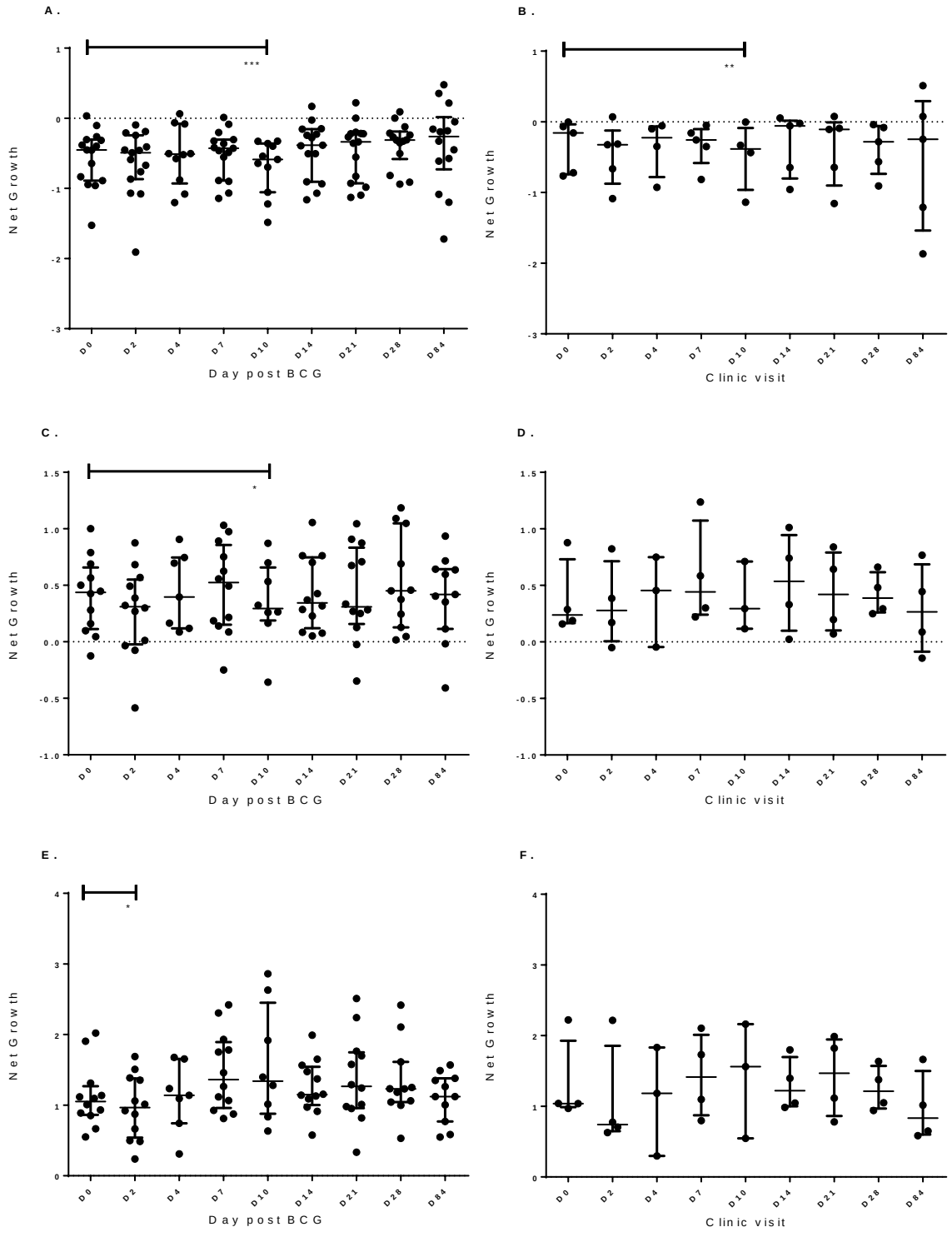


Figure 4.23 *S. aureus* GIA net growth following 24 hour incubation. Each dot represents the mean value of duplicate samples per volunteer; median and interquartile range shown. A. and B. results for GIA with 1:1 (~x10^8CFU/mL) inoculum, C. and D. results for GIA with 1:10 dilution (~x10^7CFU/mL) inoculum,

and E. and F. results for GIA with 1:100 dilution (~x10^6CFU/mL) inoculum. A. Net growth at baseline (pre-vaccination) and following BCG vaccination in Group 1 volunteers, B. Net growth over repeated sampling for non-vaccinated volunteers from Group 2, C. Net growth at baseline and following BCG vaccination in Group 1 volunteers, D. Net growth over repeated sampling for non-vaccinated volunteers from Group 2, E. Net growth at baseline and following BCG vaccination in Group 1 volunteers, F. Net growth over repeated sampling for non-vaccinated volunteers from Group 2. Oneway ANOVA with post hoc Dunn's test of multiple comparisons where appropriate, where * represents p<0.05, ** p<0.01 and *** p<0.001

4.5.1.2 E. coli: 1 hour and 24 hour incubation

Blood from volunteers enrolled into schedule 1 were also incubated with *E. coli*. Three inoculum concentrations were used on the first schedule of enrolment (n=4). On review of blinded results at D28, the decision was made to discontinue with the ~ x10^8 CFU/mL inoculum concentration as discrimination between volunteers was poor. Therefore the blood of four volunteers was processed with an inoculum of 1:1 dilution and available for analysis, however the blood of all 20 volunteers was inoculated with 1:10 and 1:100 dilutions of bacteria.

The mean inoculum CFU/mL of 1:1 dilution was 4.89 x 10^8CFU/mL (range 3.07 x 10^8 – 9.72 x x10^8CFU/mL), for 1:10 dilution it was 4.89 x 10^7 CFU/mL (range 3.04 x 10^7 – 9.33 x 10^7CFU/mL) and for the 1:100 dilution it was 4.91 x 10^6 CFU/mL (range 3.07 x 10^6 – 9.57 x 10^6CFU/mL).

Net growth of *E. coli* following 1 hour incubation is shown in Figure 4.24. As before, volunteers are divided by group and by inoculum concentration. Volunteers in Group 1 (BCG vaccinated) did not demonstrated a statistically significant change in net growth between any time-point at an inoculum dilution of 1:1 (p = 0.0719 one way ANOVA Friedmans test; Figure 4.24 A). At an inoculum dilution of 1:10, there was no significant

change in net growth between any time-point (p = 0.7910 one-way ANOVA Friedmans test; Figure 4.24 C). Again at an inoculum dilution of 1:100, there was no significant change in net growth between any time-point (p = 0.8179 one-way ANOVA Friedmans test; Figure 4.24 E).

The single control non-vaccinated volunteer in Group 2 whose blood was inoculated at an inoculum dilution of 1:1 showed no change on repeat sampling at each clinic visit, statistical analysis was not possible on only one volunteer (Figure 4.24– B). At an inoculum dilution of 1:10 and 1:100, there was no significance difference between all time-points or between D0 and all other time-points (p = 0.3893 oneway ANOVA Friedmans test, and p=0.6472 oneway ANOVA Friedmans test respectively; Figure 4.24 D and F).

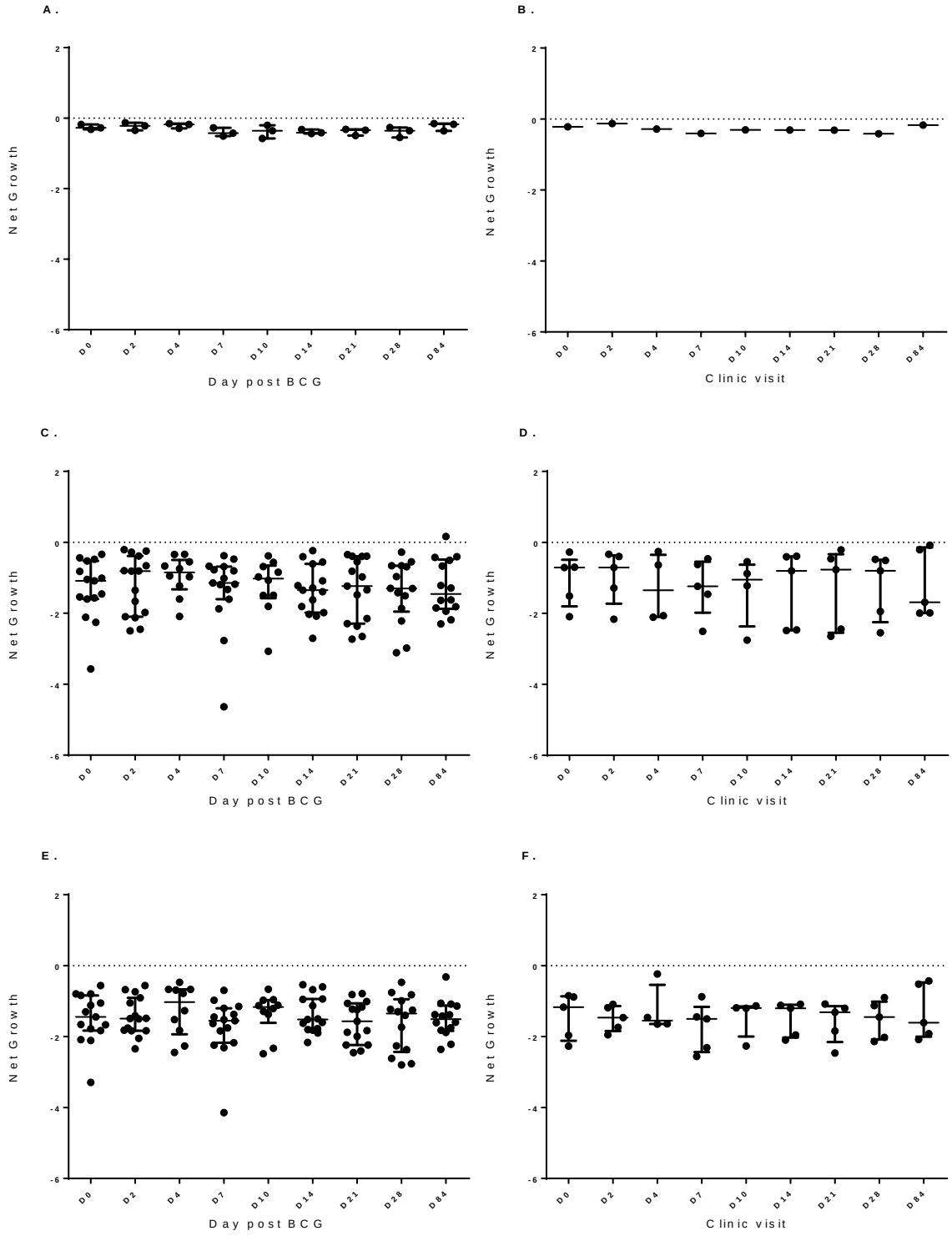


Figure 4.24 *E. coli* Net growth 1hr incubation. Each dot represents the mean value of duplicate samples per volunteer; median and interquartile range shown. A. and B. results for GIA with 1:1 dilution (~x10^8CFU/mL) inoculum, C. and D. results for GIA with 1:10 dilution (~x10^7CFU/mL) inoculum, and E.

and F. results for GIA with1:100 dilution (~x10^6CFU/mL) inoculum. A. Net growth at baseline (pre-vaccination) and following BCG vaccination in Group 1 volunteers, B. Net growth over repeated sampling for non-vaccinated volunteers from Group 2, C. Net growth at baseline and following BCG vaccination in Group 1 volunteers, D. Net growth over repeated sampling for non-vaccinated volunteers from Group 2, E. Net growth at baseline and following BCG vaccination in Group 1 volunteers, F. Net growth over repeated sampling for non-vaccinated volunteers from Group 2.

Net growth of *E. coli* following 24 hour incubation is shown in Figure 4.25. As before volunteers are divided by group and by inoculum concentration. Volunteers in Group 1 (BCG vaccinated) did not demonstrated a statistically significant change in net growth between D0 and any time-point at an inoculum dilution of 1:1 (p=0.0679 one-way ANOVA Friedmans test; Figure 4.25 A). At an inoculum dilution of 1:10, there was no significant change in net growth between D0 and any other time-point (p=0.7910 one-way ANOVA Friedmans test, Figure 4.25 C). Again at an inoculum dilution of 1:100, there was no significant change between D0 and any other time-point (p=0.8179 one-way ANOVA Friedmans test; Figure 4.25 E).

The single control non-vaccinated volunteer in group 2 whose blood was inoculated at an inoculum dilution of 1:1 showed no change on repeat sampling at each clinic visit, statistical analysis was not possible on only one volunteer (Figure 4.25 B). At an inoculum dilution of 1:10 and 1:100, there was no significance difference between D0 and all other time-points (p=0.3893 one-way ANOVA Friedmans test, and p=0.6472 one-way ANOVA Friedmans test respectively; Figure 4.25 D and F).

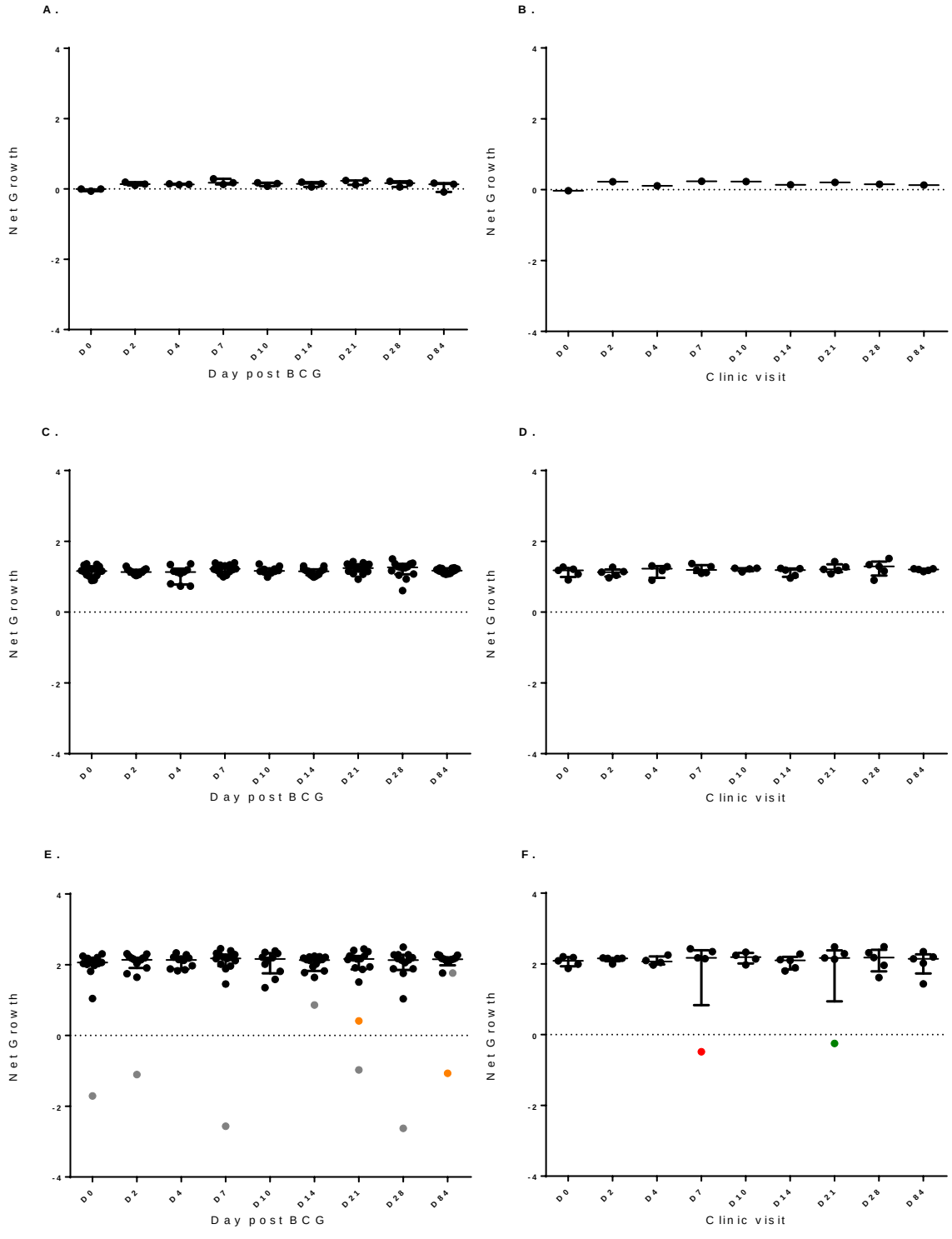


Figure 4.25 *E. coli* Net growth 24 hour incubation. Each dot represents the mean value of duplicate samples per volunteer; median and interquartile range shown. A. and B. results for GIA with 1:1 dilution (~x10^8CFU/mL) inoculum, C. and D. results for GIA with 1:10 dilution (~x10^7CFU/mL) inoculum,

and E. and F. results for GIA with 1:100 dilution (~x10^6CFU/mL) inoculum. A. Net growth at baseline (pre-vaccination) and following BCG vaccination in Group 1 volunteers, B. Net growth over repeated sampling for non-vaccinated volunteer from Group 2, schedule 1, C. Net growth at baseline and following BCG vaccination in Group 1 volunteers, D. Net growth over repeated sampling for non-vaccinated volunteers from Group 2, E. Net growth at baseline and following BCG vaccination in Group 1 volunteers, F. Net growth over repeated sampling for non-vaccinated volunteers from Group 2.

In group 1 (BCG vaccinated) at the 24 hour incubation of the 1:100 dilution inoculum, there was one volunteer (TBT-038122) who consistently appeared to demonstrate better control of *E. coli* growth than all other volunteers. This volunteer was female, but there were no obvious clinical findings to explain the improved control (volunteer highlighted in grey; Figure 4.25 E). Under those conditions, another volunteer (TBT-038117 – highlighted in orange; Figure 4.25 E) had improved control at D21 and D84. In group 2 (non-vaccinated controls) at the 24 hour incubation of 1:100 dilution inoculum, there were two volunteers who stood out as outliers. One volunteer appeared to have better control of growth at D7, and the other had better control at D21 (red and green; Figure 4.25 F).

4.5.2 BACTEC FX time to positivity

As discussed in Chapter 1: Introduction, several MGIA methods were considered during the optimisation of a standardised approach to the mycobacterial system, and in our group it was the direct MGIA using the MGIT tubes which was selected due to its simplicity and increased sensitivity in detecting differences in bacterial growth. A pilot BACTEC FX method was undertaken during this study to ascertain whether this system would also be able to detect differences and correlate with the more traditional CFU/mL plating method. Due to the volume of samples generated during this study and combined with the use of

the NHS facilities, it was not possible to process all samples using this second method. BACTEC FX bottles were prepared for all samples (*S. aureus* and *E. coli*) during schedule 1 of phase 1, and for all *E. coli* samples in phase 1. BACTEC FX bottles were not inoculated during phase 2.

Results from the four volunteers in phase 1 schedule 1 are shown in Figure 4.26. There was no significant difference in mean or between D0 and any subsequent time-point in group 1 (BCG vaccinated) volunteers (1hour incubation p=0.3213 one-way ANOVA, 24 hour incubation p=0.0954 one-way ANOVA; Figure 4.26 A and C respectively). The non-vaccinated volunteer in group 2 appeared to show improved control at D7 following 24 hour incubation (Figure 4.26 D) There was no difference in the control of *S aureus* TTP following 1 hour incubation (Figure 4.26).

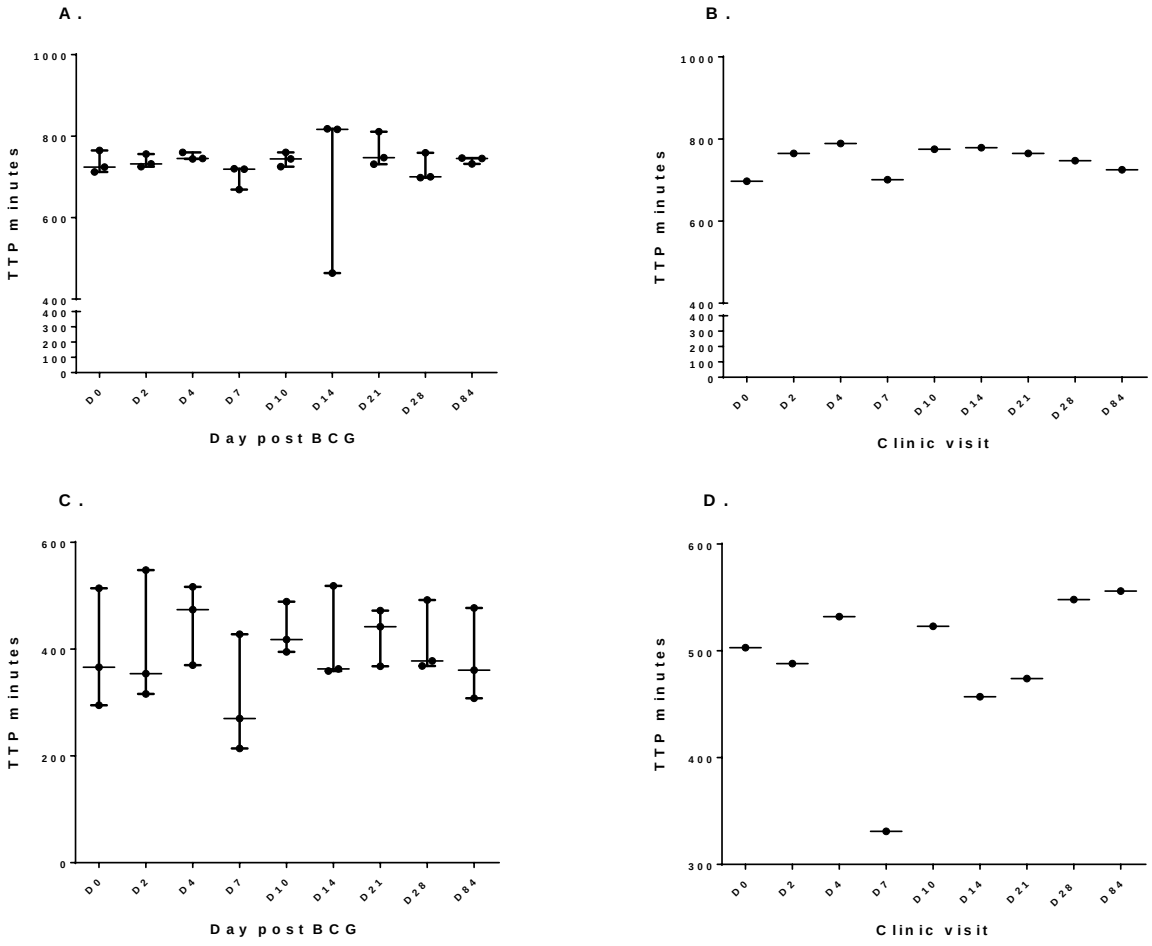


Figure 4.26 *S. aureus* BACTEC FX – Each dot represents mean of duplicate values for each volunteers, median and interquartile range shown. Inoculum concentration ~x10^8CFU/mL A. group 1 (n=3) BCG vaccinated, B. single group 2 volunteer (non-vaccinated) C. group 1, 24 hour incubation D. Group 2, 24 hour incubation

Results are shown in Figure 4.27 for BACTEC FX TTP for *E. coli* GIA. As with CFU/mL results, 1:1 dilution (~ x10^8CFU/mL) inoculum was used in schedule 1 of enrolment (n=4), but blood drawn from all 20 volunteers was processed with 1:10 (~ x10^7CFU/mL) and 1:100 (~ x10^6CFU/mL) dilution of inoculum). Five volunteers in schedule 2 at D0 were identified as outliers using the ROUT method in Graphpad Prism and excluded from analysis.

TTP results following 1 hour incubation are shown in Figure 4.27. As before volunteers are divided by group and by inoculum concentration. Volunteers in Group 1 (BCG vaccinated) at 1:1 dilution of inoculum there was a significant difference in TTD across time-points (p=0.0402 one-way ANOVA Friedmans test, Figure 4.27 A). At an inoculum dilution of 1:10 and 1:100, there was no significant change in TTP between D0 and any post-vaccination time-point (p=0.0620 one-way ANOVA Friedmans test, and p=0.3245 one-way ANOVA Friedmans test respectively, Figure 4.27 C and E).

The single non-vaccinated control volunteer in Group 2 whose blood was inoculated at an inoculum dilution of 1:1 is shown in Figure 4.27 B. At an inoculum dilution of 1:10 and 1:100, there was no significance difference between D0 and all other time-points (p=0.3893 one-way ANOVA Friedmans test, and p=0.2865 one-way ANOVA Friedmans test respectively; Figure 4.27 D and F).

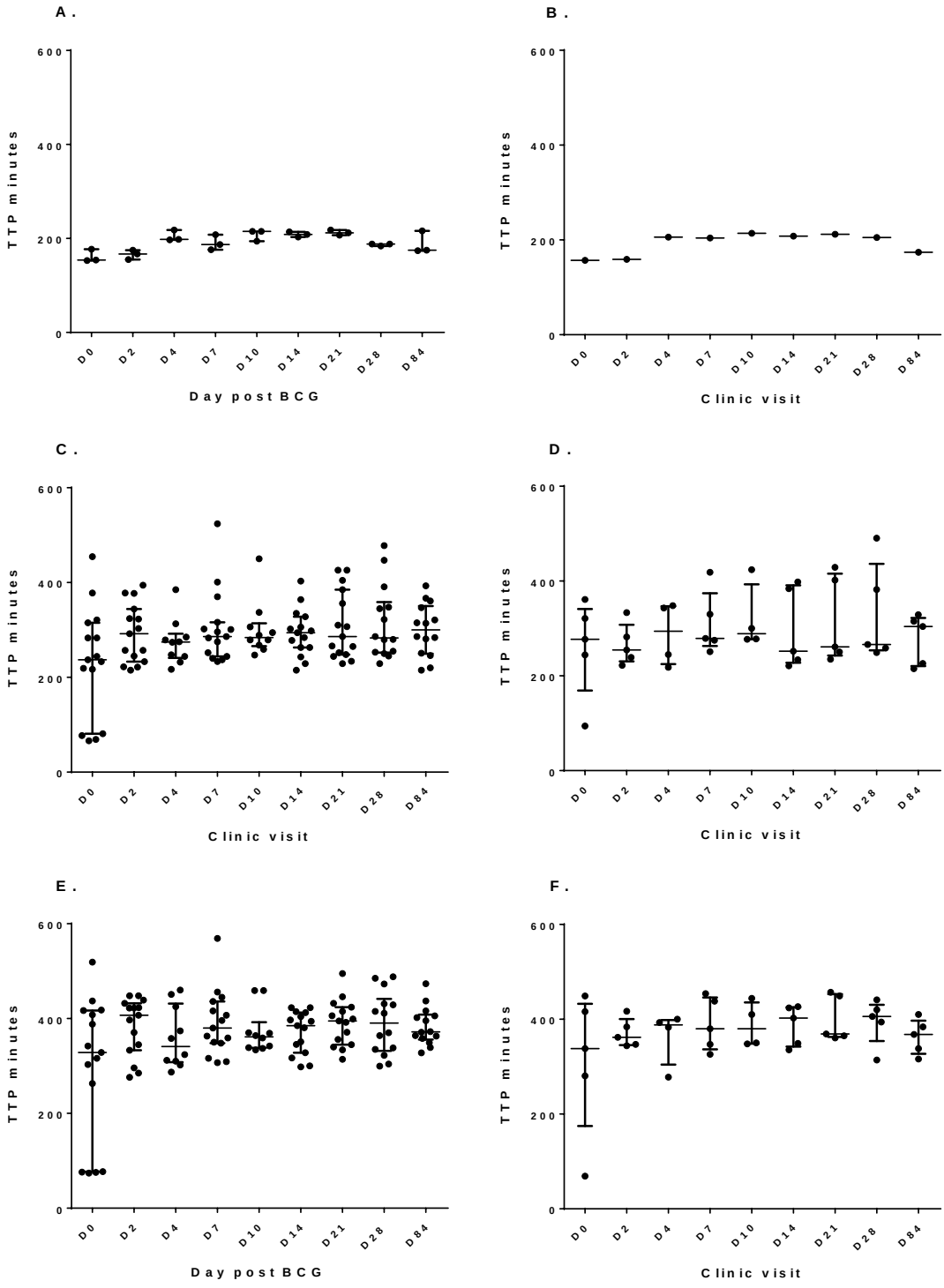


Figure 4.27 *E. coli* BACTEC FX following 1 hour GIA incubation. Each dot represents mean of duplicate values for each volunteers, median and interquartile range shown. A. 1:1 dilution of inoculum group 1 volunteers (n=3), B. 1:1 dilution of inoculum group2 volunteer (n=1), C. 1:10 dilution of inoculum, group

1 volunteers (n=20), D. 1:10 dilution of inoculum group 2 volunteers (n=5), E. 1:100 dilution of inoculum, group 1 volunteers (n=20), F. 1:100 dilution of inoculum group 2 volunteers (n=5)

TTP results following 24 hour incubation are shown in Figure 4.28. As before volunteers are divided by group and by inoculum concentration. Volunteers in Group 1 (BCG vaccinated) demonstrate a statistically significant change in TTP between D0 and other post-vaccination time-points ($p = 0.0042$ one way ANOVA Friedmans test, Figure 4.28 A). At an inoculum dilution of 1:10, there was a significant increase in TTP between D0 and D4 ($p=0.0471$ Dunn's test of multiple comparisons; Figure 4.28 C). At an inoculum dilution of 1:100, there was no significant change in TTP between D0 and any other time-point ($p = 0.2948$ one-way ANOVA Friedmans test; Figure 4.28 E).

The single non-vaccinated control volunteer in Group 2 whose blood was inoculated at an inoculum dilution of 1:1 is shown in Figure 4.28 B. At an inoculum dilution of 1:10 and 1:100, there was no significant difference between D0 and all other time-points ($p=0.8956$ one-way ANOVA Friedmans test, and $p=0.8393$ one-way ANOVA Friedmans test respectively; Figure 4.28 D and F).

There was one volunteer in group 1 (TBT-038122) who demonstrated better control of *E. coli* growth following 24 hour incubation with the 1:100 dilution inoculum, than all other volunteers, with longer TTP (volunteer highlighted in grey Figure 4.28 E). Also, another volunteer (TBT-038117 orange dots; Figure 4.28 E) had improved control at D21 and D84. In group 2 (non-vaccinated) at the 24 hour incubation of 1:100 dilution inoculum, there were two volunteers who stood out as outliers. One volunteer appeared to have better control of growth at D7, and the other had better control at D21 (red and green; Figure 4.28 F).

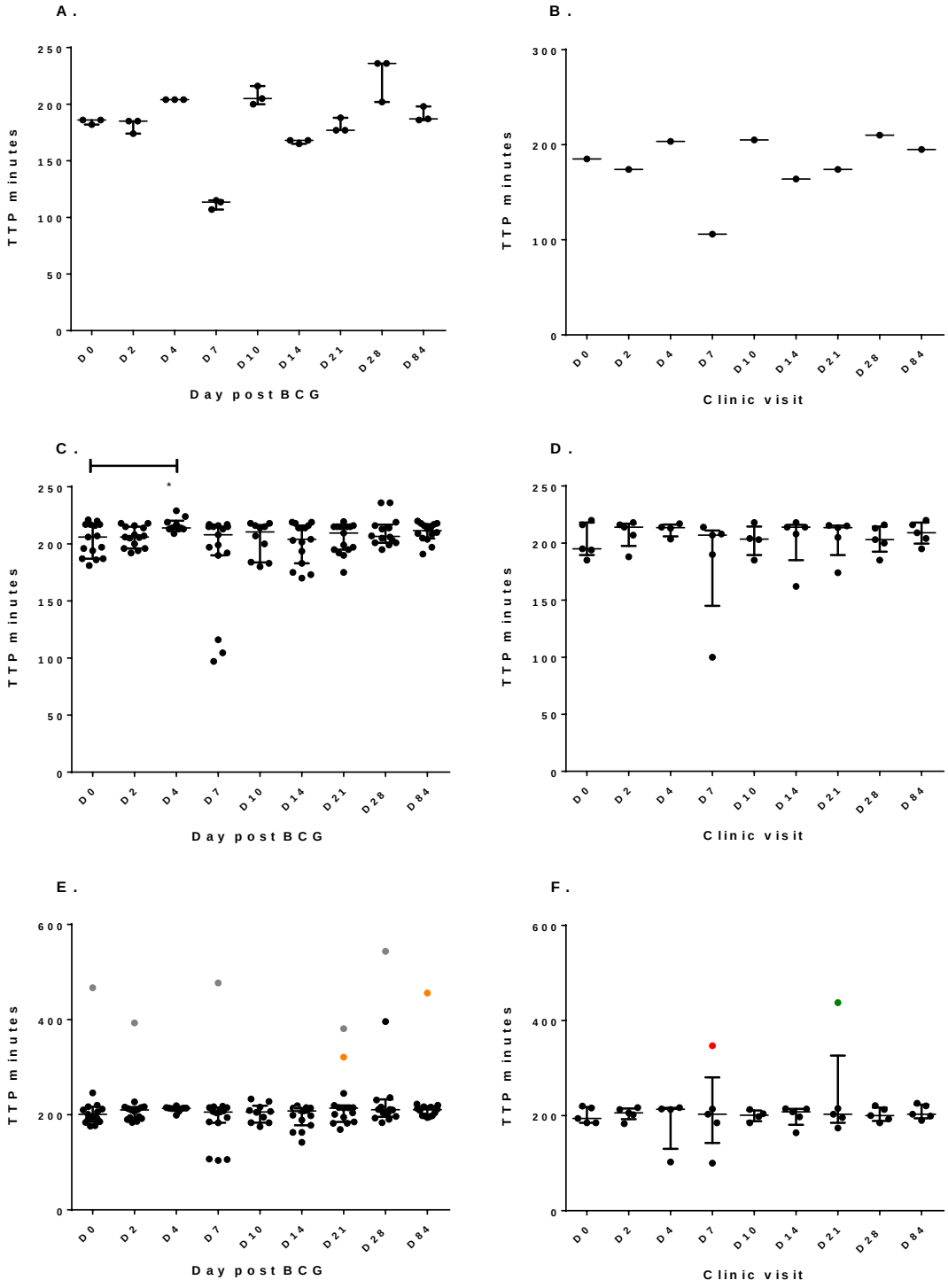


Figure 4.28 *E. coli* BACTEC FX following 24 hour GIA incubation. Each dot represents mean of duplicate values for each volunteers, median and interquartile range shown. A. 1:1 dilution of inoculum group 1 volunteers (n=3), B. 1:1 dilution of inoculum group2 volunteer (n=1), C. 1:10 dilution of inoculum, group

1 volunteers (n=20), D. 1:10 dilution of inoculum group 2 volunteers (n=5), E. 1:100 dilution of inoculum, group 1 volunteers (n=20), F. 1:100 dilution of inoculum group 2 volunteers (n=5)

Correlation analysis between CFU/mL and TTP was performed. There was significant inverse correlation for *S. aureus* at 1 hour (Spearman r -0.6024 (95% CI -0.7832 to -0.3276) p=0.0001; Figure 4.29 A) and 24 hours incubation (Spearman r -0.7016 (95% CI -0.8402 to -0.4771) p<0.0001; Figure 4.29 B).

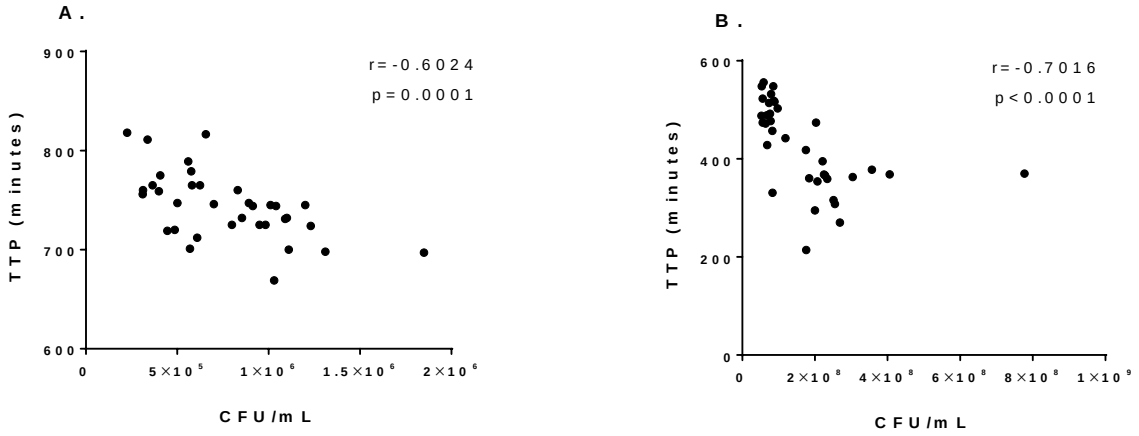


Figure 4.29 Correlation analysis between *S. aureus* GIA CFU/mL and BACTEC FX TTP. Each dot represents the mean of duplicate values for each volunteer. A. 1 hour incubation, B. 24 hour incubation. Spearman r and p value shown.

There was a significant inverse correlation for *E.coli* at 1hour incubation (Spearman r -0.5694 (95% CI -0.7607 to -0.2871) p=0.0003; Figure 4.30 A) but not at 24 hours (Spearman r -0.1546 (95% CI -0.4858 to -0.2154) p=3982; Figure 4.30 B).

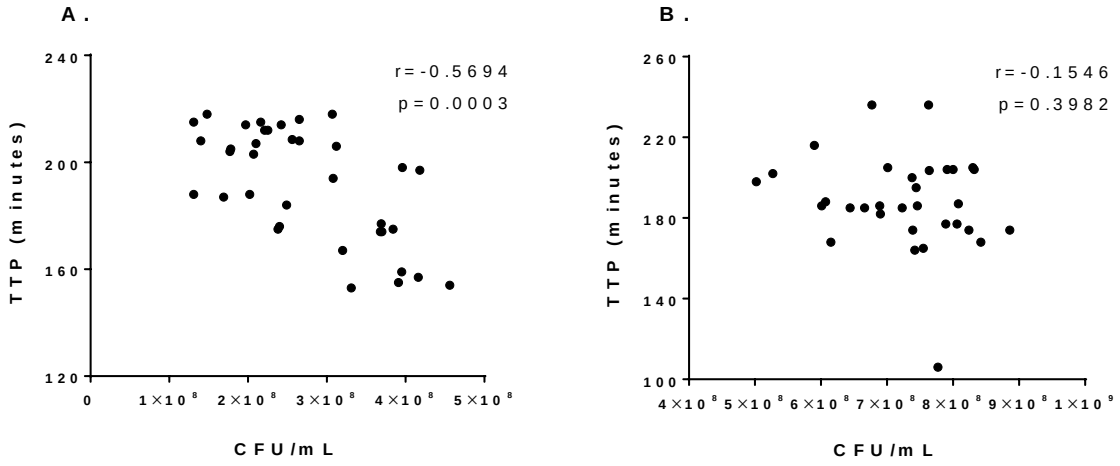


Figure 4.30 Correlation analysis between *E. coli* 1:1 dilution inoculum GIA CFU/mL and BACTEC FX TTP. Each dot represents the mean of duplicate values for each volunteer. A. 1 hour incubation, B. 24 hour incubation. Spearman r and p value shown.

At 1:10 dilution of inoculum, there was a significant inverse correlation between CFU/mL and TTP at both 1 hour (Spearman r -0.8793 (95% CI -0.9105 to -0.838) p<0.0001; Figure 4.31 A) and 24 hour (Spearman r -0.22 (95% CI -0.3668 to -0.0625) p=0.0052; Figure 4.31 B).

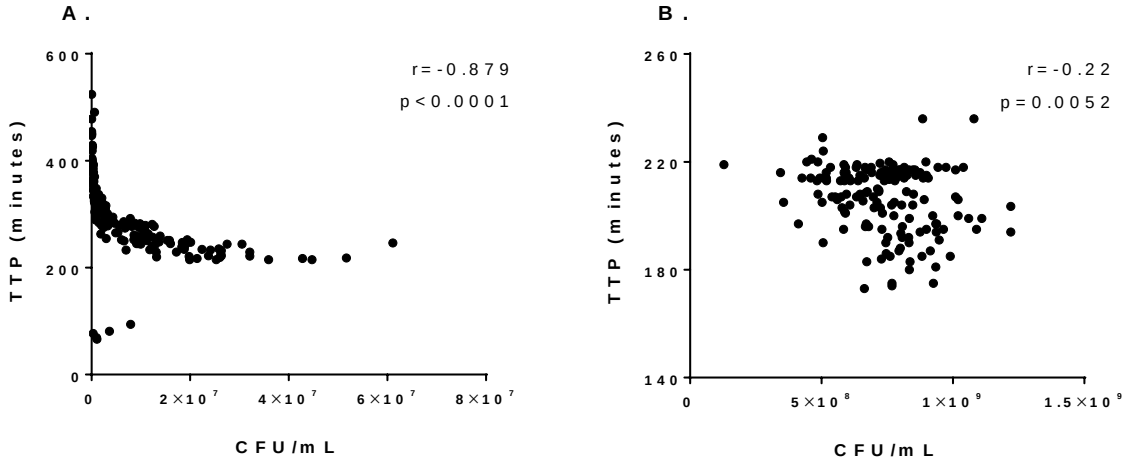


Figure 4.31 Correlation analysis between *E. coli* 1:10 dilution GIA CFU/mL and BACTEC FX TTP. Each dot represents the mean of duplicate values for each volunteer. A. 1 hour incubation, B. 24 hour incubation. Spearman r and p value shown.

Correlation analysis for a *E. coli* 1:100 dilution of inoculum also revealed a significant inverse correlation between CFU/mL and TTP following 1 hour (Spearman r -0.9034 (95% CI -0.929 to -0.8629) p<0.0001; Figure 4.32 A) and 24 hour (Spearman r -0.4133 (95% CI -0.5413 to -0.2665) p<0.0001; Figure 4.32 B).

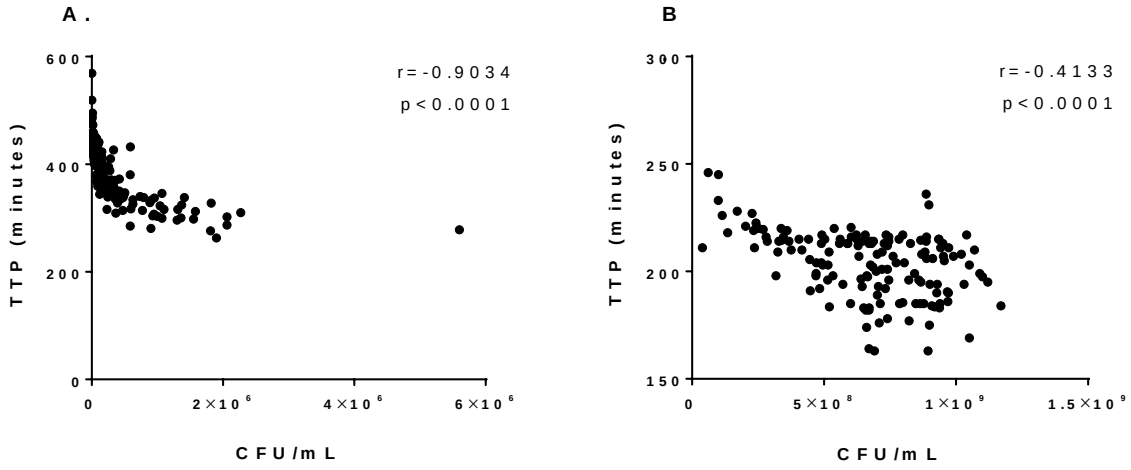


Figure 4.32 Correlation analysis between *E. coli* 1:100 dilution GIA CFU/mL and BACTEC FX TTP. Each dot represents the mean of duplicate values for each volunteer. A. 1 hour incubation, B. 24 hour incubation. Spearman r and p value shown.

The close relationship between TTP and CFU/mL is further demonstrated in Figure 4.33. The *E. coli* GIA net growth output as measured by the plating method identified four volunteers from the 1:100 dilution inoculum incubated at 24 hours, two from group 1 and 2 from group 2 (Figure 4.33 A and B). The TTP system also identified these volunteers in

the same pattern of distribution (the lower the net growth the longer the TTP Figure 4.33 C and D).

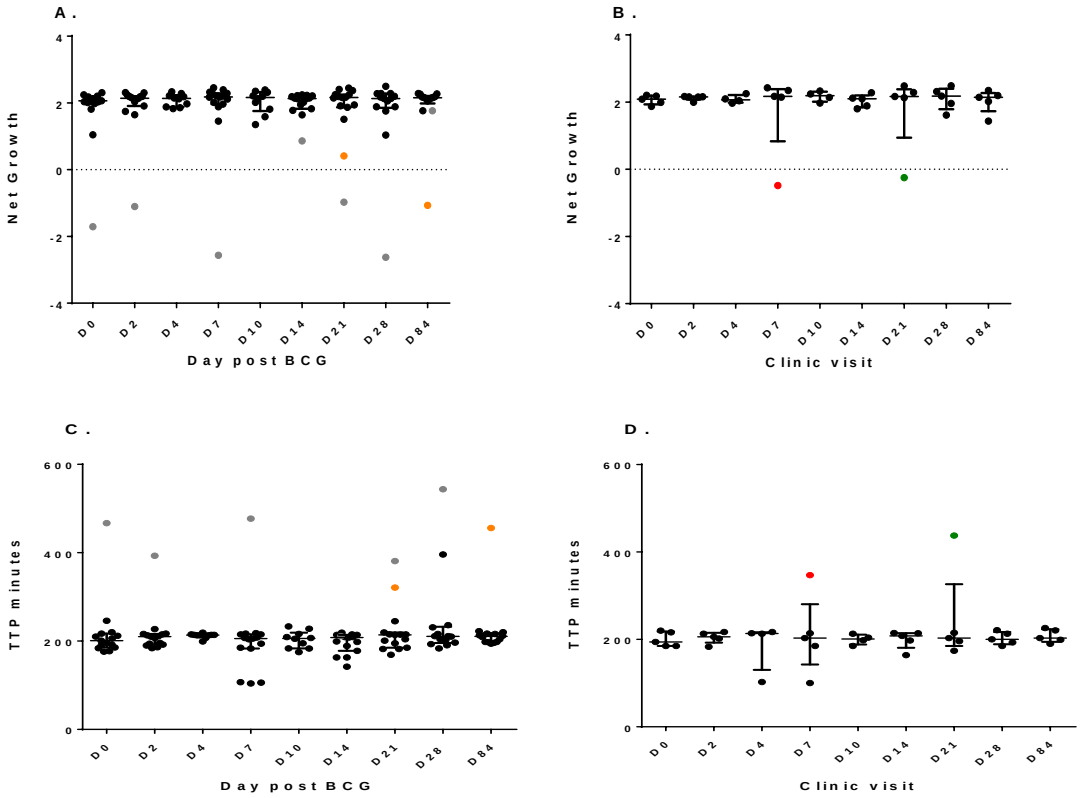


Figure 4.33 *E coli* 1:100 dilution comparison of net growth and BACTEC FX TTP. Each dot represents the mean of duplicate measures per volunteers. Volunteers marked by colour grey, orange, red and green in A and B (net growth) correspond with same volunteers in C and D (TTP)

4.5.3 *S. aureus* nasal swab colonisation status and GIA net growth

Nasal swabs had been performed at screening, D0, and D14 to ascertain *S. aureus* nasal swab carrier status in the enrolled volunteers and the results are presented in Chapter 3. Possible association between *S. aureus* carrier status on net growth of *S. aureus* from the whole blood GIA was explored. Net growth from GIA on D0 and D14, were compared with carrier status. Results are shown in Figure 4.34. There was no difference between carriers

and non-carriers of *S. aureus* in their ability to control growth in the GIA at D0 or D14 (D0 p=0.8679 Mann Whitney test; D14 p=6997 Mann Whitney test).

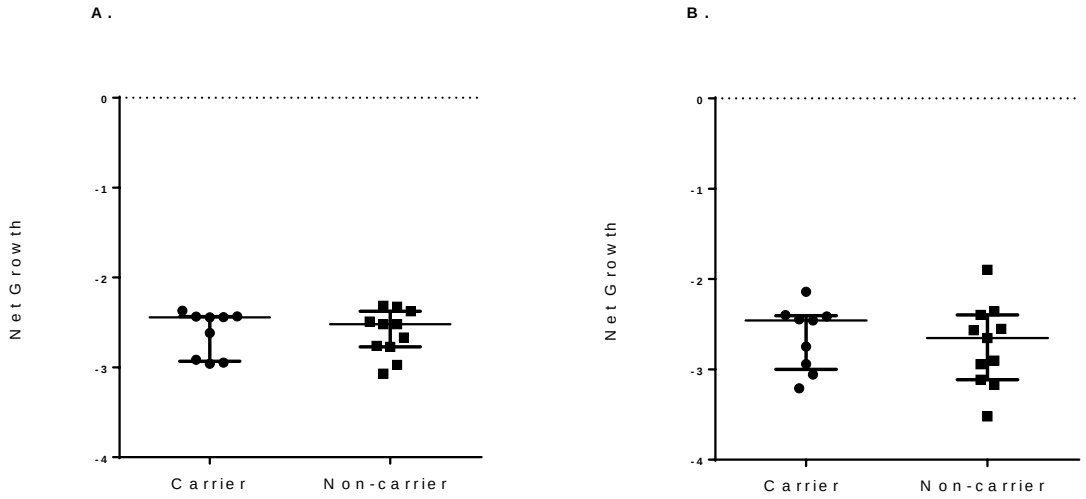


Figure 4.34 Net growth from *S. aureus* whole blood GIA compared with *S. aureus* nasal carriage status. Each dot represents mean of duplicate measures for each volunteers, median and interquartile range shown. A. D0 net growth, B. D14 net growth. Mann Whitney test performed.

4.5.4 Phase 2

15 volunteers were enrolled into phase 2 over three schedules, 12 were randomised to receive BCG vaccination and three remained non-vaccination inoculum controls. One volunteer was enrolled from Birmingham therefore did not have blood drawn on D4 and D10. One volunteer randomised to receive BCG vaccination was withdrawn at their D84 visit due to concurrent antibiotic use. One of the volunteers missed their D21 follow-up visit due to a family bereavement but completed all other study visits as per protocol. As in phase 1, volunteers attended clinic between 8am and 9am for sample collection and bloods were processed within 2-3 hours after draw depending on daily laboratory logistics.

Blood was collected into lithium heparin tubes, and was incubated with inoculum for 1 hour only.

4.5.4.1 K. pneumoniae

All blood samples drawn from the 15 volunteers enrolled in phase 2 were incubated with three dilutions of *K. pneumoniae* inoculum. 1:1 dilution had a mean concentration of 2.67 x 10^8CFU/mL (range 1.48 x 10^8 – 3.85 x 10^8CFU/mL), 1:10 dilution was 2.52 x 10^7CFU/mL (range 1.45 x 10^7 – 3.28 x 10^7CFU/mL) and 1:100 dilution was 2.58 x 10^6CFU/mL (range 1.39 x 10^6 – 3.46 x 10^6CFU/mL).

The net growth of *K. pneumoniae* following 1 hour incubation is shown in Figure 4.35. As with *S. aureus* and *E. coli*, volunteers are divided by group and by inoculum concentration. Volunteers in Group 1 (BCG vaccinated) demonstrated a statistically significant increase in net growth between D0 and D14 at an inoculum dilution of 1:1 (p=0.0471 Dunn's test of multiple comparisons; Figure 4.35 A). At an inoculum dilution of 1:10, there was a significant decrease in net growth between D0 and D2 (p=0.0361 Dunn's test of multiple comparisons; Figure 4.35 C). At an inoculum dilution of 1:100, there was significant variability in net growth across time-points (p = 0.0004 one-way ANOVA Friedmans test, Figure 4.35 E).

The three non-vaccinated control volunteers in Group 2 whose blood was inoculated at an inoculum dilution of 1:1 showed no obvious change on repeat sampling at each clinic visit (p = 0.7018 one-way ANOVA Friedmans test; Figure 4.35 B). At an inoculum dilution of 1:10 and 1:100, again there was no significance difference between D0 and all other time-points (p = 0.3518 one-way ANOVA Friedmans test, and p = 0.1391 one-way ANOVA Friedmans test respectively; Figure 4.35 D and F).

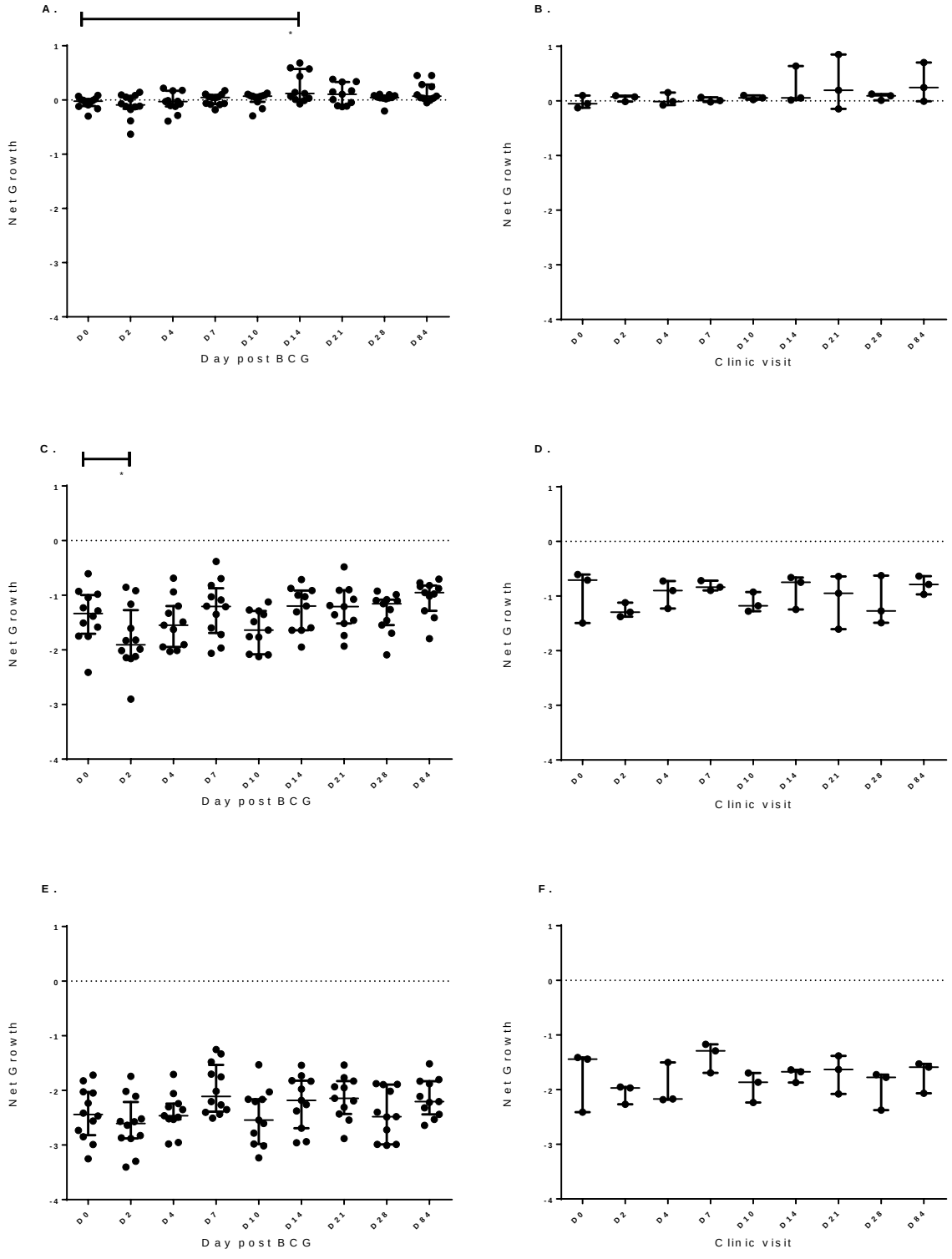


Figure 4.35 *K. pneumoniae* net growth following 1hr incubation. Each dot represents the mean value of duplicate samples per volunteer; median and interquartile range shown. A. and B. results for GIA with 1:1 dilution (~x10^8CFU/mL) inoculum, C. and D. results for GIA with 1:10 dilution (~x10^7CFU/mL)

inoculum, and E. and F. results for GIA with1:100 dilution (~x10^6CFU/mL) inoculum. A. Net growth at baseline (pre-vaccination) and following BCG vaccination in Group 1 volunteers, B. Net growth over repeated sampling for non-vaccinated volunteers from Group 2, C. Net growth at baseline and following BCG vaccination in Group 1 volunteers, D. Net growth over repeated sampling for non-vaccinated volunteers from Group 2, E. Net growth at baseline and following BCG vaccination in Group 1 volunteers, F. Net growth over repeated sampling for non-vaccinated volunteers from Group 2. Oneway ANOVA with post hoc Dunn's test of multiple comparisons where appropriate, where * represents $p<0.05$

4.5.4.2 S. agalactiae

All blood samples drawn from the 15 volunteers enrolled in phase 2 were incubated with two dilutions of *S. agalactiae* inoculum. The 1:1 dilution had a mean concentration of 8.29 x 10^7CFU/mL (range 1.59 x 10^7 – 2.37 x 10^8CFU/mL), and 1:10 dilution was 4.79 x 10^6CFU/mL (range 9.84 x 10^5 – 1.44 x 10^7CFU/mL).

The net growth of *S. agalactiae* following 1 hour incubation is shown in Figure 4.36. As with the previous three pathogens, volunteers are divided by group and by inoculum concentration. Volunteers in Group 1 (BCG SSI vaccinated) showed no significant change in net growth between time-points or between D0 and any other time-point at an inoculum dilution of 1:1 ($p = 0.1360$ one-way ANOVA Friedmans test; Figure 4.36 A). At an inoculum dilution of 1:10, there was a significant variation in net growth between time-points ($p=0.0017$ one-way ANOVA Friedmans test; Figure 4.36 C).

The three non-vaccinated volunteers in group 2 whose blood was inoculated at an inoculum dilution of 1:1 showed no obvious change on repeat sampling at each clinic visit ($p = 0.2381$ one-way ANOVA Friedmans test; Figure 4.36 B). At an inoculum dilution of 1:10, again there was no significance difference between D0 and all other time-points or between time-points ($p = 0.7694$ one-way ANOVA Friedmans test; Figure 4.36 D).

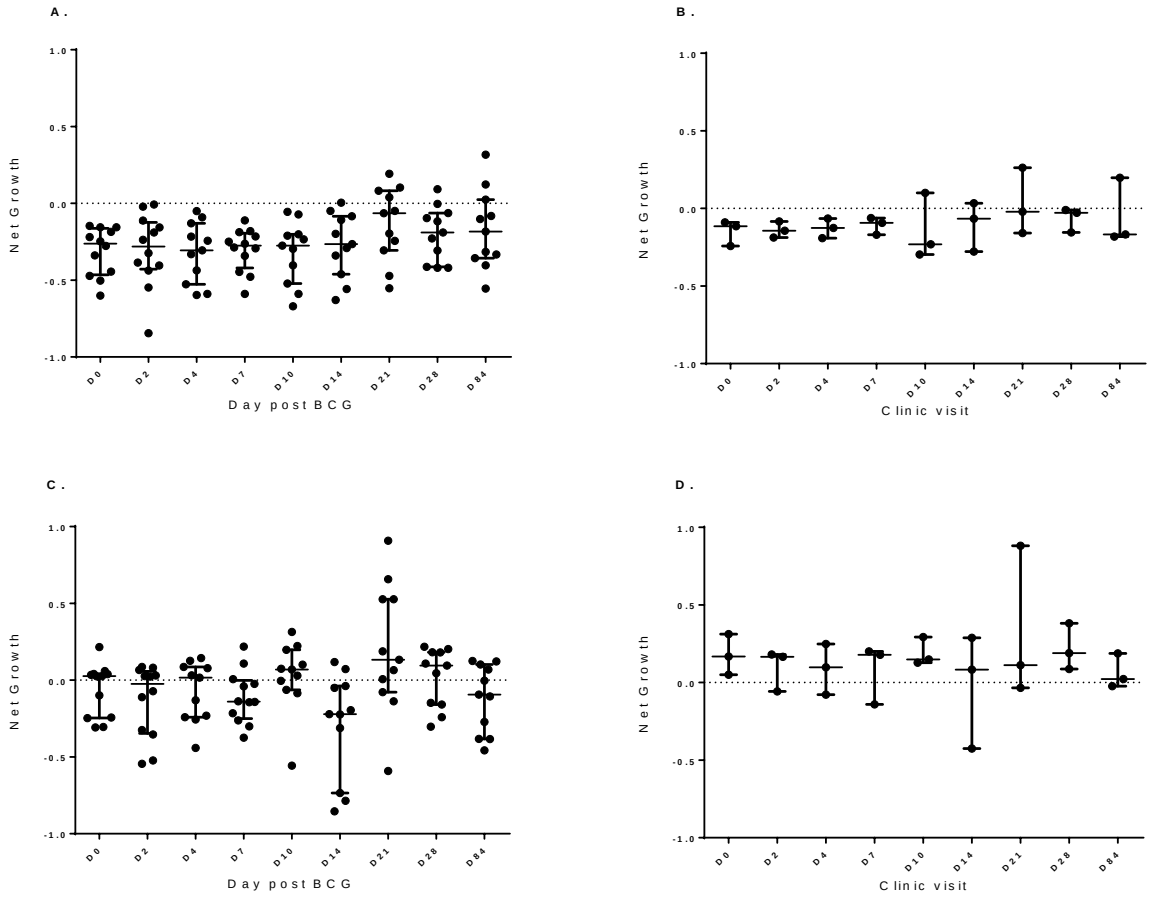


Figure 4.36 *S. agalactiae* net growth following 1hr incubation. Each dot represents the mean value of duplicate samples per volunteer; median and interquartile range shown. A. and B. results for GIA with 1:1 dilution (~x10^7CFU/mL) inoculum, C. and D. results for GIA with 1:10 dilution (~x10^6CFU/mL) inoculum. A. Net growth at baseline (pre-vaccination) and following BCG vaccination in Group 1 volunteers, B. Net growth over repeated sampling for non-vaccinated volunteers from Group 2, C. Net growth at baseline and following BCG vaccination in Group 1 volunteers, D. Net growth over repeated sampling for non-vaccinated volunteers from Group 2.

4.5.5 Confounding variables – Haemoglobin

Linear mixed models were used to assess for potential variables which may confound the GIA output. Gender, colonisation status, Hb, WCC, Platelets, Haematocrit, Neutrophils,

Lymphocytes, Monocytes, Eosinophils and Basophils, ML ratio, *S. aureus* IgG1 (4E3), and GIA inoculum were assessed. The only variable with showed consistency across all whole blood GIAs was Hb concentration. Summarised data is shown in

Table 4.1 for Hb only.

Variable	Coefficient	Standard error	P value	95% Lower CI	95% Upper CI
S. aureus GIA – 1:1 inoculum dilution – 1 hour incubation					
Hb	-0.009	0.008	0.249	-0.024	0.006
S. aureus GIA – 1:10 inoculum dilution – 1 hour incubation					
Hb	-0.028	0.006	<0.001	-0.040	-0.016
S. aureus GIA – 1:100 inoculum dilution – 1 hour incubation					
Hb	-0.027	0.009	0.004	-0.045	-0.009
S. aureus GIA – 1:1 inoculum dilution – 24 hour incubation					
Hb	0.015	0.009	0.102	-0.003	0.032
S. aureus GIA – 1:10 inoculum dilution – 24 hour incubation					
Hb	0.027	0.009	0.002	0.010	0.044
S. aureus GIA – 1:100 inoculum dilution – 24 hour incubation					
Hb	0.028	0.013	0.032	0.002	0.053
E. coli GIA – 1:1 inoculum dilution – 1 hour incubation					
Hb	-0.009	0.013	0.517	-0.035	0.017
E. coli GIA – 1:10 inoculum dilution – 1 hour incubation					
Hb	0.015	0.022	0.493	-0.028	0.057
E. coli GIA – 1:100 inoculum dilution – 1 hour incubation					
Hb	0.041	0.016	0.011	0.009	0.073
E. coli GIA – 1:1 inoculum dilution – 24 hour incubation					
Hb	0.002	0.006	0.774	-0.011	0.014
E. coli GIA – 1:10 inoculum dilution – 24 hour incubation					
Hb	0.010	0.003	<0.001	0.005	0.016
E. coli GIA – 1:100 inoculum dilution – 24 hour incubation					
Hb	0.023	0.024	0.338	-0.025	0.071
K. pneumoniae GIA – 1:1 inoculum dilution – 1 hour incubation					
Hb	0.004	0.005	0.395	-0.005	0.014
K. pneumoniae GIA – 1:10 inoculum dilution – 1 hour incubation					
Hb	0.015	0.009	0.100	-0.003	0.032
K. pneumoniae GIA – 1:100 inoculum dilution – 1 hour incubation					
Hb	0.026	0.012	0.032	0.002	0.051
S. agalactiae GIA – 1:1 inoculum dilution – 1 hour incubation					
Hb	0.004	0.005	0.351	-0.005	0.014
S. agalactiae GIA – 1:1 inoculum dilution – 1 hour incubation					
Hb	-0.011	0.006	0.072	-0.023	0.001

Table 4.1 Summary of association of volunteer Hb concentration with whole blood GIA outcome of net growth for all GIAs

Net growth from the *S. aureus* GIA had a negative correlation with Hb concentration following 1 hour incubation and a positive association following 24 hour incubation. At the lowest *E. coli* and *K. pneumoniae* inoculum concentration, Hb concentration was positively associated with GIA outcome of net growth.

4.6 Discussion

The TB vaccine community strives for the same goal; the development of a safe and effective TB vaccine for the prevention of TB disease and transmission, and the total eradication of this global scourge[2]. The current strategies, as discussed in Chapter 1: Introduction, include boosting an already BCG primed immune system with a recombinant viral vector or subunit vaccine, or, developing an attenuated whole-cell mycobacterial vaccine to replace BCG altogether[6]. A key concern around BCG replacement strategies is the potential for a non-specific effect with the current BCG vaccines. The current evidence for the non-specific effects of vaccines is strongest for BCG[83, 188, 189], and combined with the biological plausibility[115, 116, 118, 135, 190-195], if we are to replace BCG altogether, then the vaccine with which we replace it, must be non-inferior in this regard, in addition to being superior or non-inferior on safety and efficacy against *M. tb*.

There is growing interest in the development of a reliable functional GIA system to assess both vaccine and drug efficacy against a variety of pathogens including bacteria and parasites [140, 141, 186, 196-198]. Such a system would be very valuable in evaluating the non-specific effects of BCG; and could furthermore provide a tool to explore any potential underlying immunological mechanisms, a system with a reproducible measurable outcome to support large expensive field studies, and an outcome measure which would allow us to assess BCG replacement vaccines in this regard.

Optimisation of whole blood *in-vitro* GIAs is complex. The approach to standardisation of such a system requires an in-depth exploration of the potential factors which may contribute to confounding of the measurable outcome (net growth). The most important questions which needed addressing in approaching this assay were:

- Can the method used for the MGIA be applied to other pathogens?
- What is the impact of time delay in blood processing on the GIA outcome measure?
- Can wild type clinical bacterial isolates behave in a reproducible manner?
- Will growth media impact on bacterial growth kinetics?
- What impact might different growth conditions created by warm air incubator versus water bath have on bacterial growth kinetics?
- Does the anticoagulant in the blood collection tube impact on the outcome of the whole blood GIA?

A set of experiments were performed in order to answer these questions.

S. aureus is a gram positive cocci and commensal organism, which under the right conditions is capable of causing a variety of skin and soft tissue infections and less commonly invasive disease such as septic arthritis, endocarditis and bacteraemia. *S. aureus* WTNM was originally obtained from a clinical isolate, therefore known to have caused disease in humans, but due to repeated passage was considered less virulent, but fully sensitive to flucloxicillin, glycopeptides and macrolides[199]. Collaboration with Dr D Wyllie and the *S. aureus* group allowed me the opportunity to evaluate the whole blood GIA using a pathogen commonly associated with neonatal sepsis[102, 200-203]. The primary pilot work was performed by Dr R Tanner (4.4.1.1). This initial set of experiments performed on blood from healthy volunteers from a single bleed, demonstrated that the principle of the whole blood GIA was possible. Whole blood could be inoculated with *S. aureus,* and the recovery of bacteria quantified using a blood-lysis

plating method. By incubating for different periods of time and quantifying CFU/mL, it was established that there was peak killing at 1 hour incubation with steady growth thereafter. The logistical constraint of designing TB038 meant that by factoring in the time required to enrol and draw blood from volunteers in clinic and the time taken for running the assay, the most reasonable incubation periods were 1 hour and 24 hours.

Initial optimisation of the assay was undertaken using single blood draws from healthy BCG-naïve individuals. Whilst this could provide valuable information relating to assay function, it could not answer whether the bacterial counts recovered at a single time-point in an individual, would change following BCG vaccination. The opportunity was taken during a separate BCG challenge study to collect blood during the first 14 days following vaccination and process using the whole blood GIA[187]; appropriate ethical approval for the additional exploratory assay was obtained (NRES South Central B, Rec ref: 14/SC/0036). This study provided the chance to explore logistical constraints surrounding the design of TB038 in regards to the execution of the whole blood GIA, but also the opportunity of assessing whether the whole blood GIA could detect improved control of *S. aureus* growth following BCG vaccination. It was limited in that blood was only being collected on D0 (pre-vaccination), D2, D7 and D14 post vaccination. Timing of samples was challenging. The volunteer clinic appointments were varied with often up to 3 hours between bleeding the first volunteer and bleeding the last volunteer for that visit. This resulted in a disparity in the time blood was sitting on the bench prior to being processed. The net growth from the GIA suggested that there was an improvement in control of *S. aureus* growth following BCG vaccination by D14, but this did not reach statistical significance. The volunteers in this study had a skin biopsy from the site of vaccination at D14 and no blood was taken following that. It was therefore impossible to know what effect on *S. aureus* net growth BCG vaccination would have had after this time point. Whilst no conclusions could be made regarding the non-specific effects of BCG vaccination of *S. aureus* growth *in-vitro*, it did provide valuable logistical insight.

It was clear during the pilot study that timing of blood samples could be key to ensuring the optimal conditions for the assay, especially considering that blood drawn from volunteers in Birmingham could have up to a 6 hour delay in samples being taken at the NIHR WTCFR and arriving in Oxford. Therefore, blood was drawn from five healthy volunteers and samples left for 2 and 5 hours before processing in the GIA, and samples inoculated and incubated with bacteria for 1 and 24 hours. There was no difference following either 1 or 24 hour incubation for either condition. This finding supported the decision to bring volunteers enrolled in Birmingham to Oxford for all visits. Also, in order to minimise variability in processing times, but ensure a logistically workable time-frame, both in the clinic and the laboratory, volunteers were asked to attend clinic between 8 am and 9 am and bloods were processed within 3 hours of draw.

The species of bacteria chosen (*S. aureus*, *E. coli*, *K. pneumonia* and *S. agalactiae*) are common causes of neonatal sepsis particularly in the hospital setting[204]. In order to best mimic the *in-vivo* setting, wild type bacteria were chosen which had been cultured from patients with clinical infection but where the bacteria were known to be fully antibiotic sensitive and non-toxin producing making them suitable for use in a Category 2 setting. The bacteria were provided by the microbiology department at the John Radcliffe Hospital Oxford. Bacteria can be influenced by their environment[205] and it was important to characterise the growth kinetics of these wild type bacteria to ensure that they would behave in a reproducible manner on repeated culture. Frozen bacterial stocks were prepared for use before the start of the study. Bacteria were then plated from frozen onto blood agar and fresh colonies grown in liquid media for 14 hours overnight. Growth curves were then evaluated from transfer of 100μL of overnight culture into fresh media. OD_{600} and CFU/mL were measured for each curve. OD_{600} provided an indication of when the bacteria had progressed through lag and exponential growth phase, and when they had reached plateau indicating termination of the growth curve measurements. Each bacterium was evaluated at least twice from separate colonies to ensure reproducibility.

All four bacteria were reproducible and there was a strong positive correlation between OD_{600} and CFU/mL.

Experiments were performed on *E. coli* to ensure that the choice of media did not impact on bacterial growth. TSB was the media of choice for *S. aureus* therefore this was not changed for the study conditions. LB (Lysogeny broth/Luria broth/Lennox broth) is a standard, nutritionally rich media and contains peptides, casein peptones, trace elements, minerals and vitamins. TSB (tryptone soya broth) containing tryptone and soytone is also nutritionally rich and has long been used for routine culture of *S. aureus* but is also able to culture other aerobic and non-fastidious anaerobic organisms. *E. coli* was cultured in both LB and TSB. The growth curves demonstrated that the rate of growth of the bacteria was not influenced by the media, which is likely due to the fact that both LB and TSB are both nutritionally rich media routinely used for bacterial culture. Due to routine laboratory practice, *E.coli* continued to be cultured in LB media.

Logistical laboratory considerations were important in the setting up of this study and availability of orbital shaking incubators was limited. With the availability of a shaking water-bath, growth of *S. aureus* was assessed under both conditions. The bacteria appeared to grow faster in the water-bath than they did in the warm air orbital incubators, likely due to the more consistent temperature provided by the water-bath. This reinforced the need to ensure that the conditions were consistent and switching between incubator systems was not suitable.

Prior to starting phase 2, analysis of the growth of *K. pneumoniae* and *S. agalactiae* was performed. This was to ensure that the 1 hour and 24 hour incubation period remained appropriate. Both bacteria had different growth profiles in blood. *K. pneumoniae* had an initial lag, which was then followed by steady growth. *S. agalactiae* showed evidence of killing at 30 minutes with steady growth thereafter, but between 6 hours and 27 hours, there was evidence that a plateau had been reached and the bacteria had entered into a

death phase, likely as a result of bacteria beginning to over compete, and nutrition required for bacterial growth and division running out. It was not logistically possible to have multiple incubation periods for all bacteria without clear evidence of a difference; therefore incubation period for phase 2 of the study was kept consistent with phase 1.

An interesting finding during the course of the first schedule of phase 1 was the detection of a difficult to disperse pellet in the *S. aureus* GIA. This pellet had been noted earlier but significance was unclear. On reflection, *S. aureus* is generally a coagulase positive bacteria[206]. Coagulase is an enzyme produced by some bacteria, which enables the formation of a fibrin layer around the bacteria protecting it from phagocytosis and other host killing mechanisms, and plays a role in biofilm formation[207, 208]. The concern which I had was that the bacterial recovery from the *S. aureus* whole blood GIA was not an accurate reflection of bacterial growth, merely an output measure of the bacteria not bound within the formation of a clot. Heparin, a common anticoagulant used in clinical practice, has been found to increase *S. aureus* biofilm formation and has been linked with increased levels of intravenous catheter infection[209]. Alternatives to heparin have been evaluated such as citrate containing compounds which have been found to be significantly better at controlling biofilm layers[210]. As blood had to be anticoagulated for use in the study, EDTA was explored as an alternative anticoagulant additive which may navigate the problem of the clot formation. At a macroscopic level there was no clot formation, however, the study of five volunteers demonstrated that EDTA was an effective bacteriostatic agent. Following both 1 hour and 24 hour incubation, there was recovery of S. aureus, but the number present at 24 hours not statistically different from the recovery at 1 hour, suggesting that there had been no bacterial replication during that time. EDTA is known as an anticoagulant and chelator, and is increasingly being evaluated as a potential agent or adjuvant in the prevention of catheter associated infections [211-213] or as a therapeutic treatment for sepsis in animal models [214]. The optimal collection system would be been one which did not contain any additive, however, the short time-

frame required to process the blood before it began to clot and separate would have been too restrictive. Thus the decision was made to precede with lithium heparin blood collection tubes. The recovery of bacteria from GIA duplicates was very similar and there was likely some relationship between clot formation and the free bacteria left which was specific to the individual volunteer.

The timing of the second incubation period to prepare the inoculum was based on analysis of the growth curves. The aim was to ensure that the bacteria were all in the exponential growth phase and that the CFU/mL was approximately the same. The time taken by each bacterium to reach this point was important from a logistical point, as timings needed to coincide with timing of blood draw, but also to be feasibly reproducible over the duration of the study. *K. pneumoniae*, *E. coli* and *S. aureus* all reached the desired level within 30 minutes of each other (1.5 hour, 2 hour and 2.5 hour respectively). *S. agalactiae*, was however a much more slow growing bacteria and took 6 hours to reach this point. In order to improve the timings of the assay, the decision was made to remove the *S. agalactiae* following 3 hours incubation, where the bacteria would be in the exponential phase of growth, and accept that inoculum concentration would be approximately a log lower. As multiple dilutions of inoculum were being assessed during the whole blood GIA processing, this was not likely to impact significantly.

Over the duration of the study, the growth of the different bacteria was found to be highly reproducible, with very little variability in bacterial concentration, as long as the assay processing times were kept consistent.

The whole blood GIA was processed in two phases of enrolment. It was not possible to process all four bacteria in the same cohort of volunteers, as it was beyond the limits of what was possible from the laboratory perspective; therefore volunteers were enrolled in two phases, with two bacteria being explored at each phase. *S. aureus* and *E.coli* were undertaken first. In addition to the blood-lysis plating method used, samples were

processed using BACTEC FX tubes. This liquid culture system is similar to the BACTEC MGIT used for the direct MGIA and is in routine use in the microbiology laboratory at the John Radcliff Hospital. Traditionally used for detecting bacteraemia, the bottles contain fluorescent beads, which auto-fluoresce when bacteria reach detectable levels. The output reading of time-to-positivity (TTP) correlates with the CFU/mL of bacteria in the blood. Work undertaken by Tanner et al demonstrated that the BACTEC MGIT was a superior quantification method as it was less time consuming and accurately correlated with quantified *M. tb* and BCG growth by traditional CFU plating[186]. Thus it was important to explore whether this system could be superior to the more traditional and time consuming blood-lysis plating method.

S. aureus net growth from the whole blood GIA was evaluated using three different inoculum concentrations. It was important to ensure that the right concentration of inoculum was used, as too high a concentration would overwhelm the assay and fail to demonstrate an impact of recent BCG vaccination on bacterial growth, and too little would likely not discriminate between individuals with better control. Additionally the concentration of bacteria needed to be sufficient in concentration to be countable on the plate reader on recovery following incubation. This could only be tested once the volunteers were enrolled into the study.

An interesting finding was that *S. aureus* net growth decreased at D10 in both the 1:1 and 1:10 inoculum dilutions following 1 hour incubation. At the lowest dilution (1:100) there was a significant decrease in net growth at D4. However, the control group of volunteers also demonstrated the same statistically significant pattern of control at the 1:1 inoculum dilution, and at 1:10 and 1:100 dilutions, there was a similar pattern of control seen, but which did not reach statistical significance. This finding was mirrored following the 24hour incubation period suggesting that the effect seen in the volunteers in group 1 is not biological effect, but more likely due to batch effect created by the inoculums, which

was not fully corrected for despite the inoculum control being taken into account in calculating net growth value.

E. coli net growth following both 1 hour and 24 hour incubation at three inoculum concentration did not show any significant difference in net growth over time in either group 1 or group 2. On completion of the first schedule of volunteers, the discrimination between volunteers using the highest concentration of inoculum was poor and raised concern that the concentration of bacterial inoculum may be overwhelming the assay, therefore it was not continued for subsequent schedules.

BACTEC FX TTP results did not show any significant change in TTP of either *S. aureus* or *E. coli* following both 1 and 24 hour incubation. All bottles became positive, and samples were cultured to confirm bacteria. No contamination had taken place. Of note, on correlation analysis, there was a significant inverse correlation between CFU/mL and TTP. This data provides some validation for the direct plating method employed throughout this study.

Both methods were able to identify the same volunteers as having improved control of *E. coli* net growth following 24 hours incubation and 1:100 inoculum dilution. This again supported the consistency between the two methods. The relevance of volunteers TBT-038122 and TBT-038117 are unclear. TBT-038122 had better control from D0, was identified as being female, but otherwise there were no significant features to explain this finding. One hypothesis is that they may have had frequent urinary infections, therefore perhaps having a robust host immune response to *E. coli*, but they did not report this on direct questioning. Volunteer TBT-038117 did not have consistently improved control and there was no evident clinical reason for this. It could be possible that there were subtle fluctuations in their immune system to account for this, but impossible to identify within the scope of this study.

The impact of *S .aureus* nasal swab carriage was assessed. There was no association found between S. aureus carriage and net growth at D0 and D14. Potential future work would be to do nasal flora microbiome analysis before and after BCG vaccination to ascertain whether there is a change in the bacteria present.

Having reviewed the results, the decision was made to drop the 24 hour time-point moving forward as regardless of starting inoculum, CFU/mL recovery was almost identical for all volunteers and one of the aims was to be able to discriminate between volunteers in ability to control bacterial growth. Furthermore it was necessary to improve laboratory logistics. On discussion with the microbiology department, due to the volume of work generated, it was not possible to continue with the BACTEC FX method. However, moving forward, this would be a method worth pursuing as it significantly improves laboratory time, although increases the cost of the project.

In phase 2 of enrolment, there was a significant increase in *K. pneumoniae* net growth at D14 at the highest inoculum concentration (1:1 dilution) and 1 hour incubation. Whilst the volunteers in the non-vaccinated control group did not demonstrate this, the number of volunteers in this group was smaller and there was a small, non-significant increase in net growth at D14. At the middle inoculum concentration (1:10 dilution) there was a significant decrease in net growth at D2, again not statistically significant in group 2, but the pattern across the two groups was similar. There was no statistically significant difference between D0 and any other time-point at the lowest inoculum concentration.

For the *S. agalactiae* GIA net growth, there was no significant difference between D0 and any later time-point in either group, but there was a non-significant trend between time-points in both groups.

The trends seen in all four bacteria are suggestive of a significant batch effect. What is evident is that the control volunteers do not demonstrate a stable output measure from the GIA on repeated sampling. As they did not receive the intervention (BCG vaccination),

there are clearly other factors impacting on, and contributing to the fluctuations seen between blood draws in individual volunteers. Such factors could be biological or due to assay variability, but impossible to differentiate at this time. It is possible, that as for many tests, the 'natural fluctuation' seen in a volunteers on repeated measure, is larger than the effect of BCG vaccination, therefore any difference caused by BCG is not detectable due to other external factors.

One such variable is Hb concentration. Volunteer FBC parameters were used in a linear mixed model to assess whether they, along with volunteer gender or colonisation status may impact on the GIA outcome. There was a pattern seen across three of the bacterial GIAs at the lower inoculum concentrations. The negative association seen with *S. aureus* may reflect the coagulase positive nature of the bacteria, with greater clot formation and binding of available viable bacteria in the first hour of incubation. The positive association with *E. coli* and *K. pneumoniae* may reflect the ability of the bacteria to utilise Iron for metabolism and growth[156, 158]. The numbers are small and the association was not seen for all bacteria or across all inoculum concentrations and so further work is required to understand the potential impact further.

Controlling for batch effect is challenging. Ideally a volunteer would have all their blood samples processed from the same batch of inoculum; however this is impossible in this setting with the whole blood assay. If group numbers had been more equally matched, controlling for this mathematically may have been possible.

As a functional measure of the 'sum of the parts' of the non-specific or heterologous effects of BCG vaccination in a healthy UK adult population, the whole blood *in-vitro* GIA using *S. aureus*, *E. coli*, *K. pneumoniae* and *S. agalactiae*, was unable to demonstrate any effect of recent BCG vaccination on bacterial growth. There are many possible explanations for this, which include that the lack of effect in the assay is biologically real, and BCG does not exert a non-specific effect. However the impact of batch effect creates a sufficient level of

uncertainty as to the performance of the assay, and that under the pre-specified conditions used in the protocol, whether the assay is sufficiently reliable and sensitive to fully exclude this possibility. It may have been the wrong study population to see this effect in. As discussed in Chapter 1, non-specific effects of BCG are predominantly seen in African infants[9-12, 14, 145], whereas this study was conducted in UK adults. The volume of blood necessary to optimise the assay was not possible from an infant study group. Ideally, if the assay was sufficiently optimised, and a peak time-point for a reduction in net growth post BCG vaccination was identified, it could in theory be performed on no more than 600μL of blood, making undertaking this type of assay in infants possible.

The spectrum of pathogens used in the assay may have been insufficient despite the four pathogens selected providing robust gram negative and gram positive bacterial coverage. Current available research does not specify the pathogens that the non-specific effects of BCG may be protecting against. Whilst the strongest observed effect is seen in respiratory infection and fever, there is an extensive list of pathogens responsible, including but not limited to: Measles virus, Respiratory Syncytial Virus (RSV), Influenza virus, Rotavirus, *Haemophilus influenza* (*H. influenza*), *Streptococcus pneumoniae* (*S. pneumoniae*), *Salmonella typhi* (*S.* typhi) and *paratyph*i (*S. paratyphi*), and fungal infections. If we could identify the exact cause of the respiratory infections or fever potentially being augmented by BCG, then we could target our assay more optimally.

Finally, the inability to see a non-specific effect of BCG using this assay might also be because the assay was not sufficiently optimised. Assay optimisation is improved when you have a positive and negative control by which to gauge your outcome measure by. I did not have individuals that were known to be good or poor controllers for this assay. Therefore maximising the window of difference was all the more challenging. Further work could be done to explore the use of added antibiotics or antibodies to generate a

positive control and a whole blood substitute such as a biochemically similar media, lacking the human cellular and serum components, to form a negative control.

4.7 Conclusions

- Undertaking a study of this size with several logistical constraints was feasible
- The whole blood GIA as a surrogate marker for an individual's ability to control growth of bacteria following BCG vaccination did not detect significant improved control in *S aureus*, *E. coli K. pneumoniae* or *S. agalactiae*
- Assay performance was significantly influenced by inoculum batch effect
- Blood collection tube additive for preventing coagulation of blood is important and can impact both assay functionality and bacterial growth
- Wild type bacteria are, under the same conditions, able to behave in a reproducible and reliable manner
- Assay conditions such as bacterial growth settings and timing from blood draw to processing are important factors which may influence assay outcome and must be kept consistent
- There is no association between *S. aureus* nasal swab carriage status and net growth from the GIA
- Volunteer Hb concentration appears to correlate with GIA outcome measure of net growth and is bacteria specific and inoculum concentration dependent

5 PBMC GIA results

5.1 Statement of Authorship

Initial optimisation of the GIA was performed by M Wilkie, R Tanner and S Harris. Study PBMC samples were separated from whole blood and frozen by D Wright. PBMCs for use during the optimisation steps were separated from whole blood and frozen by M Wilkie. Bacterial cultures were prepared and maintained by M Wilkie. Processing of volunteer samples in the PBMC GIA was performed by M Wilkie, R Tanner, S Harris and I Satti. Data analysis was performed by M Wilkie.

5.2 Introduction

Chapter 4 explored the utility of a whole blood GIA, using four common bacterial pathogens involved in neonatal sepsis (*S. aureus*, *E. coli*, *K. pneumoniae* and *S. agalactiae*), in the identification of a potential non-specific effect of recent BCG vaccination on bacterial growth in a whole blood *in-vitro* functional assay. As discussed in Chapter 4, I was unable to detect an effect of recent BCG vaccination using the whole blood bacterial GIA. Furthermore, I demonstrated a significant batch effect using this assay.

During the development of the whole blood MGIA, the identification of an effect of Hb on the growth of mycobacteria led to the optimisation of this assay with PBMCs alone[186, 215]. From a mechanistic perspective, there is a solid foundation to this; *M. tb* specific antigen targeting by primed, IFN- γ producing, $CD4^+$ and $CD8^+$ T cells is vital for the destruction of intracellular *M. tb*[216], both of which are present in the PBMC extract of whole blood[217]. The PBMC MGIA assay demonstrated a clearer effect on mycobacterial killing than the whole blood MGIA[215]. Increasing interest in the mechanistic basis for the innate effects of BCG in the epigenetic reprogramming and 'training' of innate cells such as monocytes and NK cells, with exploration of assays specifically utilising both fresh

PBMCs and monocytes cultured in the laboratory[134, 135, 138] supports a rationale for exploring this option. Therefore I chose to refine this bacterial killing assay and develop a PBMC based assay. This would have the effect of removing any potential influence of variables such as Hb, whilst additionally allowing for the control of differences in individual volunteer cell numbers, and fluctuations in bacterial inoculum caused by repeated culture.

5.3 Aim of Chapter

The aim of the experiments detailed in this chapter were to:

- Optimise the assay to explore the best PBMC concentration and bacterial inoculum concentration before proceeding with volunteer samples from the study
- Assess whether the replacement of whole blood with PBMCs in the GIA would allow the detection of an effect of BCG vaccination on the control of growth of *S. aureus*, *E. coli*, *K. pneumoniae* and *S. agalactiae* from the volunteers enrolled in the study
- Understand the impact of possible confounding factors such as gender and bacterial colonisation status

5.4 Optimisation strategy

As before, it was important to define the bacterial growth kinetics, when inoculated into a PBMC culture, and if it was possible to identify a peak incubation period when bacteria were being maximally inhibited by the PBMCs. In addition to this, identification of the optimal bacterial and PBMC concentration needed to be identified prior to processing the study samples. Blood was obtained from 10 healthy volunteers and PBMCs separated.

PBMCs were frozen prior to use in order to replicate the conditions under which the study samples would be processed.

5.4.1 Assay optimisation for PBMCs

The whole blood GIA protocol included a water lysis step following incubation in order to break down red and white blood cells and maximise recovery of all viable bacteria. The samples were then centrifuged, supernatant removed and the samples re-suspended in PBS. This step was used in the whole blood MGIA but not necessary in the PBMC MGIA, therefore the first experiments undertaken were to confirm whether this step could be removed, and to identify the best solution to re-suspend the PBMC and bacteria in, following the removal of the supernatant.

PBMCs were used at a concentration of 1.5 x 10^6 cells/well with the lowest concentration of bacterial inoculum that had been used in the whole blood GIA (*S. aureus* ~ x 10^5CFU/mL, *E. coli* ~ x 10^6CFU/mL, *K. pneumoniae* ~ x 10^6CFU/mL and *S. agalactiae* ~ x 10^5CFU/mL). The reason for this inoculum was that from experience with the MGIA; the PBMC MGIA required much lower concentrations of inoculum than were required for the whole blood equivalent. PBMCs and bacterial inoculum were incubated for 1 hour and 3 hours to assess bacterial recovery. Three conditions were then assessed following both incubations:

A. Following incubation, sample centrifuged, supernatant removed and pellet re-suspended in water
B. Following incubation, sample centrifuged, supernatant removed and pellet re-suspended in PBS
C. Following incubation, sample centrifuged, supernatant removed, sample then re-suspended in water and incubated at room temp for 10 minutes before undergoing a

second centrifugation, removal of supernatant and re-suspension in PBS (same as steps in whole blood GIA)

The CFU/mL recovery of *S. aureus* under the defined conditions for the PBMCs of two volunteers is shown in Figure 5.1. The highest recovery of CFU/mL at both time-points of *S. aureus* was condition A (re-suspension in water; Figure 5.1 A and B). Under condition B (re-suspension in PBS), recovery from volunteer 1 was the same as for condition A, however, recovery from volunteer 2 was less at 3 hours (Figure 5.1 B). Under condition C, there was less recovery of *S. aureus* particularly following 3 hour incubation for both volunteers. As there was no difference in the way the assay was performed other that the post incubation lysis and re-suspension steps, this difference is very unlikely to be due to PBMC killing of bacteria.

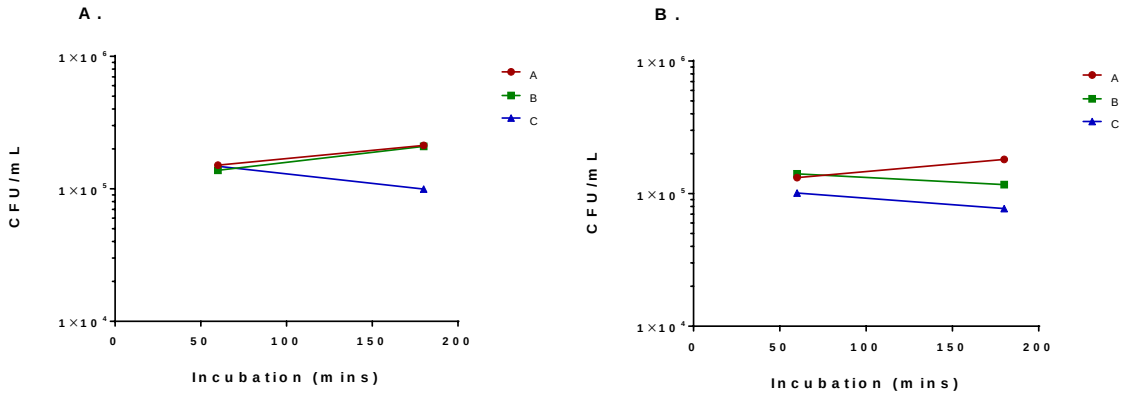


Figure 5.1 *S. aureus* recovery following incubation with PBMCs. CFU/mL measured at 1 hour and 3 hours under three conditions: A. water resuspension; B. PBS resuspension; C. water lysis then resuspension in PBS. Each dot represents the mean of duplicates. A. PBMCs from volunteer 1; B. PBMCs from volunteer 2.

In the recovery of *E.coli* following the three conditions, there was no difference seen (Figure 5.2). Nor was there any difference in recovery of *K. pneumoniae* (Figure 5.3) or *S. agalactiae* (Figure 5.4).

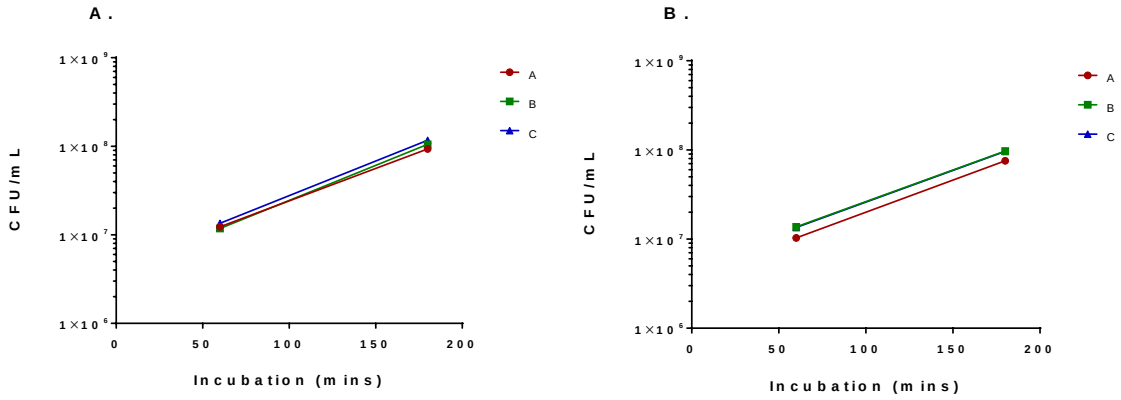


Figure 5.2 *E. coli* recovery following incubation with PBMCs. CFU/mL measured at 1 hour and 3 hours under three conditions: A. water resuspension; B. PBS resuspension; C. water lysis then resuspension in PBS. Each dot represents the mean of duplicates. A. PBMCs from volunteer 1; B. PBMCs from volunteer 2.

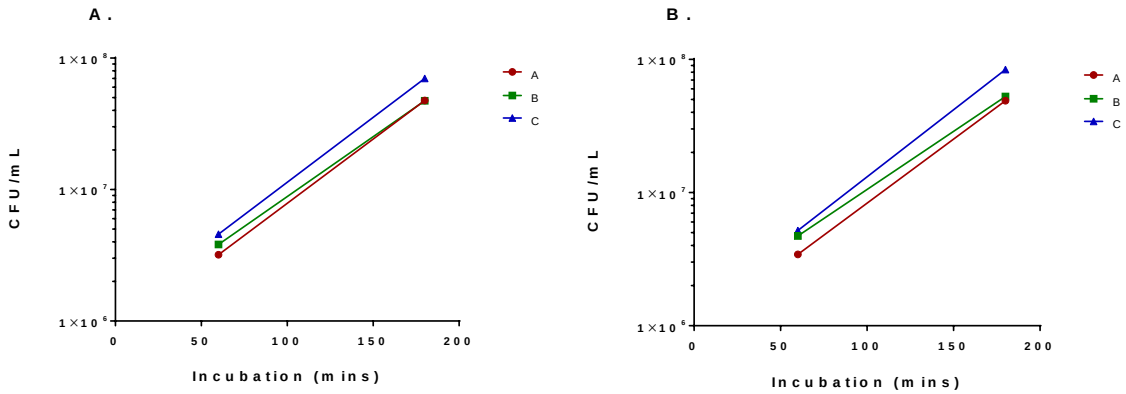


Figure 5.3 *K. pneumoniae* recovery following incubation with PBMCs. CFU/mL measured at 1 hour and 3 hours under three conditions: A. water resuspension; B. PBS resuspension; C. water lysis then resuspension in PBS. Each dot represents the mean of duplicates. A. PBMCs from volunteer 1; B. PBMCs from volunteer 2.

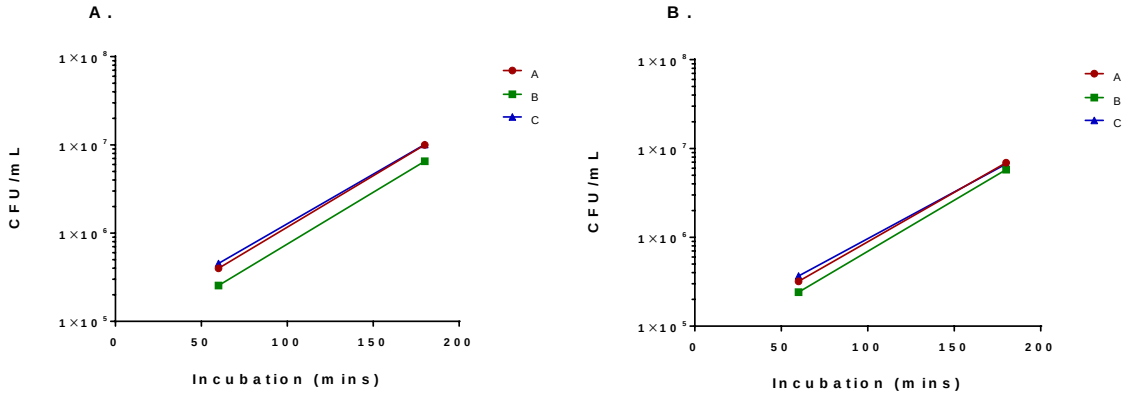


Figure 5.4 *S. agalactiae* recovery following incubation with PBMCs. CFU/mL measured at 1 hour and 3 hours under three conditions: A. water resuspension; B. PBS resuspension; C. water lysis then resuspension in PBS. Each dot represents the mean of duplicates. A. PBMCs from volunteer 1; B. PBMCs from volunteer 2.

To ensure consistency across the four pathogens, the final step chosen was to centrifuge samples post incubation, remove supernatant and re-suspend pellet in water.

5.4.2 Checkerboard experiment: optimisation of PBMC and bacterial inoculum concentration

Following refinement of the post incubation steps of the assay, the next set of experiments were designed to evaluate which concentration of PBMCs would be optimal and to identify which concentration of bacterial inoculum would be sufficient to see killing, whilst not overwhelming the assay, or providing too few bacteria to distinguish between samples. Three different concentrations of PBMCs per well were chosen: 5 x 10^5cells/well, 1 x 10^6cells/well and 2 x 10^6cells/well. Three bacterial inoculums concentrations were also chosen: ~ x 10^4CFU/mL, ~ x 10^5CFU/mL and ~ x 10^6CFU/mL. Bacterial inoculums were lower than had been used during the whole blood bacterial GIA experiments. Experiments with the MGIA had shown that when switching from whole blood to PBMCs, the concentration of inoculum needed to be much lower as a high multiplicity of infection (MOI) overpowered any effect induced by the vaccine[186]. Outlines of the checkerboard of conditions explored are detailed in Table 5.1.

Conditions	~ x 10^6CFU/mL	~ x 10^5CFU/mL	~ x 10^4CFU/mL
5 x 10^5cells/well	A	B	C
1 x 10^6cells/well	D	E	F
2 x 10^6cells/well	G	H	I

Table 5.1 Schematic representation of checkerboard layout detailing conditions tested

PBMCs from the same volunteer were processed for each condition, with the PBMCs from 1 volunteer being used per bacteria. Samples were processed in duplicate. Samples from each condition were incubated for 1, 4 and 24 hours, and CFU/mL recovered from each time-point and plotted to assess whether there was less bacterial recovery under any of the pre-specified conditions suggesting conditions for optimal bacterial killing.

S. aureus recovery was observed to be most significantly influenced by inoculum concentration rather than PBMC concentration as shown in Figure 5.5. Under all conditions, there was steady growth in bacteria between 1 and 24 hours. There was some distinction demonstrated by PBMC concentrations, particularly using 1 x 10^6cells/well and 2 x 10^6 cells/well, with the greatest spread following 24 hour incubation. Growth of bacteria under all nine conditions was similar, although recovery at 24 hours was lowest for condition I (highest PBMC concentration/well and lowest inoculum concentration).

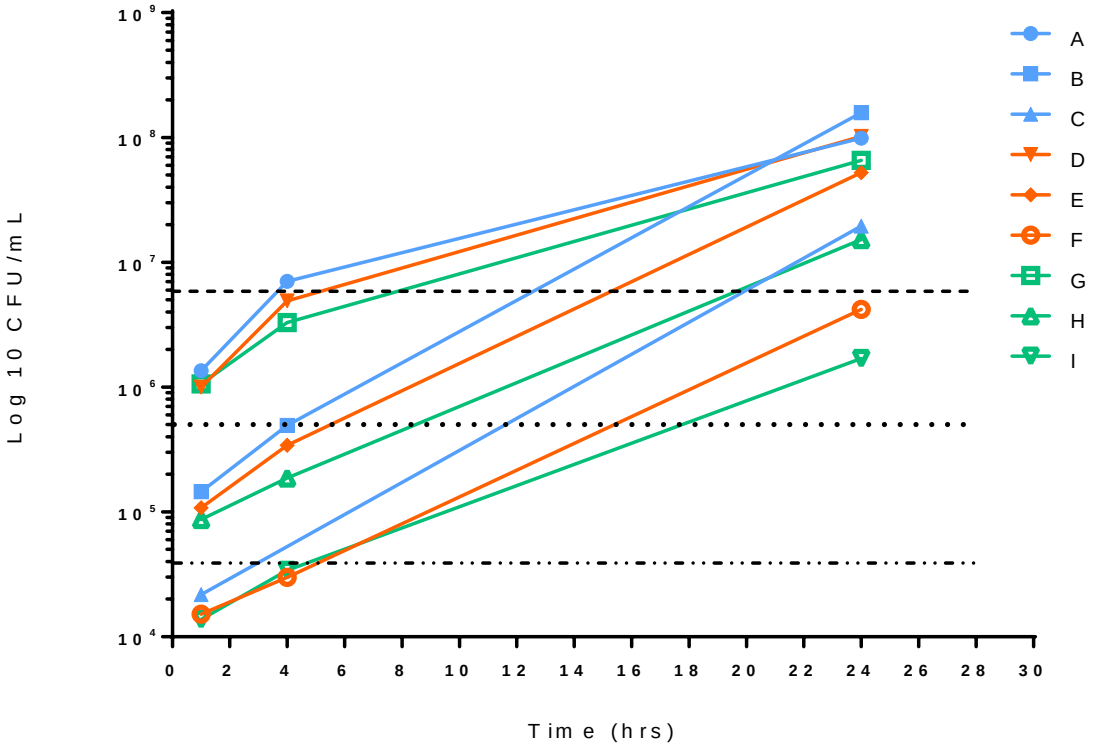


Figure 5.5 *S. aureus* recovery at 1 hour, 4 hour and 24 hours incubation in the PBMC GIA measured as Log10 CFU/mL. Nine conditions tested (A-I) and detailed in Table 5.1. Each dot represents the mean of duplicate readings. The dotted lines crossing from the Y axis represent the CFU/mL of the three different inoculums used.

In the case of *E. coli*, again the greatest impact was by the inoculum concentration rather than the influence of the concentration of PBMCs (Figure 5.6). Bacterial growth kinetics from all time-points under all conditions was very similar, with most reaching that same concentration of bacteria, regardless of starting inoculum concentration. Condition C (lowest PBMC concentration and lowest bacterial inoculum concentration), did not plateau at the same CFU/mL compared with all other conditions at 24 hours.

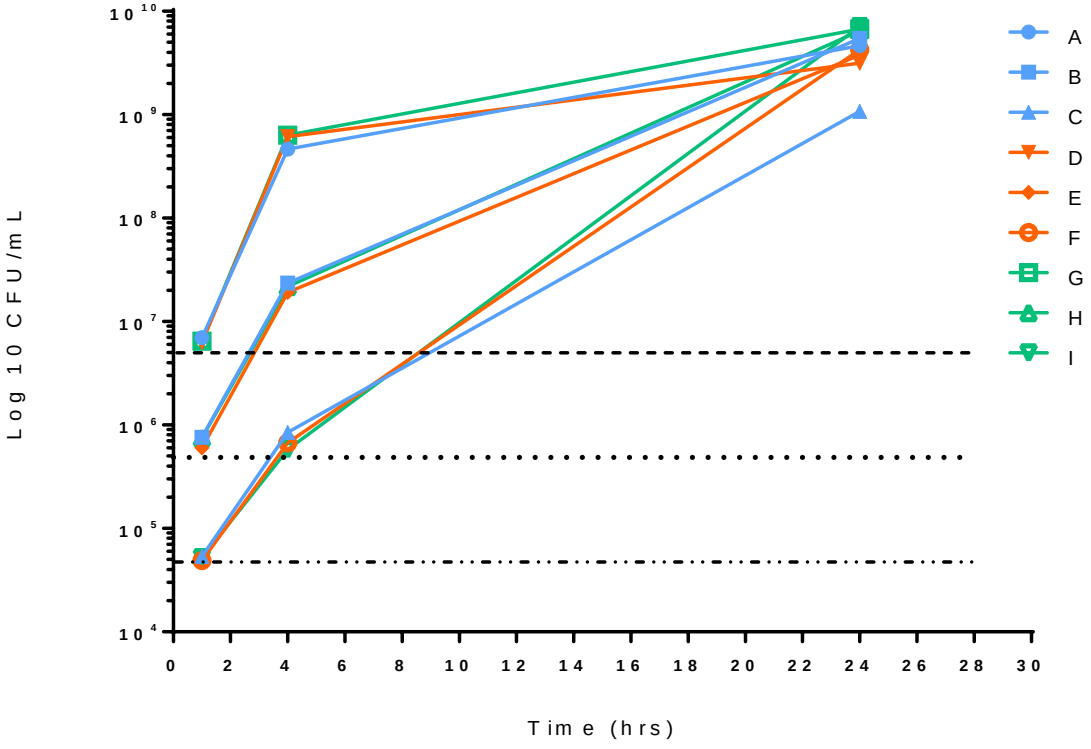


Figure 5.6 *E. coli* recovery at 1 hour, 4 hour and 24 hour incubation in the PBMC GIA measured as Log10 CFU/mL. Nine conditions tested (A-I) and detailed in Table 5.1. Each dot represents the mean of

duplicate readings. The dotted lines crossing from the Y axis represent the CFU/mL of the three different inoculums used.

K. pneumoniae, like *S. aureus* and *E. coli* was influenced the greatest by bacterial inoculum concentration, with no suggestion of an influence of PBMC concentration at any time-point under any condition. In addition, regardless of starting concentration of inoculum, bacterial recovery at 24 hours had plateaued at the same concentration. No one condition demonstrated superiority.

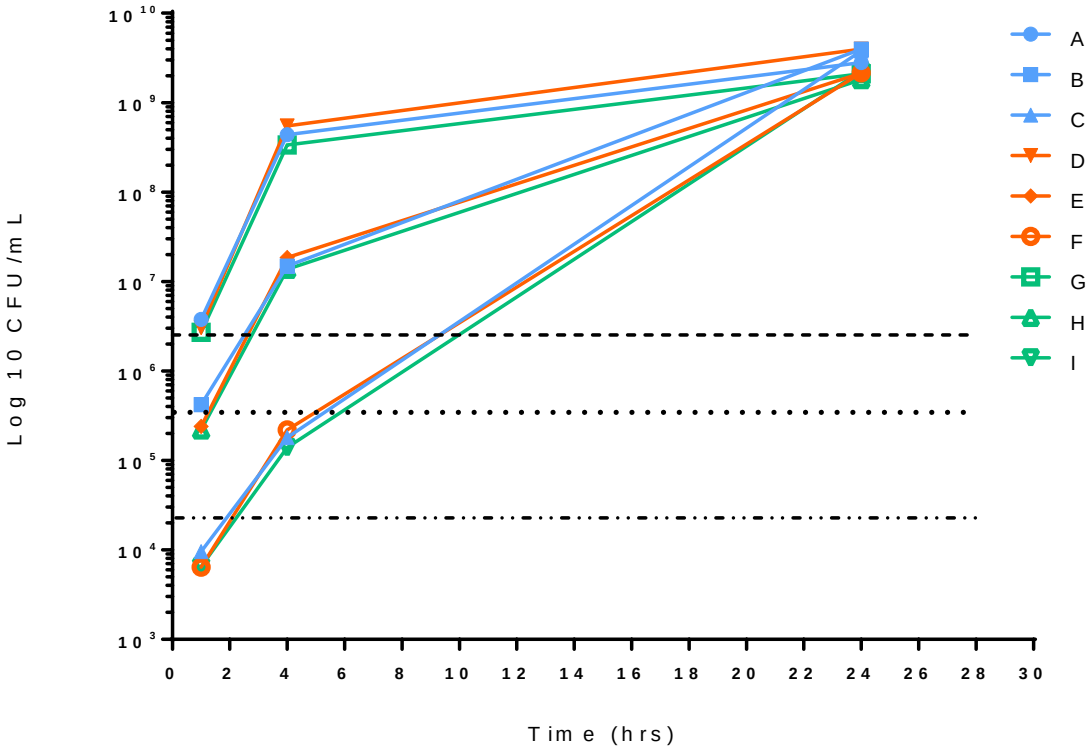


Figure 5.7 *K. pneumoniae* recovery at 1 hour, 4 hour and 24 hour incubation in the PBMC GIA measured as Log10 CFU/mL. Nine conditions tested (A-I) and detailed in Table 5.1. Each dot represents the mean of duplicate readings. The dotted lines crossing from the Y axis represent the CFU/mL of the three different inoculums used.

S. agalactiae followed the same pattern of growth over time as *K. pneumoniae* for all conditions, and again, the greatest influence was caused by bacterial inoculum concentration. There was some separation of bacterial recovery between different concentrations of inoculum shown following the 4 hour incubation, and at the lowest bacterial inoculum concentration. The highest concentration of PBMCs had the lowest bacterial recovery, with the middle concentration next and the lowest concentration showing the highest recovery at that time-point.

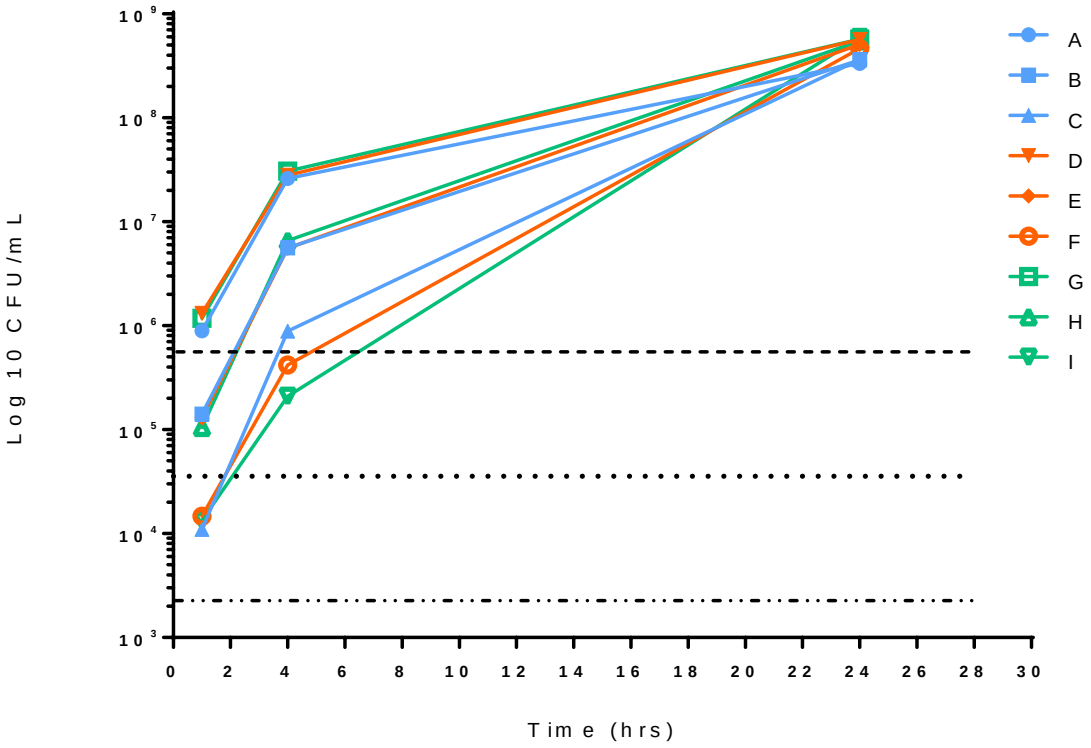


Figure 5.8 *S. agalactiae* recovery at 1 hour, 4 hour and 24 hour incubation in the PBMC GIA measured as Log10 CFU/mL. Nine conditions tested (A-I) and detailed in Table 5.1. Each dot represents the mean of duplicate readings. The dotted lines crossing from the Y axis represent the CFU/mL of the three different inoculums used.

As there was no consistency across the pathogens, and no convincing evidence that one condition was superior to the other at demonstrating either peak killing or a significant reduction in bacterial recovery, the decision was made to proceed with 1 x 10^6cells /well, as stored PBMCs from the study were limited and this was the best utility of the cells available, and a bacterial inoculum concentration of ~ x 10^5CFU/mL. It was not possible to know the impact of vaccination over time prior to commencing with stored samples from the study, therefore a review of the data following every set of four volunteers conducted would allow for this concentration to be adjusted as necessary.

5.5 PBMC GIA results

Stored PBMCs were thawed and rested for two hours prior to processing in sets of four volunteers. Due to logistics and to balance Oxford and Birmingham volunteer time-points, time-points processed were D0, D2, D7, D14, D21, and D84 (Figure 5.9). All time-points for each individual volunteer were processed at the same time from the same batch of bacterial inoculum to eliminate the impact of inoculum batch effect.

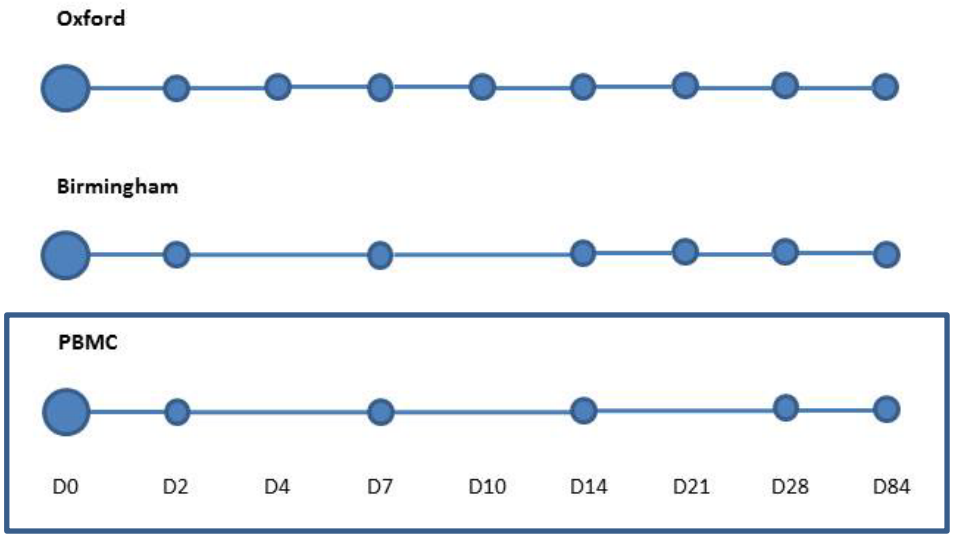


Figure 5.9 Schematic representation of sample collection time-line for Oxford and Birmingham volunteers and selection of samples used for PBMC GIA.

There were 1 x 10^6 cells/well in a volume of 300µL (3.33 x 10^6 cells/mL). Starting bacterial inoculum was ~ x 10^5CFU/mL for *S. aureus*, *E. coli*, and *K. pneumoniae*, and ~ x 10^4CFU/mL for *S. agalactiae* due to longer bacterial multiplication time as previously discussed in Chapter 4. Cells were incubated for one hour with bacterial inoculums in order for consistency between whole blood and PBMC GIA.

Bacterial net growth output measures for all four bacteria were tested for normality using Shapiro Wilks test. Not all conditions passed normality test, therefore for consistency, non-parametric, paired, one-way ANOVA (Friedmans test), with post-hoc Dunn's multiple comparisons test was performed where appropriate. The mean of each time-point was compared to mean of D0.

5.5.1 S. aureus

All 35 volunteers stored PBMCs were inoculated and incubated in the GIA. Net growth of *S. aureus* at each time-point for both group 1 and group 2 volunteers is shown in Figure 5.10. Mean inoculum concentration was 1.71 x 10^5CFU/mL (range 1.26 x 10^5 – 2.93 x 10^5CFU/mL).

The net growth of *S. aureus* following 1 hour incubation with the PBMCs from group 1 showed a significant decrease in net growth between D0 and D7 (p=0.0085; Figure 5.10 A) Once adjusted for multiple variables, the significant difference between D0 and D7 was no longer significant. There was no significant difference in non-vaccinated group 2 volunteers between time-points (p=0.3739; Figure 5.10 B).

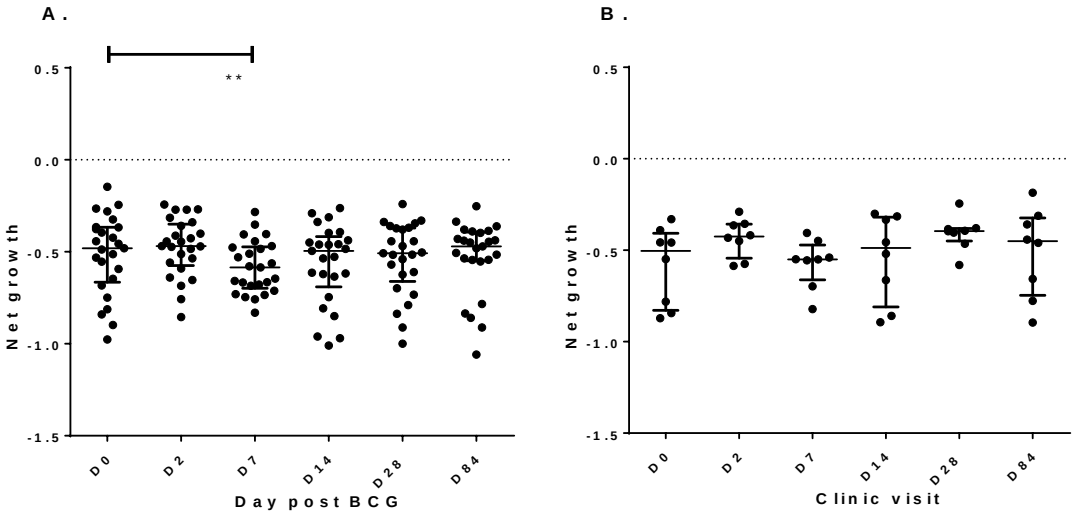


Figure 5.10 *S. aureus* PBMC GIA net growth. Each dot represents the mean of duplicate measures for each volunteer, median and interquartile range shown. A. net growth for group 1 (BCG vaccinated) and B. net growth for group 2 (non-vaccinated) volunteers. Non-parametric paired one-way ANOVA was performed where ** signifies p≤0.01. Net growth (Log10(Sample CFU/mL ÷ Control CFU/mL))

5.5.2 E. coli

All 35 volunteers stored PBMCs were inoculated and incubated in the GIA. Three inoculum concentrations were assessed. The first four volunteer PBMCs were inoculated with 5.17 x 10^5CFU/mL of inoculum. Net growth following 1 hour incubation is shown in Figure 5.11 A and B. There was no significant difference between D0 and subsequent time-points for group 1 (BCG-vaccinated) volunteers (p=0.1765; Figure 5.11 A).

Concerned that the inoculum concentration was too high with little difference between volunteers, the next 4 volunteers PBMCs were incubated with a reduced inoculum concentration of 4.48 x 10^4CFU/mL. Net growth following 1 hour incubation is shown in Figure 5.11 C and D. Again, there was no significant difference between D0 and any

subsequent post vaccination time-points for volunteers in group 1 (p=0.8363 Figure 5.11 C). Again concerned that the inoculum concentration remained too high and overwhelming any killing effect (Figure 5.11 C and D), all subsequent volunteers PBMCs were inoculated using the lowest mean inoculum concentration possible in order to calculate CFU/mL recovery using the defined protocol.

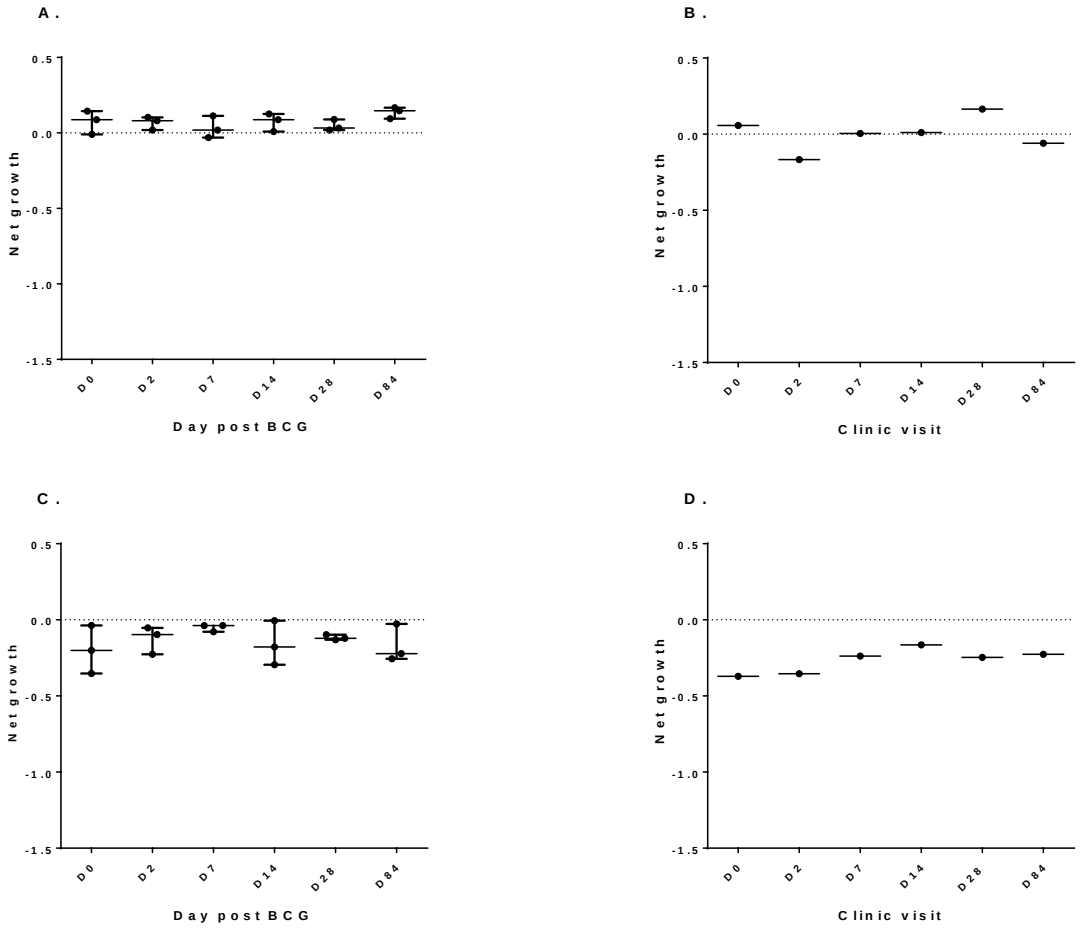


Figure 5.11 *E. coli* PBMC GIA net growth at ~x10^5 and ~x10^4 CFU/mL inoculum concentration. Each dot represents the mean of duplicate measures for each volunteer, median and interquartile range shown. A. net growth for group 1 (BCG vaccinated) volunteers inoculum concentration ~ x10^5CFU/mL, B. net growth for group 2 (non-vaccinated) volunteers inoculum concentration ~ x10^5CFU/mL. C. net growth for group 1 (BCG vaccinated) volunteers inoculum concentration ~ x10^4CFU/mL, and D. net growth for group 2 (non-vaccinated) volunteers inoculum concentration ~ x10^4CFU/mL. Non-parametric paired one-way ANOVA was performed. Net growth (Log10(Sample CFU/mL ÷ Control CFU/mL))

All 27 sets of remaining volunteer PBMCs were inoculated with a mean inoculum concentration of 4.4 x 10^3CFU/mL (range 3.96 x 10^3 – 5.34 x 10^3CFU/mL) for 1 hour incubation period. Net growth of *E. coli* at each time-point for both group 1 and group 2 volunteers is demonstrated in Figure 5.12 A and B.

The net growth of *E. coli* following 1 hour incubation with the PBMCs from volunteers in group 1 showed no significant change in net growth between D0 and any other time-point (p=0.0937; Figure 5.12 A). There was no statistically significant difference between D0 and any other time-points on repeated sampling in the non-vaccinated volunteers in group 2 (p=0.3252; Figure 5.12 B).

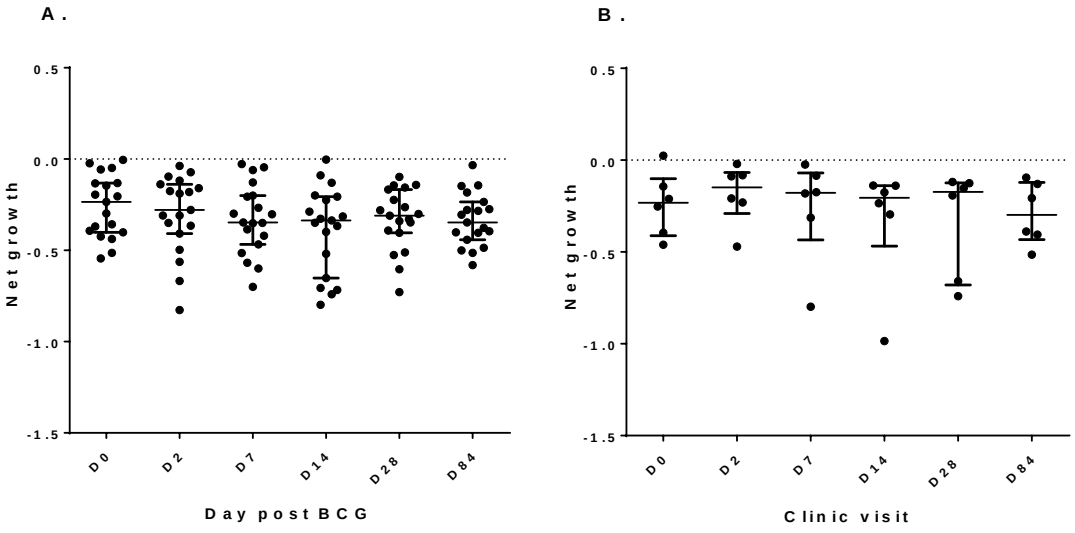


Figure 5.12 *E. coli* PBMC GIA net growth with ~ x10^3CFU/mL inoculum concentration. Each dot represents the mean of duplicate measures for each volunteer, median and interquartile range shown. A. net growth for group 1 (BCG vaccinated volunteers) and B. net growth for group 2 (non-vaccinated) volunteers. Non-parametric paired one-way ANOVA was performed. Net growth (Log10(Sample CFU/mL ÷ Control CFU/mL))

5.5.3 K. pneumoniae

All 35 volunteers stored PBMCs were inoculated and incubated in the GIA. As with *E. coli*, three inoculum concentrations were assessed. The first four volunteer PBMCs were inoculated with 2.45 x 10^5CFU/mL inoculum. Net growth following 1 hour incubation is shown in Figure 5.13 A and B. There was no significant change over time pre-vaccination at D0 and for any post vaccination time-point for volunteers in group 1 (p=0.8909; Figure 5.13 A).

Concerned that the inoculum concentration was too high the PBMCs from the next four volunteers were incubated with a reduced inoculum concentration of 2.29 x 10^4CFU/mL. No significant difference was seen between D0 and any subsequent post-vaccination time-points for volunteers in group 1 (p=0.2819; Figure 5.13 C). Concerned that the inoculum concentration remained too high and overwhelming any killing effect seen (Figure 5.13 C and D), all subsequent volunteers were analysed using the lowest mean inoculum concentration possible in order to calculate CFU/mL using the protocol as defined.

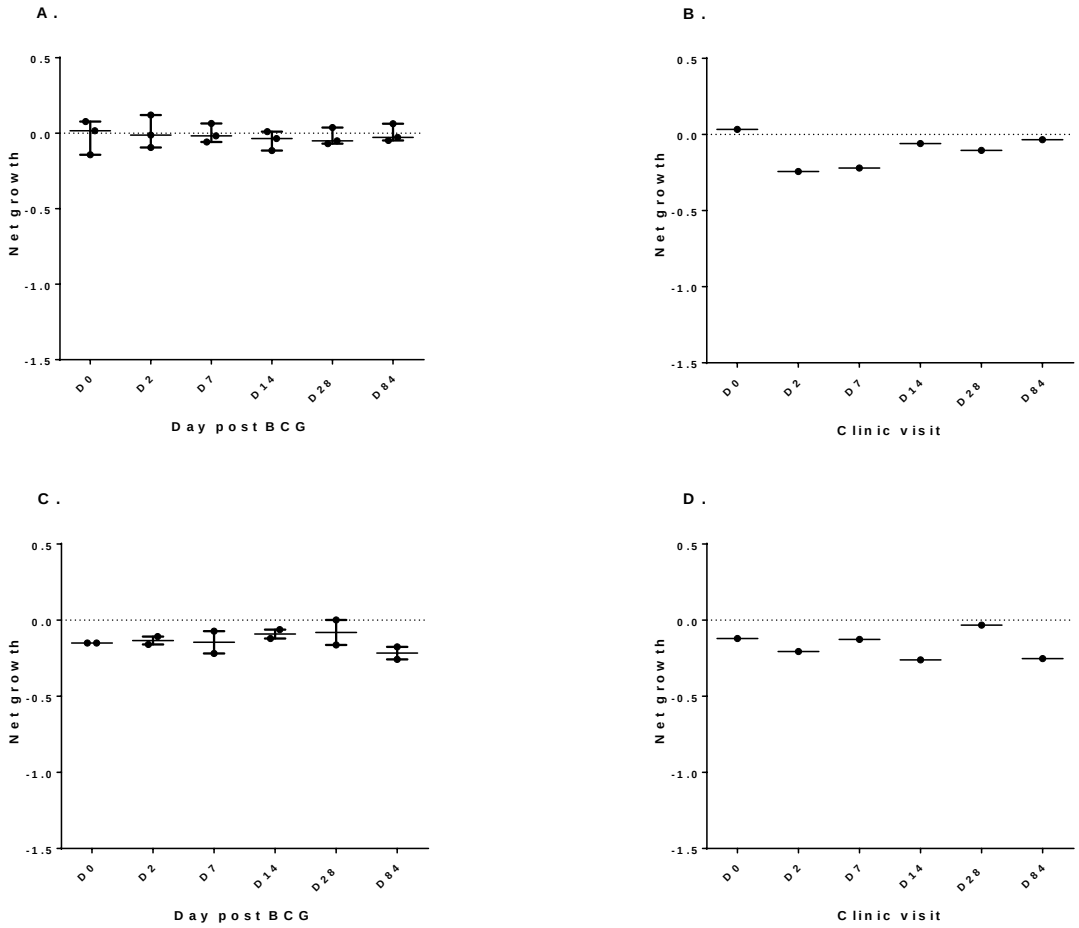


Figure 5.13 *K. pneumoniae* PBMC GIA net growth with ~x10^5 and ~x10^4 CFU/mL inoculum. Each dot represents the mean of duplicate measures for each volunteer, median and interquartile range presented. A. net growth for group 1 (BCG vaccinated volunteers) and B. net growth for group 2 (non-vaccinated) volunteers inoculum concentration ~ x10^5CFU/mL. C. net growth for group 1 (BCG vaccinated volunteers) and D. net growth for group 2 (non-vaccinated) volunteers inoculum concentration ~ x10^4CFU/mL. Non-parametric paired one-way ANOVA was performed. Net growth (Log10(Sample CFU/mL ÷ Control CFU/mL))

All 27 sets of remaining volunteer PBMCs were inoculated with a mean inoculum concentration of 2.12 x 10^3CFU/mL (range 1.59 x 10^3 – 2.38 x 10^3CFU/mL). Net growth of

K. pneumoniae at each time-point for both group 1 and group 2 volunteers is demonstrated in Figure 5.14.

The net growth of *K. pneumoniae* following 1 hour incubation with the PBMCs from group 1 volunteers (BCG vaccinated) showed no significant variability in net growth over time (p=0.0871; Figure 5.14 A). There was no statistically significant difference between time-points on repeated sampling in the non-vaccinated volunteers in group 2 (p=0.1562; Figure 5.14 B).

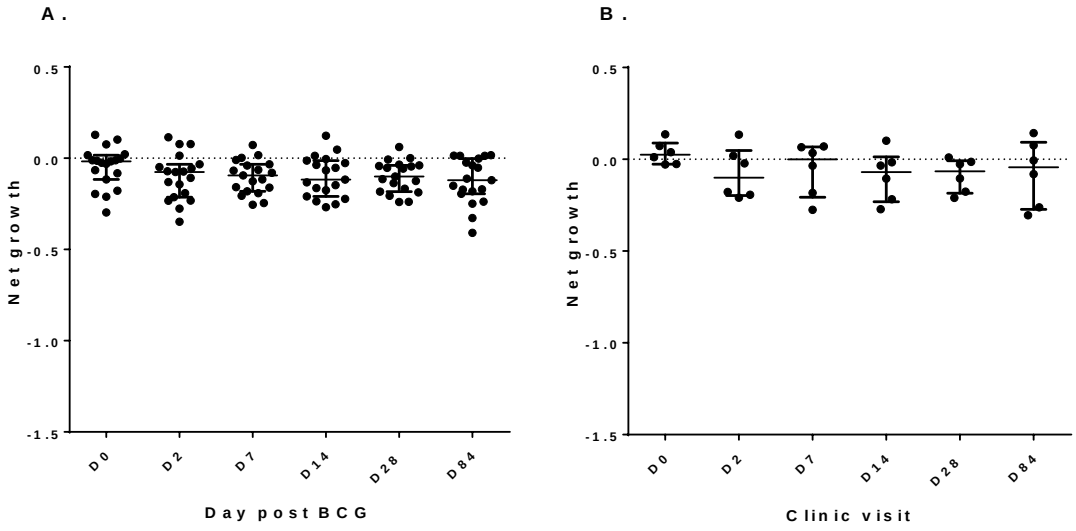


Figure 5.14 *K. pneumoniae* PBMC GIA net growth with ~x10^3 CFU/mL inoculum concentration. Each dot represents the mean of duplicate measures for each volunteer, median and interquartile range shown. A. net growth for group 1 (BCG vaccinated volunteers) and B. net growth for group 2 (non-vaccinated) volunteers. Non-parametric paired one-way ANOVA was performed. Net growth (Log10(Sample CFU/mL ÷ Control CFU/mL))

5.5.4 S. agalactiae

All 35 volunteers stored PBMCs were inoculated and incubated in the GIA. Net growth of *S. agalactiae* at each time-point for both group 1 and group 2 volunteers is demonstrated

in Figure 5.15. Mean inoculum concentration was 3.17 x 10^4CFU/mL (range 1.75 x 10^4 – 4.12 x 10^4CFU/mL).

The net growth of *S. agalactiae* following 1 hour incubation with the PBMCs from volunteers in group 1 demonstrated no trend towards a decrease or increase in net growth between D0 (pre-vaccination) and any other time-point (p=0.4646; Figure 5.15 A). There was no statistically significant difference between time-points in non-vaccinated volunteers in group 2 (0.9891; Figure 5.15 B).

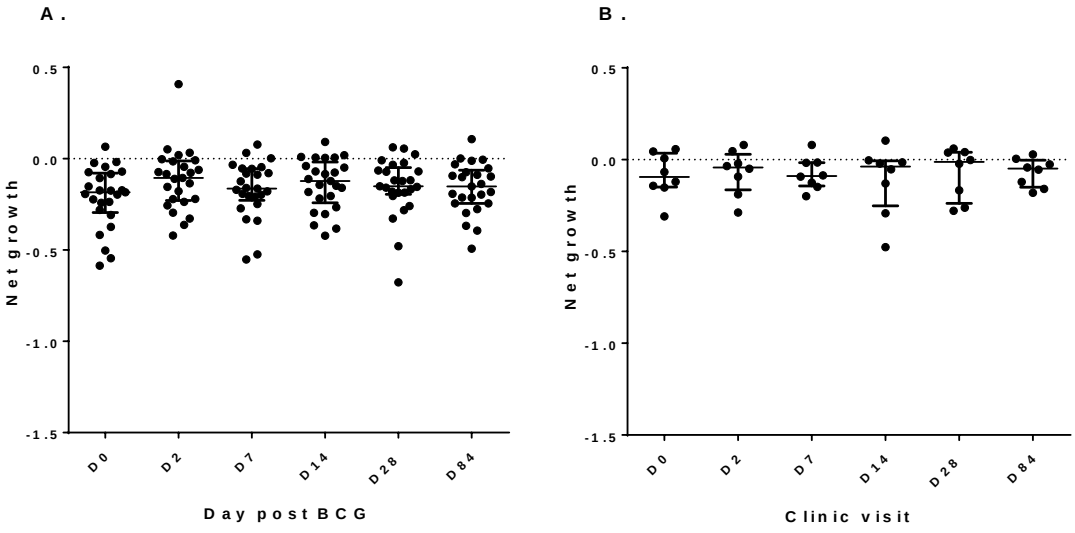


Figure 5.15 *S. agalactiae* PBMC GIA net growth with ~x10^4 CFU/mL inoculum concentration. Each dot represents the mean of duplicate measures for each volunteer, median and interquartile range shown. A. net growth for group 1 (BCG vaccinated volunteers) and B. net growth for group 2 (non-vaccinated) volunteers. Non-parametric paired one-way ANOVA was performed. Net growth (Log10(Sample CFU/mL ÷ Control CFU/mL))

5.6 Discussion

Using the whole blood *in-vitro* GIA as a functional measure of the 'sum of the parts' of the non-specific effects of BCG vaccination in a healthy UK adult population, with the four defined pathogens (*S. aureus*, *E. coli*, *K. pneumoniae* and *S. agalactiae*), I was unable to demonstrate any effect of recent BCG vaccination on inhibition of bacterial growth. As discussed in Chapter 4, there are several possible explanations for this including but not limited to:

a) The possibility that BCG vaccination does not exert a non-specific effect
b) BCG vaccination does not exert a non-specific effect in an UK adult study population
c) The non-specific effect of BCG is too low in magnitude to be detectable using this methodology and sample size, and vulnerable to the impact of confounders such as bacterial inoculum batch effect.
d) The whole blood *in-vitro* GIA does not have sufficient sensitivity to detect small improvements in control of bacterial growth in this study population or was insufficiently optimised.
e) The spectrum of pathogens chosen, whilst providing robust gram positive and gram negative coverage, may have been insufficient to target all causes of respiratory infection and fever in an infant cohort which would include: Measles virus, Respiratory Syncytial Virus (RSV), Influenza virus, Rotavirus, *Haemophilus influenzae*, *Streptococcus pneumoniae*, *Salmonella typhi* and *paratyphi*, and fungal infections.

By refining the bacterial GIA using PBMCs rather than whole blood, the aim was to limit factors influencing GIA output such as inoculum batch effect and fluctuations in volunteer Hb, in order to ascertain whether a subtle non-specific effect of BCG vaccination was being masked by such variables. As with the whole blood GIA, some optimisation steps were

necessary prior to the handling of stored volunteer PBMCs, with additional improvements to be made during sample processing.

One of the most important refinements to be made was removal of the water-lysis step. The removal of this simple step significantly improved the overall processing time of the assay and therefore some of the logistical considerations. There was no obvious difference in resuspension of the bacterial pellet in water or PBS for any of the four pathogens, and therefore for consistency across pathogens and assays, resuspension in water was chosen. As PBS is isotonic, the use of water would also allow for the lysis of internalised bacteria from PBMCs to maximise viable bacterial recovery. Ultimately it improved assay delivery by approximately one hour.

Similar to the direct MGIA, in undertaking the PBMC GIA for the four defined pathogens, after thawing the PBMCs, no additional antibiotic was used in any of the resuspension media in order to ensure no artificial lowering of bacterial recovery[140]. During development of the direct MGIA method, removal of the standard media antibiotics (Penicillin/Streptomycin) initially resulted in the effect of antigen specific T cells on mycobacterial growth being overwhelmed in the assay by the number of mycobacteria present, and subsequently it was discovered that a much lower MOI was necessary for the detection of a specific BCG vaccine effect[140, 218]. In this study, it was important to try to ascertain the optimal bacterial inoculum concentration, seeking a sufficient inoculum concentration neither to overwhelm nor underwhelm the assay. In addition to this, it was important to identify the optimal concentration of PBMCs to use per well. A checkerboard of bacterial inoculum and PBMC concentrations were used to explore this. The main limitation to this approach, as with the optimisation of the whole blood GIA, was that this was a static time-point and it was impossible to know what PBMC concentration and bacterial inoculum concentration pairing would be optimal to see any potential non-specific effects caused by BCG vaccination. This could only fully be explored once

processing the stored volunteer samples. However, the checkerboard would provide potentially useful information regarding bacterial growth kinetics in PBMC rich media, impact of incubation period following inoculation of PBMCs and also ascertain any significant differences in bacteria which may pose a challenge to the logistical considerations of performing the assay. Each checkerboard of experiments showed that the biggest influence on bacterial recovery regardless of incubation time was bacterial inoculum concentration, and with the exception of *S. aureus*, all bacteria reached the same CFU/mL plateau following 24 hour incubation regardless of starting inoculum concentration. There was a suggestion with the *S. agalactiae* GIA, that an effect of PBMC concentration was potentially seen following the 4 hour incubation, however this was subtle and could have been due to technical/operator factors. The recovery of *S. aureus* demonstrated the greatest separation in recovery at different incubation times based on the PBMC concentration and inoculum concentration, but again was most influenced by the starting inoculum and regardless of starting PBMC concentration, bacteria multiplied in a steady fashion aiming to plateau at least 2 Log higher in concentration. It is possible that a longer incubation period would have demonstrated that all starting inoculum concentrations of *S. aureus* would reach a similar plateau as was the case with the other three bacteria. With no clear optimal PBMC concentration, and being sympathetic to the limited number of stored volunteer PBMCs, it was decided to proceed with 1 x 10^6cells/well, and a lower CFU/mL (~ x 10^5CFU/mL or ~ x 10^4CFU/mL for *S. agalactiae*), with the potential to lower the CFU/mL of bacterial inoculum depending on the emerging results.

In order to negate the batch effect in each volunteer, all time-points for each volunteer were processed at the same time and from the same bacterial inoculum. Calculation of volume of work and time involved in processing the samples meant that it was possible to process four volunteers at a time. Therefore volunteers were processed in order of

enrolment with three group 1 volunteers and one group 2 volunteers per batch to reduce processing bias.

There was a significant decrease in net growth of *S. aureus* from the PBMC GIA seen between D0 and D7 in the group 1 (BCG vaccinated) volunteers which was not seen in the group 2 (non-vaccinated) volunteers. This finding was extremely interesting as it suggests a potential biological non-specific effect of BCG vaccination which cannot be explained by batch effect or technical factors as processing conditions were kept the same and each time-point for each volunteer was processed from the same inoculum. The additional increase in sample size – by combining both phase 1 and phase 2 – may also have increased the ability to see a small but significant improvement in control of bacterial growth following vaccination. However when adjusted for multiple variables including age, sex, colonisation status and FBC parameters, this potential improved control in net growth disappeared.

In contrast, results of net growth of the other gram positive bacteria *S. agalactiae* from the GIA did not show any significant improved control of bacterial growth following vaccination with BCG. There are many possible reasons for this including: no non-specific effect of BCG on *S. agalactiae*, the inoculum concentration remained too high, therefore the MOI overwhelmed the PBMCs in the assay, concentration of PBMCs per well was too low, or the GIA incubation period was too short. Whilst is was not possible to explore this within the time-frame of this study and the number of PBMCs available, it would be interesting to explore the effect of changing some of these parameters on the outcome. Within the parameters defined in the GIA protocol and using the spiral plater to quantify CFU/ml of recovered of *S. agalactiae,* it was just at the limit of detection, as was the case with the other three pathogens. Therefore successful quantification of recovered CFU/mL using this method but with a lower inoculum concentration would have been challenging and required further optimisation of the assay. The use of the BACTEC FX system here

could potentially navigate the challenge of using ultra low concentrations of bacterial inoculum as it could potentially be able to detect much lower concentrations than the spiral plating method. By calculating a standard curve and estimating the upper and lower limit of detection, this validated system could provide a more accurate readout but further work would need to be undertaken to explore this possibility.

I was concerned that for both gram negative pathogens (*E. coli* and *K. pneumoniae*), at an inoculum concentration of ~ x 10^5CFU/mL and ~ x 10^4CFU/mL, they appeared to overwhelm the assay with very little discrimination between volunteers and net growth, and little or no inhibition of bacterial replication even at baseline. Therefore, the bacterial inoculum was reduced to the lowest possible concentration, ~ x 10^3CFU/mL, which would also allow the quantification of net growth following incubation, using the defined spiral plating method. Whilst there was no significant reduction in net growth of *E. coli* or *K. pnuemoniae* from the PBMC GIA, there was a downward trend following vaccination with BCG in group 1. One way ANOVA analysis for *E. coli* PBMC GIA, although approached (p=0.0937), did not quite reach a significant decrease in bacterial net growth, which was also the case with the *K. pneumoniae* PBMC GIA (p=0.0871). As with the *S. agalactiae* PBMC GIA, there would be significant scope to optimise the assay further looking at inoculum concentrations, PBMC concentrations, incubation times and the utility of the BACTEC FX system. In addition, repeating the assay with the lower concentration for the initial 8 volunteer samples, initially done with a higher inoculum concentration, by increasing the sample size could reveal a significant difference in net growth of the bacteria following BCG vaccination as with the *S. aureus* PBMC GIA.

5.7 Conclusions

- Adaptation of the *in-vitro* GIA using cryopreserved PBMCs as opposed to whole blood was able to navigate the issue of batch effect by enabling the inoculation of volunteer samples,

obtained from different time-points, to be processed at the same time from the same inoculum under the same conditions

- The PBMC GIA was a more time-efficient technique
- There appears to be a signal of improved control of growth of *S. aureus* by PBMCs of BCG vaccinated volunteers 7 days after vaccination, but further work is necessary to validate this finding and potential mechanism
- Further optimisation of the PBMC GIA assay is required.

6 Immunological results

6.1 Statement of Authorship

Ex-vivo PPD-specific IFN-γ ELISpots were performed by D Wright, R Tanner and S Harris. BCG IgG ELISA and *S. aureus* whole bacteria ELISAs were performed by M Wilkie. *S. aureus* IgG1 specific clumping factor B (ClfB) ELISAs were performed by M Wilkie and A Flaxman. Direct MGIAs were performed by M Wilkie, S. Harris and R. Tanner. Cytokine stimulation assays were performed by M Wilkie and R Tanner. Cytokine ELISA optimisation was performed by M Wilkie and study samples processed by M Wilkie and A Jacobs. Data analysis was performed by M Wilkie.

6.2 Introduction

Whilst the aim of this study was to evaluate the non-specific effects of BCG vaccination on the growth of four selected pathogens involved in neonatal sepsis, it was important to also have an understanding of the antigen-specific immunological responses induced by BCG vaccination using both conventional approaches such as *ex-vivo* PPD-specific IFN-γ ELISpot responses; but also by using assays which may better correlate with protection such as the direct MGIA.

At the point of birth, the neonatal immune system begins a significant transformation due to the sudden removal of the infant from the sterile, protective environment of the uterus, to the pathogen rich environment which is the 'outside' world[219]. Knowledge of the neonatal immune response is incomplete but we know there are key differences between it and the adult immune response[220]. Neonatal immunity is driven predominantly by the need to protect the neonate/infant from bacterial and viral infections encountered at birth and in the short months thereafter, allow the natural colonisation of the infant by synergistic environmental microorganisms, and stave off potentially dangerous Th1 cell

responses which can lead to maternal/foetal reactions and spontaneous abortion[219, 221]. Th1 cellular immune responses are necessary for viral and bacterial killing, particularly of intracellular pathogens, by preferentially encouraging the production of opsonising antibodies (immunoglobulin G1 (IgG1)), induction of cellular cytotoxicity, and macrophage activation[222]. Th2 cellular immune responses promote the production of immunoglobulin E (IgE) and immunoglobulin G4 (IgG4) and stimulate eosinophil development [222]. In comparison, the Th17 subset have been shown to be central to the host defence against pathogens at the mucosal level, particularly extracellular pathogens and fungi, but also has a role in autoimmune disease[223]. Neonates have a Th2 dominant cellular immune response with down regulation of Th1 cellular responses[224], and it is well documented that infants have lower defence mechanisms against intracellular pathogens with lower INF-γ production[225, 226]. A robust Th1 cellular immune response is vital in the host control of mycobacterial infection [227]. In adults, BCG has been shown to induce a Th1 cellular response[228-230] and in infants, when given at birth, BCG is able to induce a memory Th1 type response[231], but when BCG vaccination is delayed, the Th1/Th17 response is reduced[232].

The role of antibodies in protection against TB is unclear; however there is growing interest in the role they may play[233]. We know that antibodies are key players in the humoral immune systems defence against a variety of different pathogens, through functions such as neutralisation of infective pathogens, assistance with phagocytosis and antibody dependent complement and cellular cytotoxicity. The antibody repertoire of an adult is significantly greater than that of a neonate[234], due to the increasing pathogen exposure following birth. It is possible that pre-existing specific Ig levels against pathogenic bacteria used in the GIA may confound the GIA results in an adult study cohort.

There is a growing body of evidence that BCG induces training of monocytes and to a lesser degree NK cells, and this 'training' may be part of the immunological mechanisms

underpinning any non-specific effects of BCG[134, 135, 139]. It was important to demonstrate evidence of training in the clinical study reported here, through measurement of key cytokines produced by innate cells, following a secondary, *in-vitro* PBMC stimulation with agents such as *C. albicans*, *S. aureus*, PPD, and *E. coli* lipopolysaccharide (EC-LPS). An absence of this training effect might explain the lack of a strong effect in the GIA.

6.3 Aim of chapter

The aims of this chapter were to:

- Evaluate the antigen-specific cellular and humoral responses induced by BCG SSI in this study population
- Evaluate specific BCG SSI vaccine induced mycobacterial growth inhibition using the PBMC direct MGIA and to determine whether this response correlates with whole cell BCG total IgG and PPD responses.
- Evaluate total IgG response to *S. aureus* and evaluate whether these responses correlate with bacterial growth in the GIA
- Evaluate the non-specific effects of BCG on training of innate immunity through detection of IFN-γ, TNF-α, IL-6, , IL-1β, IL-10 and IL-1ra in PBMCs from group 1 volunteers (BCG SSI vaccinated) following a secondary in-vitro stimulation with heat killed *S. aureus* (HKSA), heat killed *C. albicans* (HKCA), EC-LPS, and PPD

6.4 PPD-specific ex-vivo IFNγ Enzyme-linked Immunospot assay (ELISpot)

Ex-vivo PPD-specific IFN-γ ELISpot responses were assessed at baseline (D0) and at D7, D14, D21, D28 and D84 in all volunteers. Data was blinded until the final schedule of volunteers for each phase had reached D84. ELISpots were performed on fresh PBMCs. Combined results (phase 1 and phase 2 of enrolment) for group 1 and group 2 are shown in Figure 6.1. PPD ELISpot responses were tested for normality using Shapiro-Wilk normality test. Not all passed, therefore non-parametric, paired, One-way ANOVA (Friedman test) with, where appropriate, post hoc Dunn's multiple comparisons test was performed comparing the mean of D0, with the mean of all other time-points. Five volunteers in group 1 and one from group 2 were excluded from the statistical analysis due to missing time-points.

Volunteers in group 1 demonstrated a significant increase in SFC/10^6PBMC which peaked at D7 ($p<0.0001$; Figure 6.1), and was sustained at D14 ($p<0.0001$; Figure 6.1) and D28 ($p=0.0001$; Figure 6.1). There was no significant change in responses over time in group 2 ($p=0.4209$; Figure 6.1).

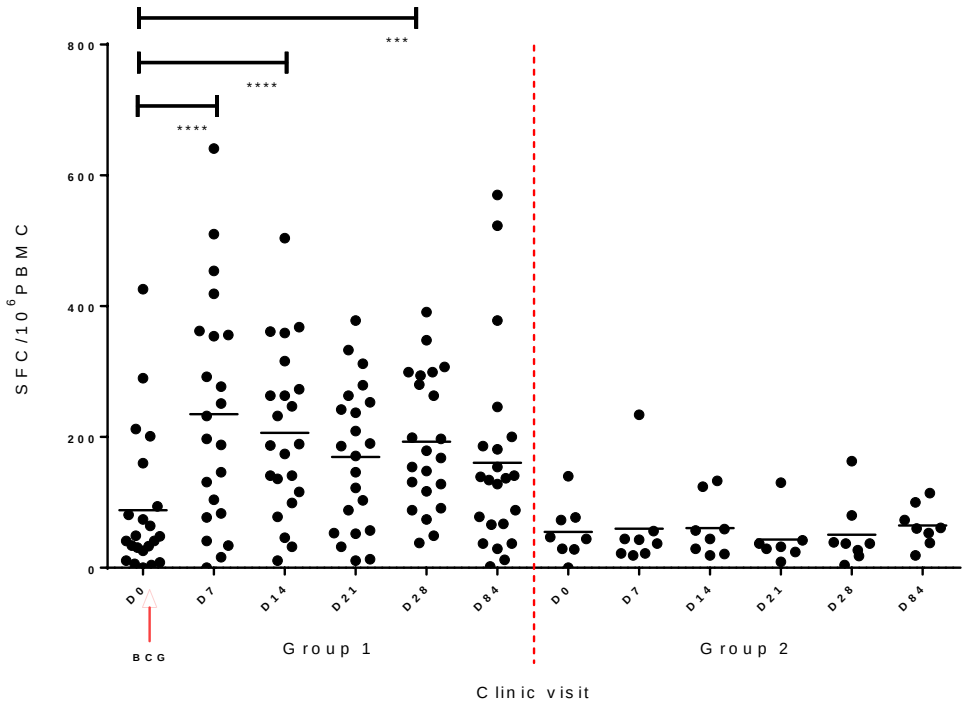


Figure 6.1 *Ex-vivo* PPD-specific IFN-γ ELISpot responses in study volunteers (phase 1 and phase 2 combined) split by Group1 (BCG SSI vaccinated n=27) and Group 2 (non-vaccinated n=8). Output is measured as spot forming cells (SFC)/10^6PBMC. Each dot represents background subtracted mean of triplicate values for each volunteer. Median line represented at each time-point for each group. Paired, non-parametric, one-way ANOVA with Dunn's multiple comparisons test was performed where *** signifies a p≤0.001 and **** signifies a p≤0.0001.

Further study was made by splitting group 1 volunteers by phase of enrolment, therefore by batch of BCG SSI used for vaccination. This was important given that the first batch used (batch no. 114015A), from phase 1, exhibited variability in dose from vial to vial on plating; and the second batch used (batch no. 114022A), from phase 2, was used during the 6 month expiry extension date granted by PHE during the global BCG shortage. Data is shown in Figure 6.2. Three volunteers from phase 1 and two from phase 2 were excluded from statistical analysis due to missing time-points.

Volunteers enrolled in phase 1 demonstrated significant increases in SFC/10^6PBMC peaking between D0 and D14 ($p<0.0001$; Figure 6.2). Volunteers in phase 2 demonstrated a significant increase in SFC/10^6PBMC between D0 and D7 only ($p=0.0206$; Figure 6.2).

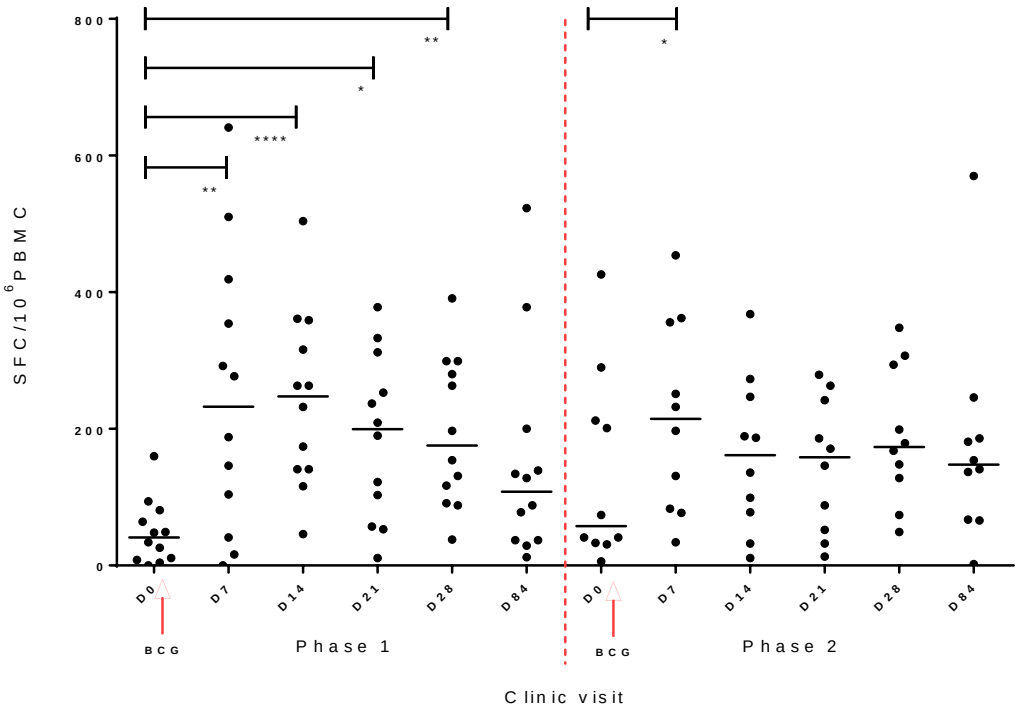


Figure 6.2 PPD-specific ex-vivo IFN-γ ELISpot responses in study volunteers in group 1 (BCG vaccinated) split by phase 1 (BCG SSI batch no. 114015A n=15) and Phase 2 (BCG SSI batch no. 114022A n=12). Output is measured as spot forming cells (SFC)/10^6PBMC. Each dot represents background subtracted mean of triplicate values for each volunteer. Median line represented at each time-point for each phase. Paired, non-parametric, one-way ANOVA with Dunn's multiple comparisons test was performed where * signifies a p≤0.05, ** signifies a p≤0.01, *** signifies a p≤0.001 and **** signifies a p≤0.0001.

Mann Whitney tests were performed on PPD-specific ELISpot responses at D0, D7, D14 and D28 between group1 (BCG vaccinated) volunteers enrolled in phase 1 and phase 2. There was no significant difference between phase 1 and phase 2 volunteers at any of the time-points. (D0 p=1745, D7 p=0.9229, D14 p=0.1747 and D28 0.9601).

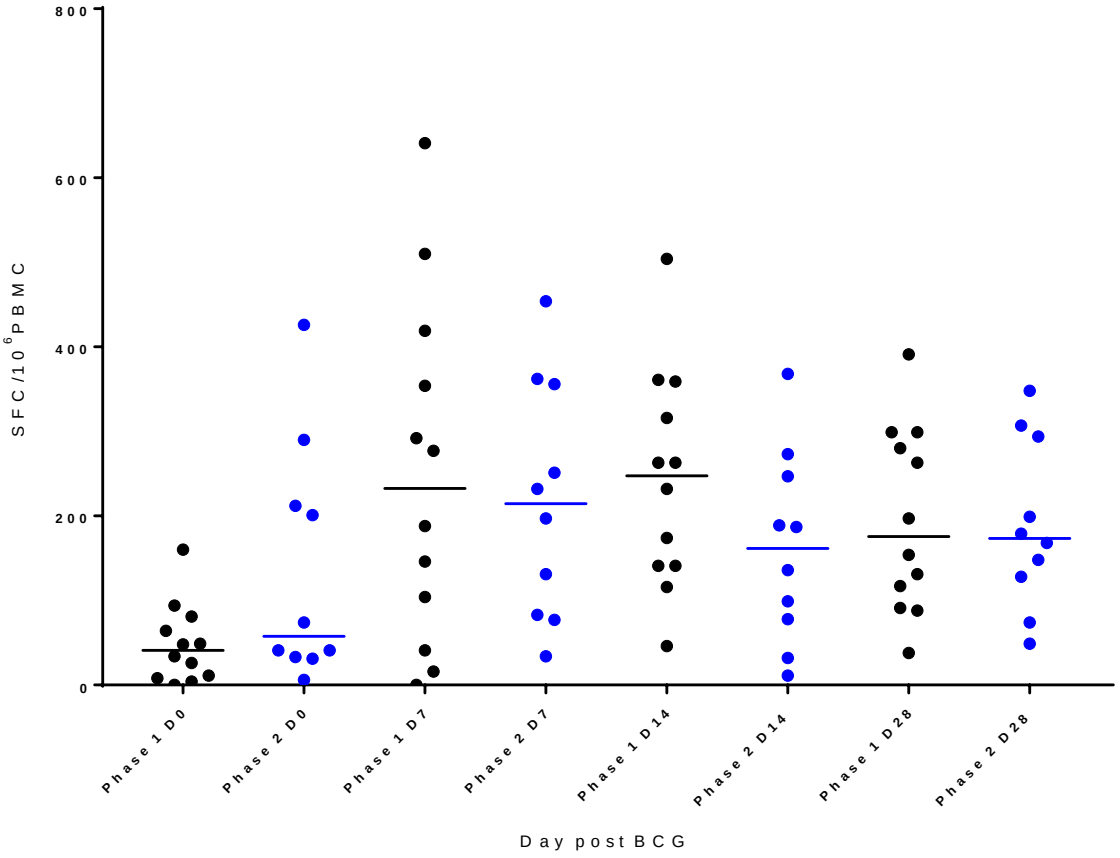


Figure 6.3 Direct comparison of PPD-specific ELISpot responses for volunteers in group 1 (BCG vaccinated) between phase 1 and phase 2. Comparison made at D0, D7, D14 and D28. Mann Whitney test performed at each time-point between phase 1 and phase 2. Each dot represents the background subtracted mean of triplicate measures for each volunteer. Median line represented.

6.5 Direct mycobacterial growth inhibition assay (MGIA)

The effect of BCG SSI vaccination on the *ex-vivo* control of BCG growth in a PBMC direct MGIA was performed as per the technique developed previously[140, 186]. Stored PBMCs were used from D0 (baseline), D28 and D84 and inoculated with BCG. Following 96 hour incubation, samples were then transferred to the BACTEC MGIT system. Output was

measured as time to detection (TTD) and converted into net growth using a standard curve.

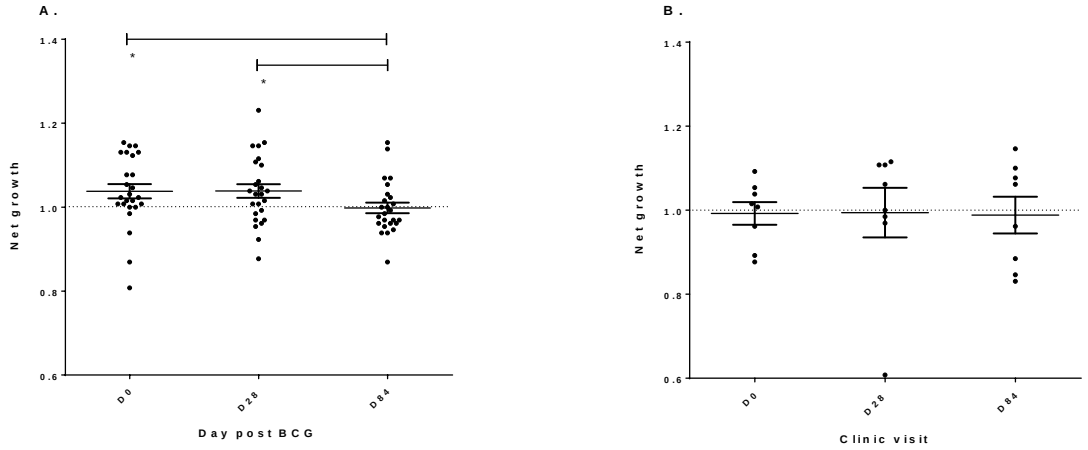


Figure 6.4 BCG direct MGIA – group 1 and group 2. Each dot represents the mean of duplicate measure for each volunteer. Shown is median and interquartile range and differences between time-points measured by Wilcoxon matched-pairs signed rank test where * signifies $p<0.05$. A. volunteers in Group 1 (BCG SSI vaccinated) at D0 (pre-vaccination), D28 and D84 post vaccination. B. volunteers in group 2 (non-vaccinated) on repeated sampling.

In volunteers in group 1 (Figure 6.4 A) there was a significant decrease in net growth between D0 and D84 ($p=0.0240$, Wilcoxon matched-pairs signed rank test) and D28 and D84 ($p=0.0372$, Wilcoxon matched-pairs signed rank test). There was no difference between D0 and D28 ($p=0.7259$, Wilcoxon matched-pairs signed rank test).

Volunteers in group 2 demonstrated no significant difference in net growth between any time-point. (D0 and D28 $p=0.7422$, D0 and D84 $p=0.9453$, and D28 and D84 $p=0.8438$ Wilcoxon matched-pairs signed rank test).

The impact of the batch of BCG SSI used was explored as before. Volunteers from phase 1 demonstrated a much wider range of responses at each time-point. Following BCG SSI

vaccination, there was a trend towards a decrease in net growth (correlating with improved control of BCG growth) but this did not reach statistical significance (p=0.0703, Wilcoxon matched-pairs signed rank test; Figure 6.5 A). Volunteers in phase 2 of enrolment had much less inter-volunteer variability and demonstrated a significant decrease in net growth between D0 and D84 (p=0.0322, Wilcoxon matched-pairs signed rank test; Figure 6.5 B).

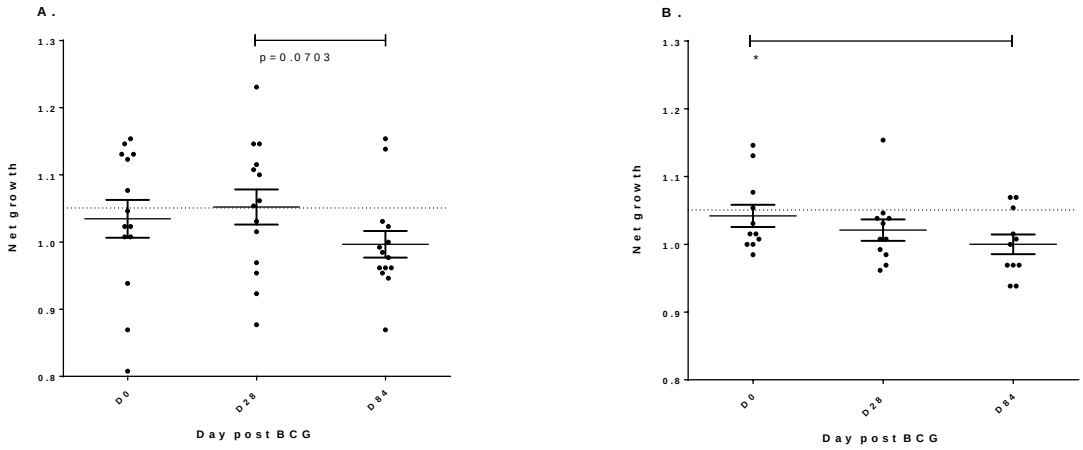


Figure 6.5 BCG direct MGIA – phase 1 and phase 2. Each dot represents the mean of duplicate measure for each volunteer. Shown is median and interquartile range and significant differences between time-points as measured by Wilcoxon matched-pairs signed rank test. A. volunteers from phase 1, group 1 (BCG SSI vaccinated) at D0 (pre-vaccination), D28 and D84 post vaccination. B. volunteers from phase 2, group 1 (BCG SSI vaccinated) at D0 (pre-vaccination), D28 and D84 post-vaccination.

6.6 Bacteria IgG Enzyme-linked immunosorbent assays (ELISA)

6.6.1 IgG BCG-specific ELISA

Whole mycobacteria BCG ELISA was performed on all volunteers from D0 and D28 serum samples and analysis of fold change performed. Volunteers in group 1 showed a significant increase in total IgG response to BCG at D28 post BCG SSI vaccination

(p=0.0002, Wilcoxon matched-pairs signed rank test). There was no significant increase at D28 in volunteers in group 2 (p=0.5469, Wilcoxon matched-pairs signed rank test) (Figure 6.6).

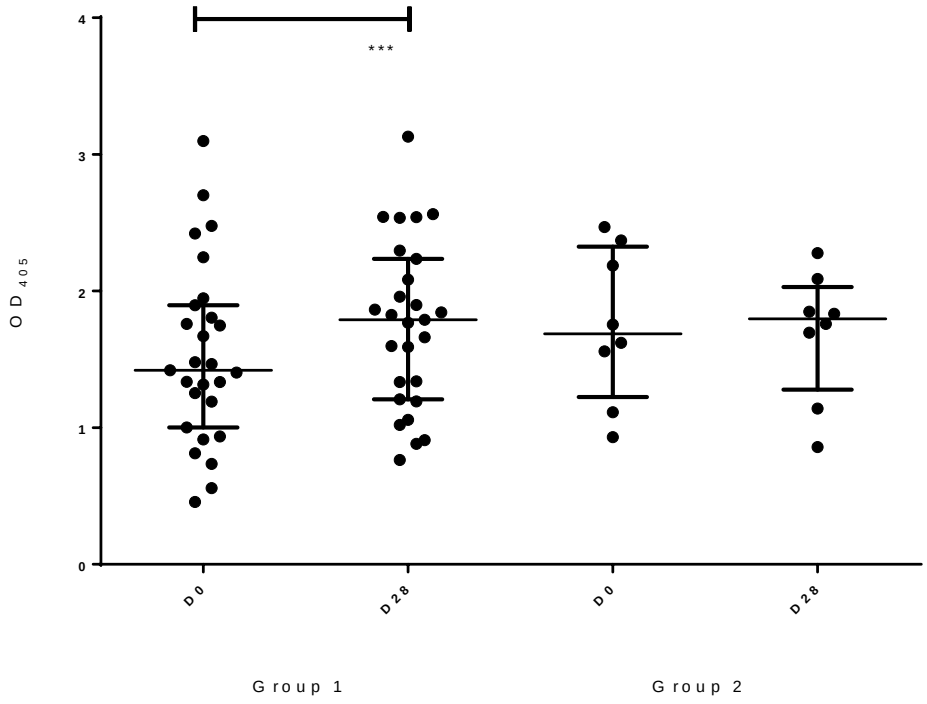


Figure 6.6 Total IgG response against whole bacteria BCG at D0 and D28 in group 1 (BCG SSI vaccinated) and group 2 (non-vaccinated). Each dot represents the mean of duplicates for each volunteer. Wilcoxon matched-pairs signed rank test performed.

Analysis of fold change between groups was performed (Figure 6.7). There was a significant difference between the fold change seen in group 1 compared with group 2 (p=0.0115, Mann Whitney test).

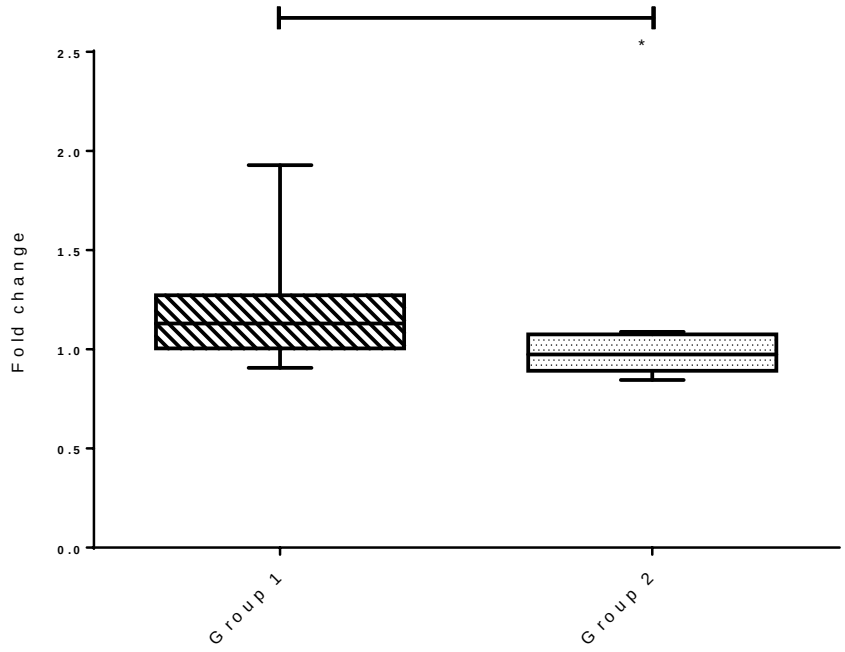


Figure 6.7 Fold change in total IgG response to whole bacteria BCG SSI for volunteers (combined phase 1 and phase 2) in group and 1 and group 2 between D0 and D28 as measured by optical density 405 (OD_{405}). Mann-Whitney test was performed.

6.6.2 S. aureus ELISA

Total IgG against whole bacteria SPA negative *S. aureus* and IgG1 subclass (4E3 clone) were performed on all volunteers from D0 serum samples. Evaluation of IgG1 subclass was performed as work in the *S. aureus* group had observed a bimodal distribution of low and high 4E3 binders in three cohorts which may have implications with individual immune response to infection (data not published).

6.6.2.1 Whole bacteria total IgG S. aureus ELISA

Total IgG against SPA negative *S. aureus* was performed on D0 stored serum samples on all volunteers. Responses were measured using OD_{405} and normalised from a standard curve from reference serum made available from the *S. aureus* group. OD_{405} readings from three

volunteers were below the range of the standard curve and therefore could not be interpolated. They were excluded from analysis. The data from all other volunteers are shown in Figure 6.8. Values did not pass normality test (Shapiro-Wilk normality test).

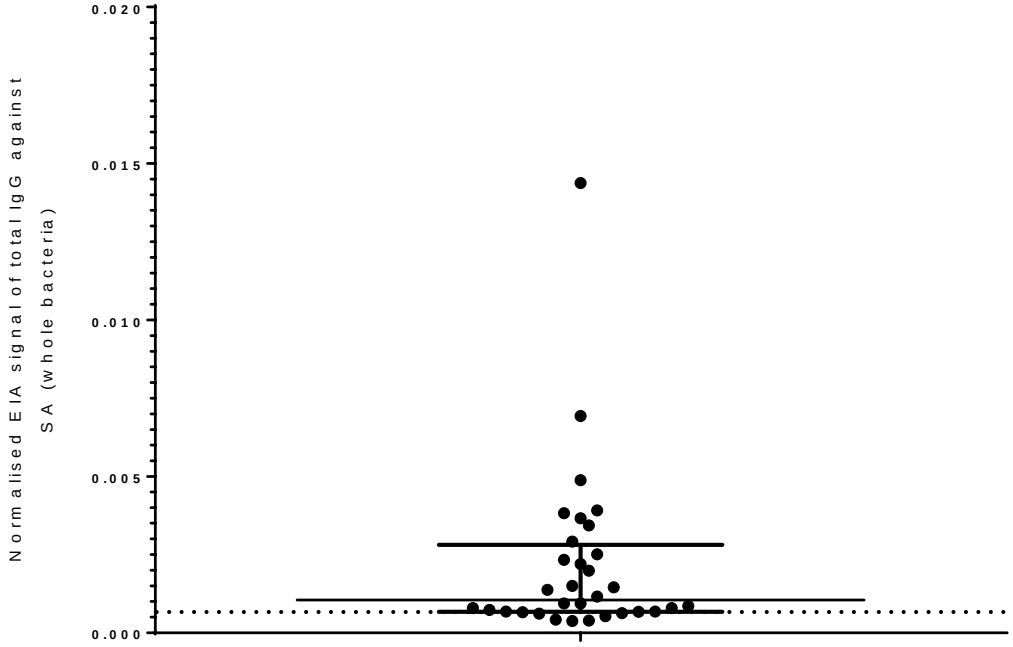


Figure 6.8 Normalised EIA signal of total IgG responses against whole bacteria *S. aureus* (SPA negative) in all study volunteers. Each dot represents the mean value of duplicates for each volunteer. Median with interquartile range is shown. Dotted line represents 1:200 dilution IgG1k negative control.

Separating responses by nasal swab carrier status is shown in Figure 6.9. There was no statistically significant difference in IgG levels between those volunteers who were nasal *S. aureus* carriers, compared with those volunteers who were not (p=0.1555, Mann Whitney test), however there was a trend towards those with the highest response having a positive *S. aureus* carrier status.

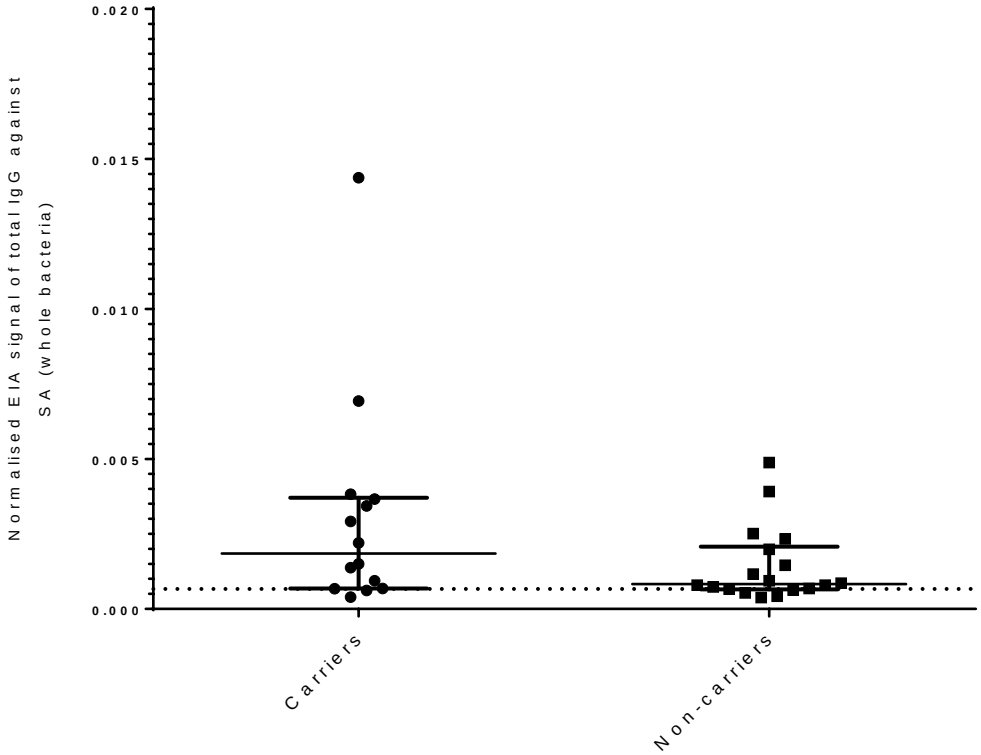


Figure 6.9 Normalised EIA signal of total IgG responses against whole bacteria *S. aureus* (SPA negative) in all study volunteers split by *S. aureus* nasal swab carrier status. Each dot represents the mean value of duplicates for each volunteer. Median with interquartile range is shown. Dotted line represents 1:200 dilution IgG1k negative control.

6.6.2.2 IgG1 4E3 S. aureus ELISA

IgG1 subclass (4E3 clone) responses to *S. aureus* clumping factor B (ClfB) were evaluated from D0 stored serum samples for all volunteers. Responses were measured using OD_{405} and normalised from a standard curve from reference serum made available from the *S. aureus* group. Results are shown in Figure 6.10. Values did not pass normality test (Shapiro-Wilk normality test).

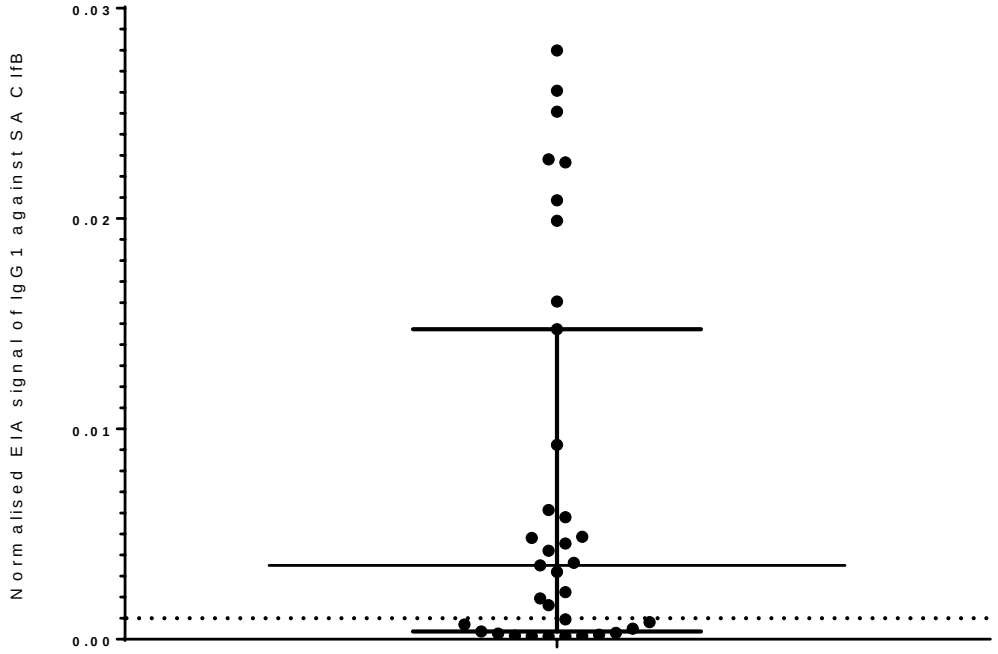


Figure 6.10 Normalised EIA signal of total IgG1 responses against *S. aureus* ClfB in all study volunteers. Each dot represents the mean value of duplicates for each volunteer. Median with interquartile range is shown. Dotted line represents 1:200 dilution IgG1k negative control.

Data where the IgG responses are separated by nasal swab *S. aureus* carrier status are shown in Figure 6.11. There was no statistically significant difference between those volunteers who were nasal *S. aureus* carriers, compared with those volunteers who were not (p=0.6535, Mann Whitney test), however there was a trend towards those with the highest response having a positive nasal *S. aureus* carrier status.

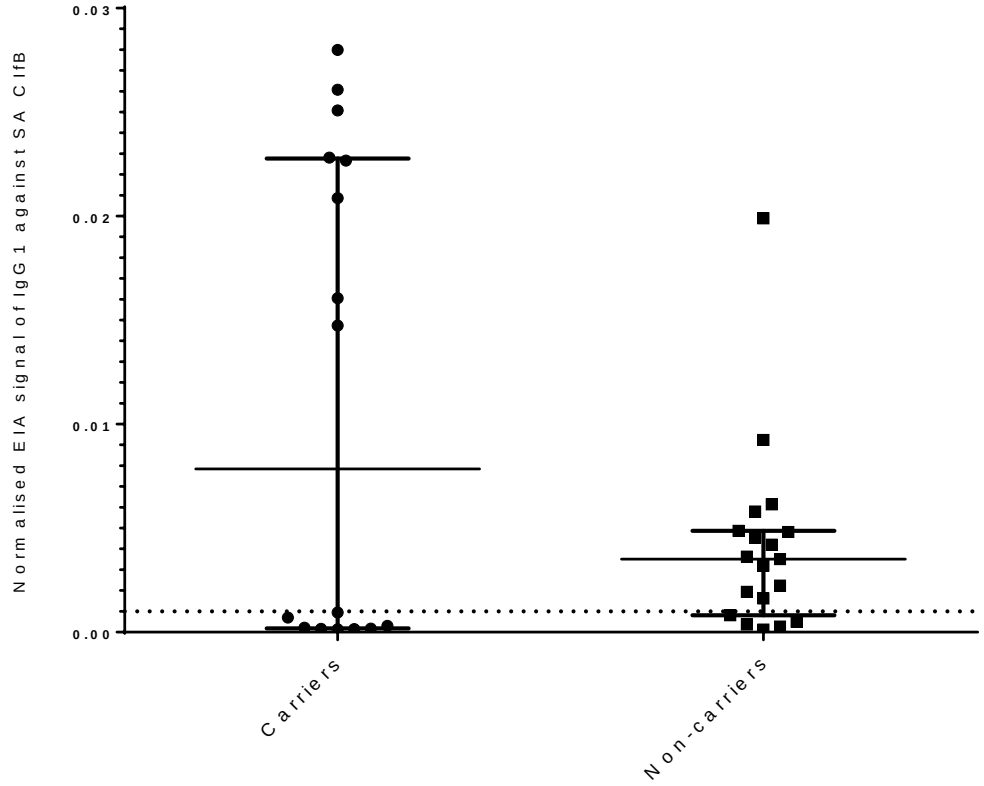


Figure 6.11 Normalised EIA signal of total IgG1 responses against *S. aureus* ClfB in all study volunteers split by *S. aureus* nasal swab carrier status. Each dot represents the mean value of duplicates for each volunteer. Median with interquartile range is shown. Dotted line represents 1:200 dilution IgG1k negative control.

Volunteers in each phase were split by low and high response to 4E3 binding status and are shown in Figure 6.12. The purpose of separation was to assess whether there was any correlation between a volunteers baseline bacterial killing ability from the whole blood GIA at D0 with 4E3 binding of *S. aureus* ClfB.

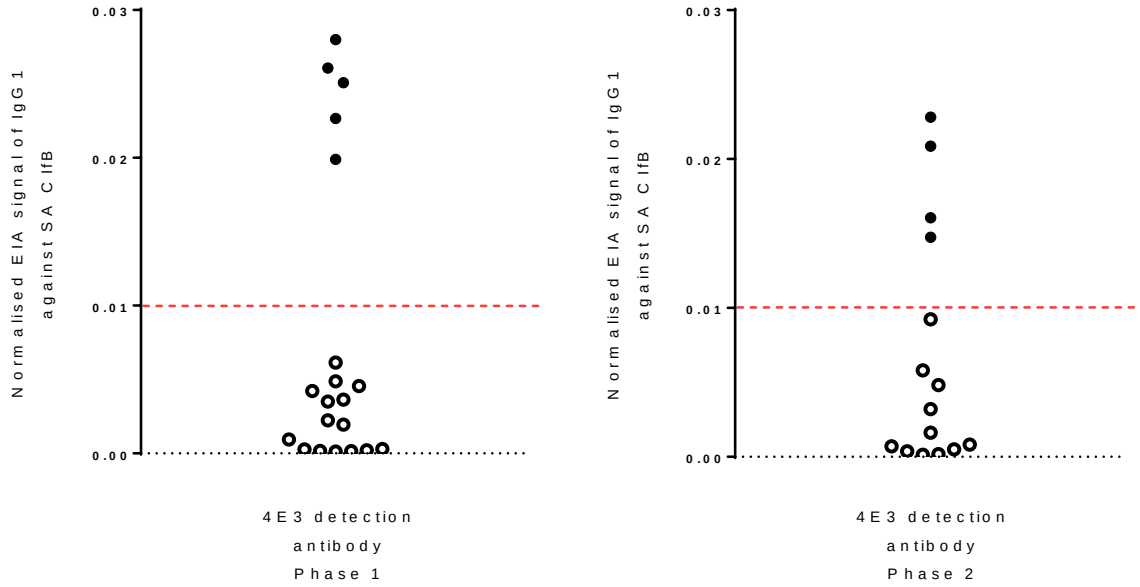


Figure 6.12 Normalised EIA signal of total IgG1 responses against *S. aureus* ClfB in all study volunteers split by phase of enrolment and divided in to low and high 4E3 responders. High responders are above dotted red line (each dot represents an individual volunteer), low responders lie below dotted red line (each circle represents an individual volunteer). Each dot/circle represents the mean of duplicate values for each volunteer.

Five volunteers enrolled in phase 1, and four volunteers from phase 2 were identified as having high binding affinity for 4E3 (Figure 6.12). Data from the S. aureus group had detected a bimodal distribution of responses in other cohorts and dictated the cut-off of 0.01 as demarcated by the red line.

Comparison of high and low 4E3 binding status with *S. aureus* recovery from the whole blood GIA at D0 is shown in Figure 6.13. Analysis was made with GIA bacterial recovery from all inoculum concentrations, ~ x 10^8, ~ x 10^7, and ~ x 10^6CFU/mL. Following a 1 hour incubation there was no difference seen between the high and low 4E3 binders (p=0.5995, p=0.1804 and p=0.2212, Mann Whitney test respectively). There was a significant difference seen following 24 hour incubation at ~ x 10^8CFU/mL inoculum

concentration (p=0.0499, Mann Whitney test Figure 6.13 A.). At an inoculum concentration of ~ x 10^7, and ~ x 10^6CFU/mL, there was no significant difference between low and high 4E3 binders following 24 hour incubation (p=0.6172 and p=0.6937, Mann Whitney respectively).

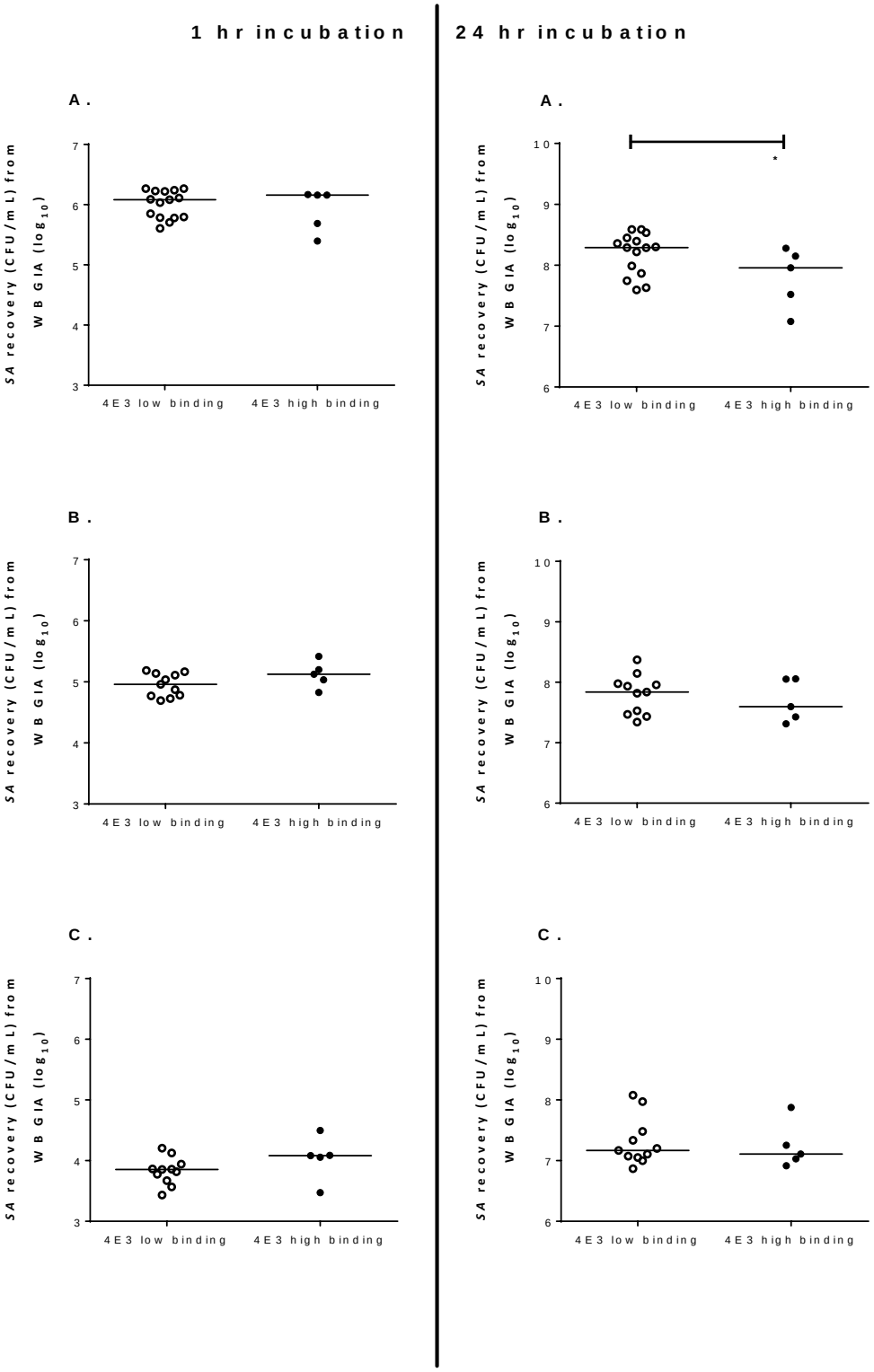


Figure 6.13 Evaluation of association of 4E3 binding status and *S. aureus* recovery from whole blood GIA at D0 (baseline) for volunteers in phase 1. 1hour and 24hour incubation represented. All inoculum concentrations evaluated (A. ~x10^8CFU/mL, B. ~x10^7CFU/mL and C. ~x10^6CFU/mL). Each circle represents a volunteer with low 4E3 binding and dot, a volunteer with high 4E3 binding. Bar denoting median shown.

I then went on to compare high and low 4E3 binding status with *E. coli* recovery from the whole blood GIA at D0, in order to assess any possible cross-reactivity[235, 236]. These data are shown in Figure 6.14. Following 1 hour incubation, there was a significant difference seen in bacterial recovery between high and low 4E3 binders at an inoculum concentration of ~ x 10^7CFU/mL (p=0.0187, Mann Whitney test). At an inoculum concentration of ~ x 10^6CFU/mL there was no significant difference demonstrated (p=0.1326, Mann Whitney test). There was no difference seen following 24 hour incubation (p=0.4970 and p=0.1627, Mann Whitney test). Notably, the volunteer who demonstrated the lowest net growth from the assay (TBT-038122) also had high binding for 4E3.

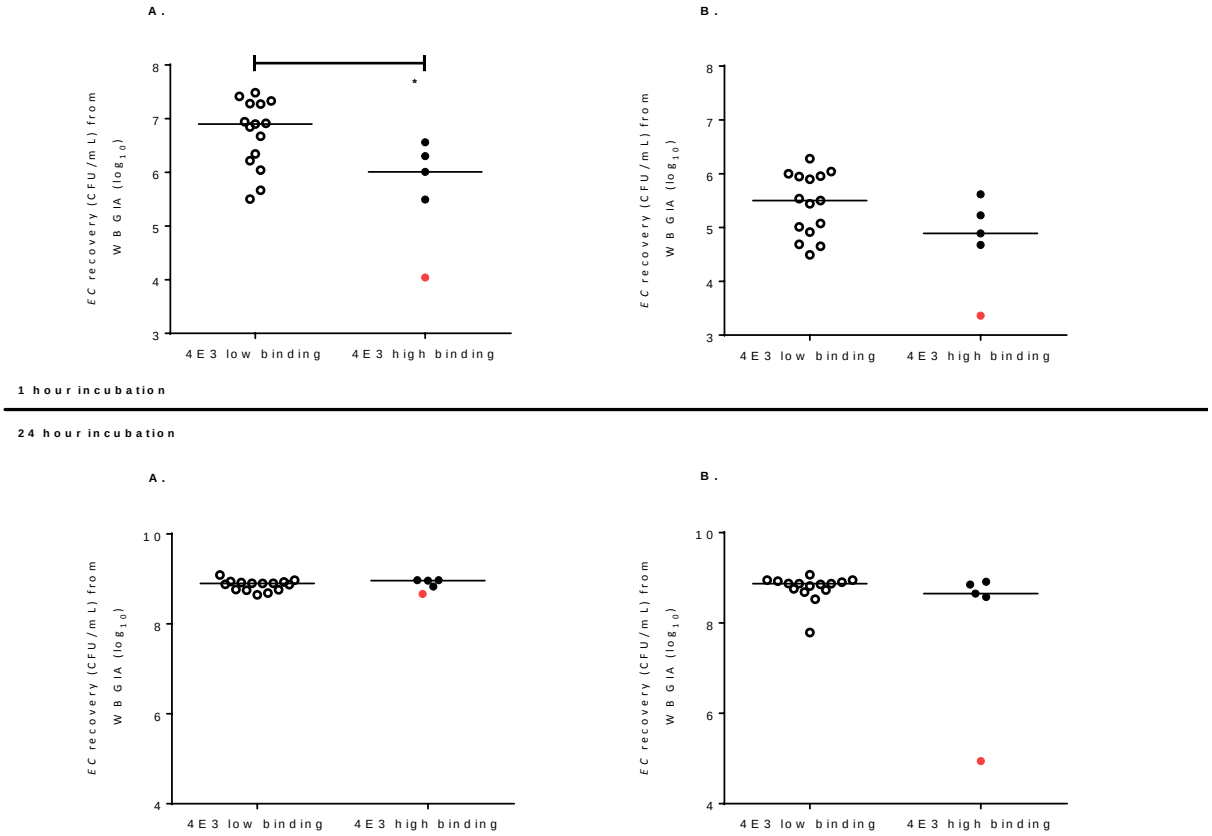


Figure 6.14 Evaluation of association of 4E3 binding status and *E. coli* recovery from whole blood GIA at D0 (baseline) for volunteers in phase 1. 1hour and 24hour incubation represented. Results from whole blood GIA using 1:10 and 1:100 inoculum concentrations shown (A. ~ x10^7CFU/mL and B. ~ x10^6CFU/mL). Each circle represents a volunteer with low 4E3 binding and dot, a volunteer with high 4E3 binding. Volunteer denoted by red corresponds with volunteer TBT-038122. Bar denoting median shown.

Comparison between *K. pneumoniae* and *S. agalactiae* recovery and 4E3 binding status was performed (Figure 6.15 and Figure 6.16 respectively). There was no association found following 1 hour incubation for recovery of either organism in phase 2 (*K. pneumoniae* inoculum concentration ~ x 10^8 CFU/mL p=0.7824; Figure 6.15 A, ~ x 10^7CFU/mL p>0.9999; Figure 6.15 B, and ~ x 10^6CFU/mL p=0.6608; Figure 6.15 C, Mann

Whitney test)(*S. agalactiae* ~ x 10^7CFU/mL p=0.8747 (Figure 6.16 A) and ~ x 10^6CFU/mL p=0.8498 (Figure 6.16 B) Mann Whitney test).

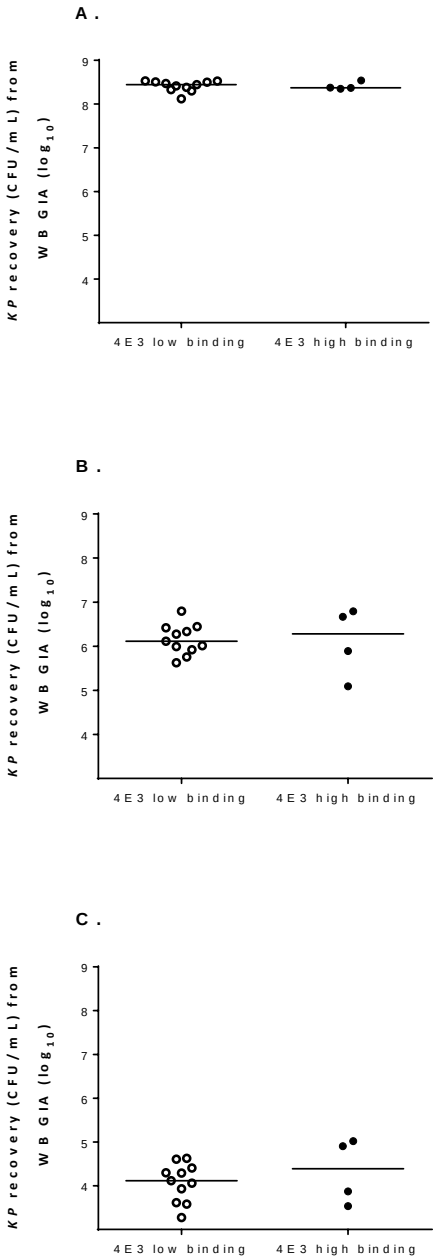


Figure 6.15 Evaluation of association of 4E3 binding status and *K. pneumoniae* recovery from whole blood GIA at D0 (baseline) for volunteers in phase 2. 1hour incubation represented. All inoculum concentrations evaluated (A. ~x10^8CFU/mL, B. ~x10^7CFU/mL and C. ~x10^6CFU/mL). Each circle represents a volunteer with low 4E3 binding and dot, a volunteer with high 4E3 binding. Bar denoting median shown.

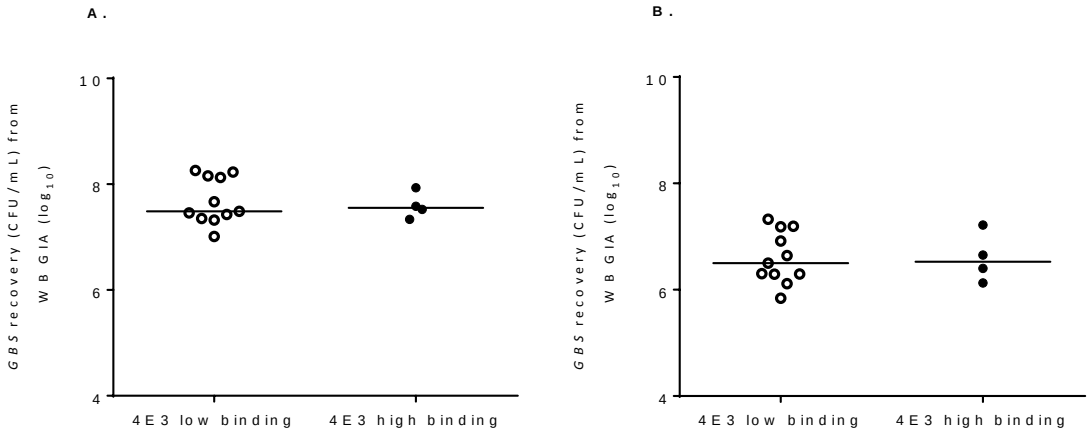


Figure 6.16 Evaluation of association of 4E3 binding status and *S. agalactiae* recovery from whole blood GIA at D0 (baseline) for volunteers in phase 2. 1hour incubation represented. All inoculum concentrations evaluated (A. ~x10^7CFU/mL and B. ~x10^6CFU/mL). Each circle represents a volunteer with low 4E3 binding and dot, a volunteer with high 4E3 binding. Bar denoting median shown.

6.7 Cytokine stimulation assay

The final experiments performed were to explore the potential training of innate immune cells in the study volunteers by BCG vaccination, as evidence of the non-specific immunological effects in this cohort. This was a pilot experiment and therefore not all stored samples were processed. Stored PBMCs from D0, D14, and D84, of 12, phase 2, group 1 volunteers (BCG SSI vaccinated), were stimulated in-vitro with EC-LPS, HKCA, HKSA, PPD and RPMI (unstimulated). Cells were stimulated for 24 hours and the supernatants analysed for cytokine production using ELISA. Stimulations were performed in duplicate to allow optimisation experiments and processing of the samples. Results are presented by cytokine of interest.

6.7.1 Optimisation

Optimisation experiments were performed to identify the optimal dilution for plating the volunteer samples. Each stimulation produced approximately 150-160μL of supernatant. D0 PBMCs from 6 volunteers were stimulated with EC-LPS, PPD, HKSA, HKCA and RPMI and plated using serial dilutions. One volunteer, with each stimulation condition, was used for each cytokine. A recombinant human standard for normalisation of results was provided (Figure 6.17). Standard curves were performed for each experiment, and each cytokine, and were reproducible each time and identical to the standard curve demonstrated in each ELISA kit (data not shown).

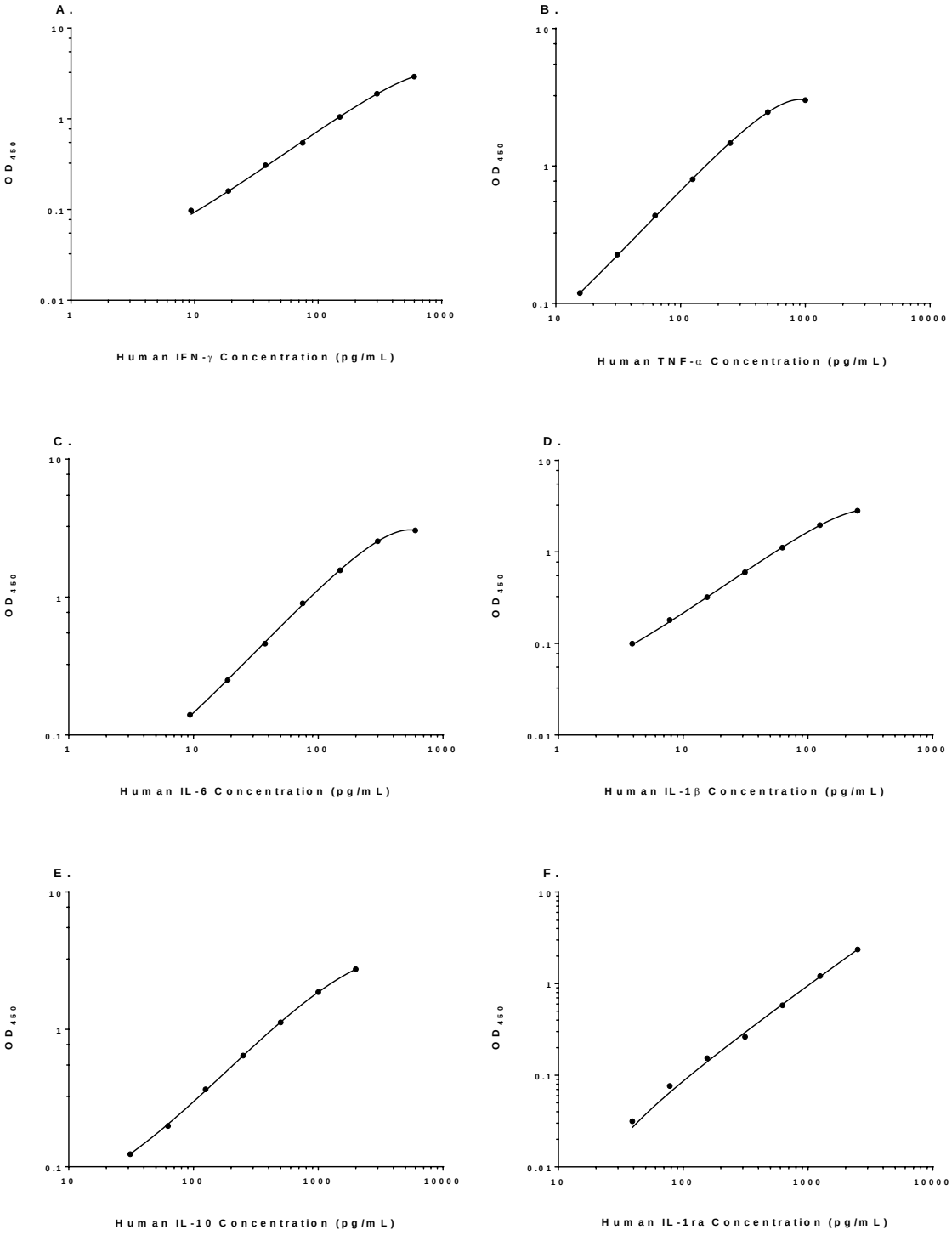


Figure 6.17 Cytokine ELISA standard curves. A. human IFN-γ, B. human TNF-α, C. human IL-6, D. human IL-1β, E. human IL-10 and F. human IL-1ra.

Unstimulated PBMCs were evaluated to assess background responses to each cytokine (Figure 6.18). A 1 in 5 dilution was prepared, with 1 in 2 serial dilutions thereafter. There was minimal detection of IFN-γ, TNF-α, IL-6, IL-1β, IL-10, or IL-1ra from PBMCs stimulated with RPMI at any dilution.

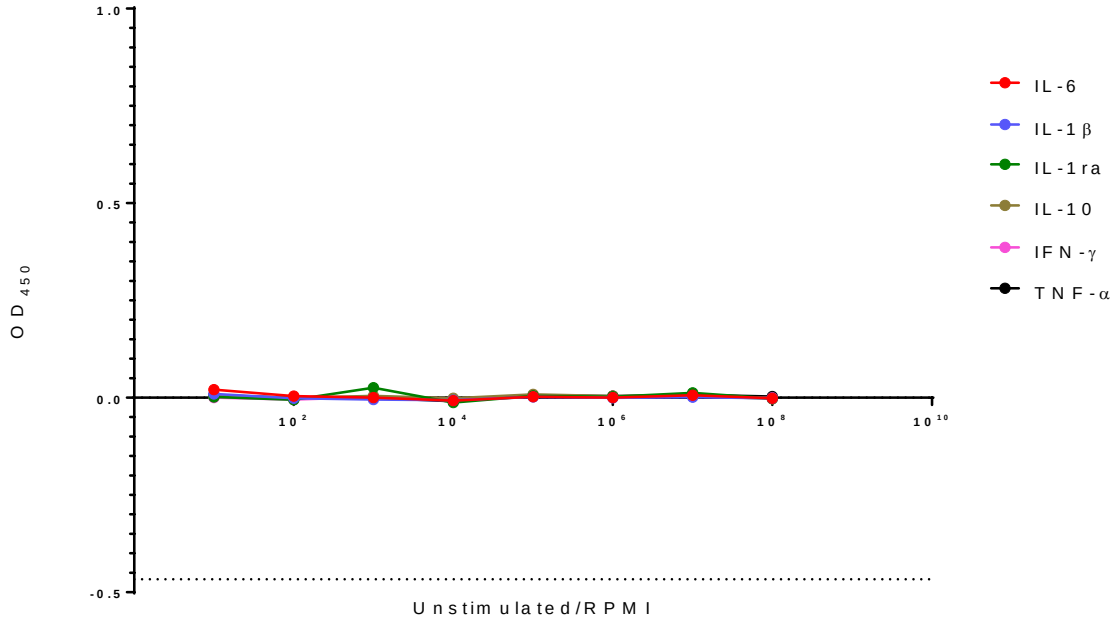


Figure 6.18 Cytokine production by PBMC following 24 hour incubation with RPMI. Standard curve of serial dilutions presented for IFN-γ, TNF-α, IL-6, IL-1β, IL-10 and IL-1ra

EC-LPS, PPD, HKSA and HKCA induced different levels of cytokine production from PBMCs. EC-LPS stimulated high levels of TNF-α, IL-10, and IFN-γ (Figure 6.19 A). IL-6, IL-1β and IL-1ra detection was low at the lowest dilution (1:5) (Figure 6.19 A).

PPD stimulated TNF-α and IFN-γ, with low levels of detection of IL-6, IL-1β, IL-1ra and IL-10 at the lowest dilution (1:5) (Figure 6.19 B).

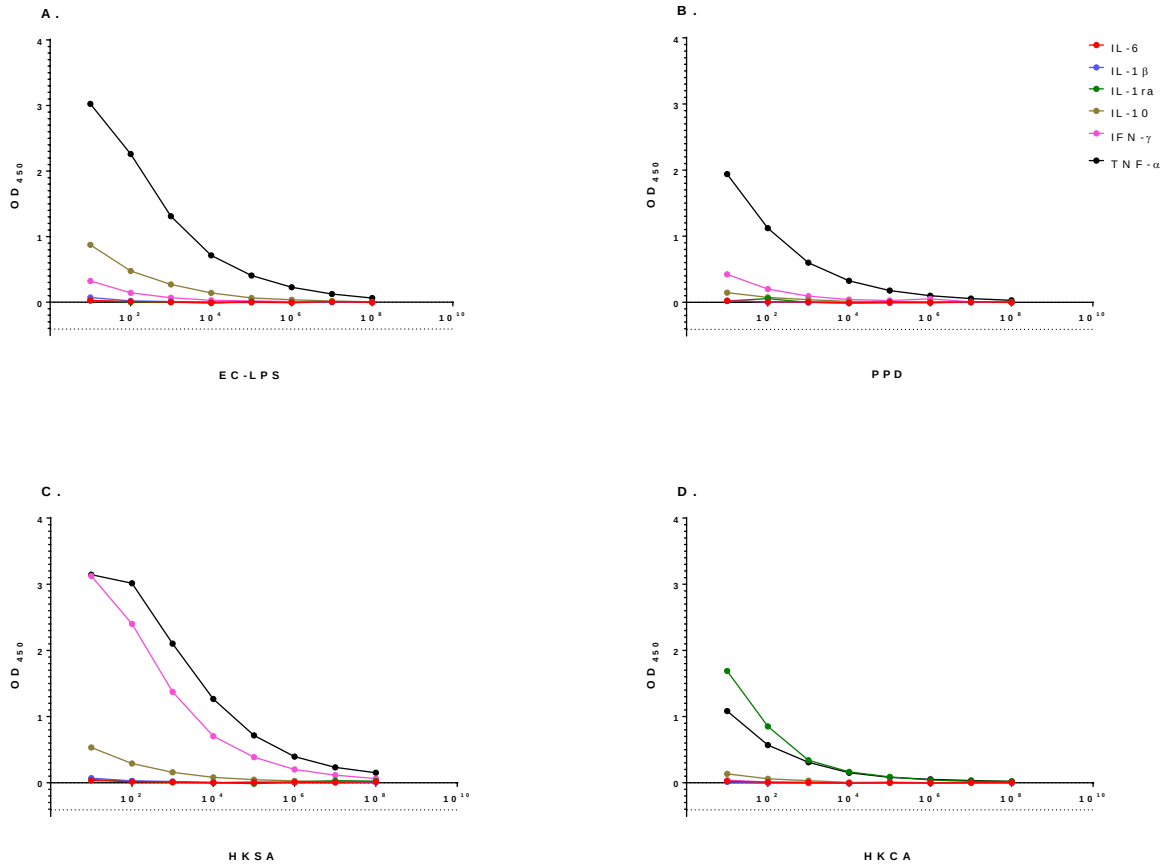


Figure 6.19 Cytokine production by PBMC following 24 hour incubation with EC-LPS, PPD, HKSA and HKCA. Standard curve of serial dilution presented

HKSA was a potent inducer of TNF-α and IFN-γ, with moderate IL-10 detection (Figure 6.19 C). IL-6, IL-1β and IL-1ra were all at low levels of detection at the lowest dilution (1:5) (Figure 6.19 C). HKCA induced IL-1ra and TNF-α, but IL-6, IL-1β, IL-1ra and IL-10 detection was low at the lowest dilution (1:5) (Figure 6.19 D).

To ensure the best utilisation of the limited supernatant available from the assay, with the best chance of detecting a change in cytokine stimulation between time-points, the following supernatant dilutions were chosen based on the optimisation experiment (Table 6.1). 100μL of sample was required per well in duplicate following dilution.

	HKSA	HKCA	EC-LPS	PPD	RPMI
IL-6	1:5 – 20:80 Total 40µL	1:5 – 20:80 Total 40µL	1:5 – 20:80 Total 40µL	1:5 – 20:80 Total 40µL	1:5 – 20:80 Total 40µL
IL-1β	1:5 – 20:80 Total 40µL	1:5 – 20:80 Total 40µL	1:5 – 20:80 Total 40µL	1:5 – 20:80 Total 40µL	1:5 – 20:80 Total 40µL
IL-1Ra	1:5 – 20:80 Total 40µL	1:160 – 1:160 Total 2µL	1:5 – 20:80 Total 40µL	1:5 – 20:80 Total 40µL	1:5 – 20:80 Total 40µL
IL-10	1:20– 5:95 Total 10µL	1:20– 5:95 Total 10µL	1:20– 5:95 Total 10µL	1:20– 5:95 Total 10µL	1:20– 5:95 Total 10µL
IFN-γ	1:160 – 1:160 Total 2µL	1:10 – 10:90 Total 20µL	1:10 – 10:90 Total 20µL	1:10 – 10:90 Total 20µL	1:10 – 10:90 Total 20µL
TNF-α	1:160 – 1:160 Total 2µL	1:160 – 1:160 Total 2µL	1:160 – 1:160 Total 2µL	1:160 – 1:160 Total 2 µL	1:160 – 1:160 Total 2µL
Total	134µL	154µL	152µL	152µL	152µL

Table 6.1 Supernatant dilutions for each cytokine and stimulation condition used.

Using the defined dilutions, cytokine ELISAs were performed on all stored supernatants for the 12 phase 2, group 1 volunteers.

6.7.2 IFN-γ

Fold change in IFN-γ production between baseline (D0) and D14 and D84 post BCG SSI vaccination is shown in Figure 6.20. Samples were processed in duplicate with mean result presented. Samples were adjusted for plate background and each individual value adjusted for their unstimulated response. Data presented is in OD_{450}.

There was no significant fold change in IFN-γ detection following 24 hour incubation of PBMCs with EC-LPS, PPD, HKSA and HKCA. Following stimulation with PPD, IFN-γ fold change between D0 and D84 was approaching significance (p=0.0990; Figure 6.20 B). Following stimulation with HKSA, IFN-γ fold change between D14 and D84 was approaching significance (p=0.0990; Figure 6.20 C).

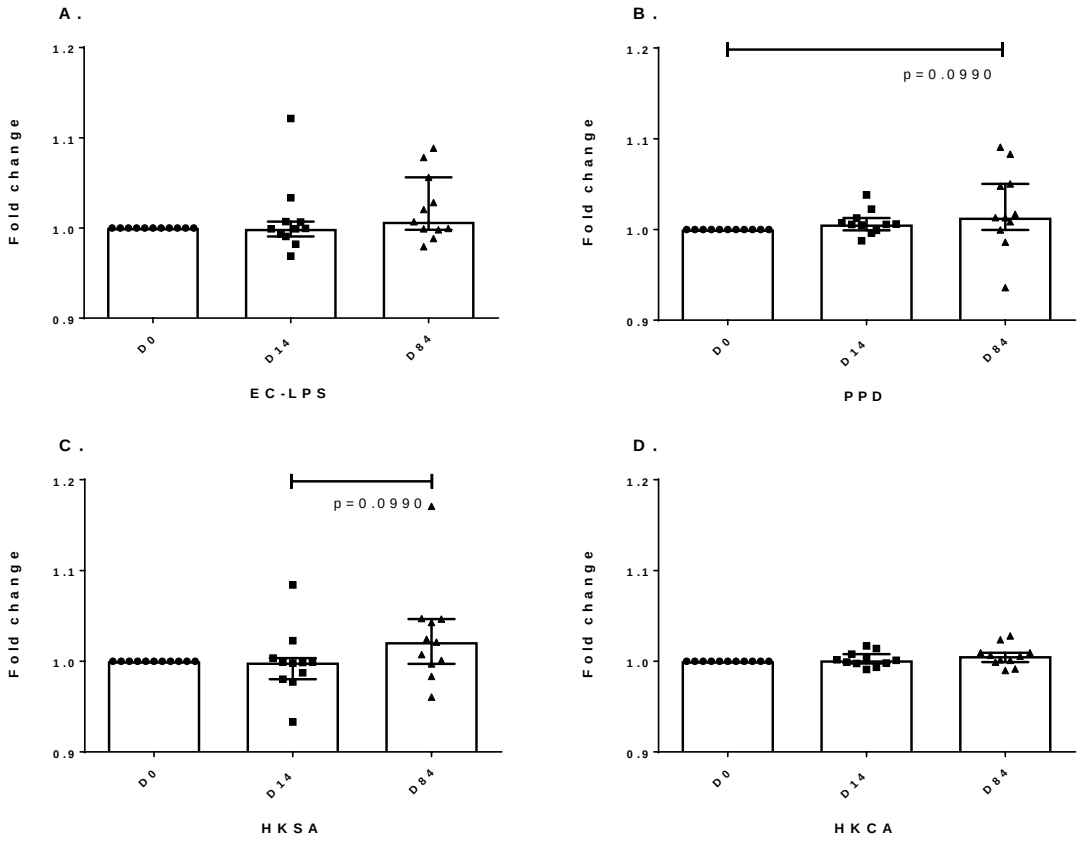


Figure 6.20 Fold change in IFN-γ production following secondary *in-vitro* stimulation of volunteer PBMCs at D14 and D84 compared with baseline (D0). Secondary stimulant: A. EC-LPS, B. PPD, C. HKSA and D. HKCA. Bar represents median with interquartile range shown. Each dot corresponds with mean of duplicate values for each volunteer (n=12). One-way ANOVA and Dunn's multiple comparisons test performed.

6.7.3 TNF-α

Fold change in TNF-α production between baseline (D0) and D14 and D84 post BCG SSI vaccination is shown in Figure 6.21. Samples were processed in duplicate with mean result presented. Samples were adjusted for plate background and each individual value adjusted for their unstimulated response. Data presented is in OD_{450}.

There was no significant fold change in TNF-α detection following 24 hour incubation of PBMCs with EC-LPS, PPD, HKSA and HKCA, although two volunteers showed an increase in TNF-α production at D14 and D84 post vaccination following secondary stimulation with HKSA (Figure 6.21).

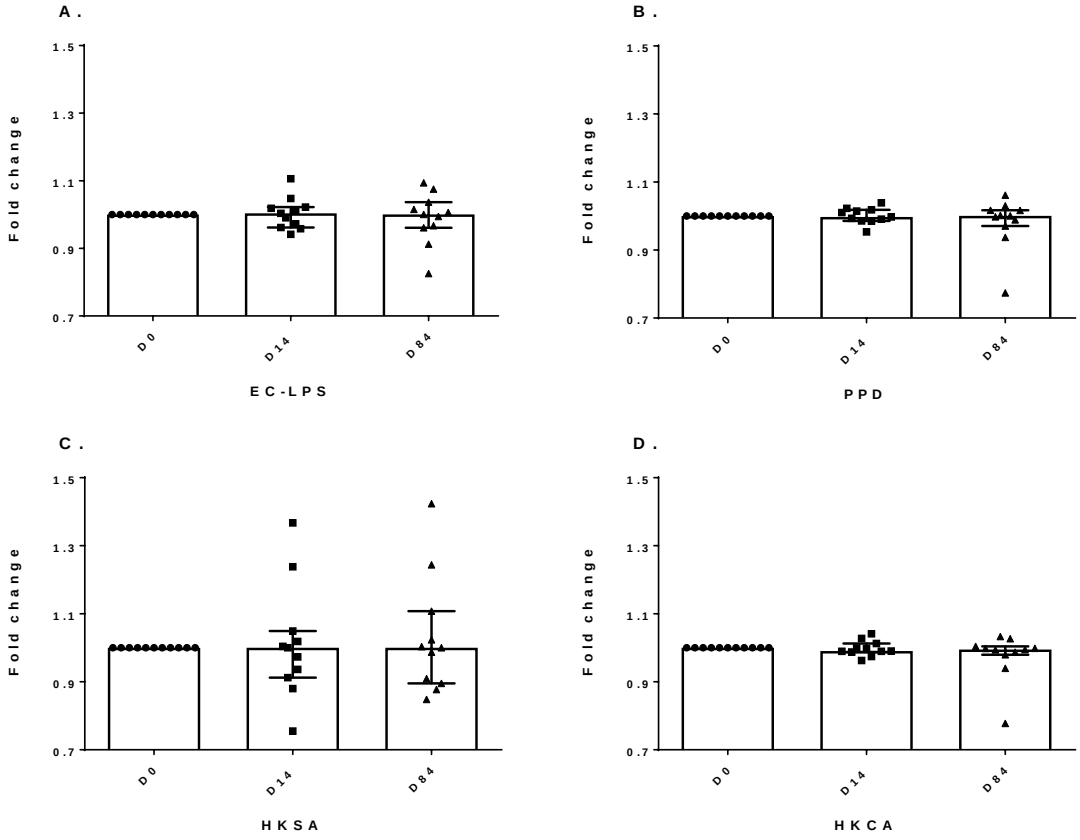


Figure 6.21 Fold change in TNF-α production following secondary *in-vitro* stimulation of volunteer PBMCs at D14 and D84 compared with baseline (D0). Secondary stimulant: A. EC-LPS, B. PPD, C. HKSA and D. HKCA. Bar represents median with interquartile range shown. Each dot corresponds with mean of duplicate values for each volunteer (n=12). One-way ANOVA and Dunn's multiple comparisons test performed.

6.7.4 IL-6

Fold change in IL-6 production between baseline (D0) and D14 and D84 post BCG SSI vaccination is shown in Figure 6.22. Samples were processed in duplicate with mean result presented. Samples were adjusted for plate background and each individual value adjusted for their unstimulated response. Data presented is in OD_{450}.

There was no significant fold change in IL-6 detection following 24 hour incubation of PBMCs with EC-LPS, PPD, HKSA and HKCA (Figure 6.22). Following stimulation with EC-LPS, two volunteers demonstrated a 4 fold increase in IL-6 production at D14 and three volunteers by D84 (Figure 6.22 A). Following PPD stimulation, three volunteers had a 4 fold increase in IL-6 by D14, and four volunteers by D84 (Figure 6.22 B). HKSA stimulation led to 2 volunteers having a 4 fold increase in IL-6 at D14, and three by D84 (Figure 6.22 C). This did not result in a significant fold change overall for the group as all others demonstrated no change. There was little change induced by HKCA.

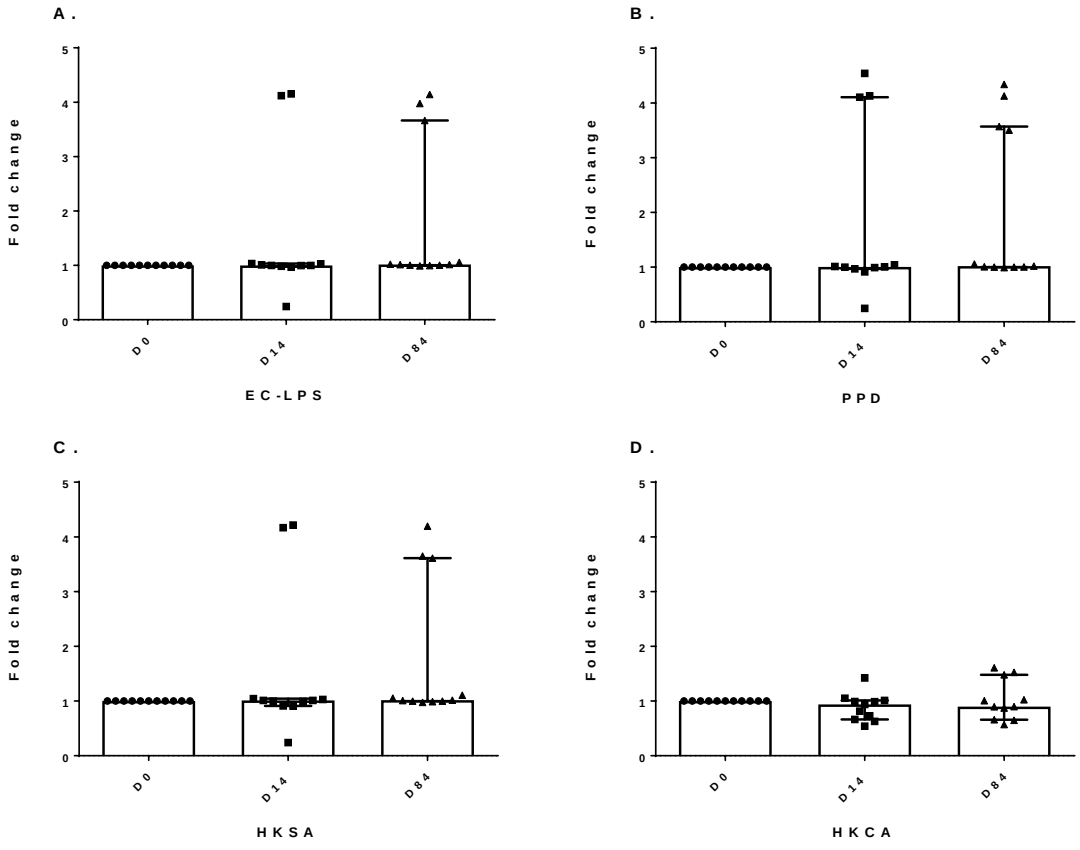


Figure 6.22 Fold change in IL-6 production following secondary *in-vitro* stimulation of volunteer PBMCs at D14 and D84 compared with baseline (D0). Secondary stimulant: A. EC-LPS, B. PPD, C. HKSA and D. HKCA. Bar represents median with interquartile range shown. Each dot corresponds with mean of duplicate values for each volunteer (n=12). One-way ANOVA and Dunn's multiple comparisons test performed.

6.7.5 IL-1β

Fold change in IL-1β production between baseline (D0) and D14 and D84 post BCG SSI vaccination is shown in Figure 6.23. Samples were processed in duplicate with mean result presented. Samples were adjusted for plate background and each individual value adjusted for their unstimulated response. Data presented is in OD_{450}.

There was no significant fold change in IL-1β detection following 24 hour incubation of PBMCs with EC-LPS, PPD, HKSA and HKCA. Following stimulation with EC-LPS, two volunteers demonstrated a 2.5-3 fold increase in IL-1β production at D14 and three volunteers a 3-4 fold increase by D84. Following PPD stimulation, one volunteer had an approximately 2 fold increase in IL-1β by D14, and one volunteer a 3 fold increase by D84. HKSA stimulation led to 2 volunteers having a 3.5-4 fold increase in IL-1β at D14, and three by D84. This did not result in a significant fold change overall for the group as all others demonstrated no change. There was no change induced by HKCA.

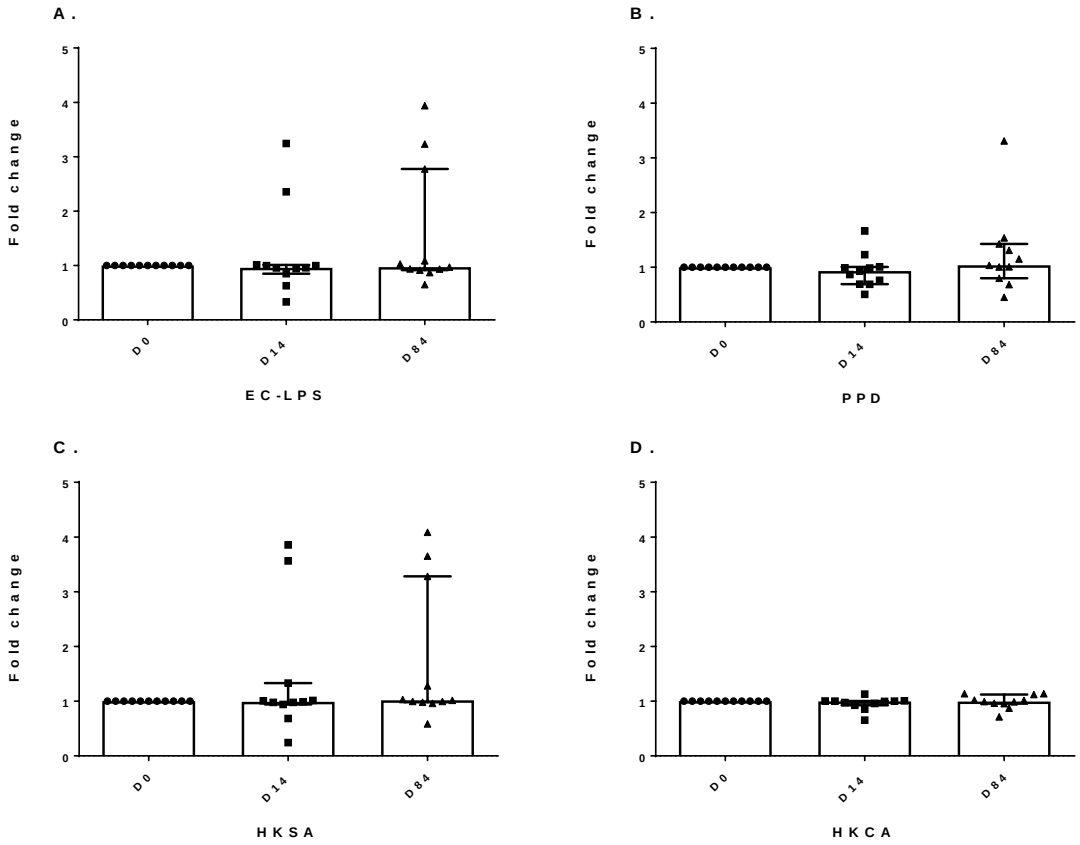


Figure 6.23 Fold change in IL-1β production following secondary *in-vitro* stimulation of volunteer PBMCs at D14 and D84 compared with baseline (D0). Secondary stimulant: A. EC-LPS, B. PPD, C. HKSA and D. HKCA. Bar represents median with interquartile range shown. Each dot corresponds with mean of duplicate values for each volunteer (n=12). One-way ANOVA and Dunn's multiple comparisons test performed.

6.7.6 IL-10

Fold change in IL-10 production between baseline (D0) and D14 and D84 post BCG SSI vaccination is shown in Figure 6.24. Samples were processed in duplicate with mean result presented. Samples were adjusted for plate background and each individual value adjusted for their unstimulated response. Data presented is in OD_{450}.

There was no significant fold change in IL-10 detection following 24 hour incubation of PBMCs with EC-LPS, PPD, HKSA and HKCA.

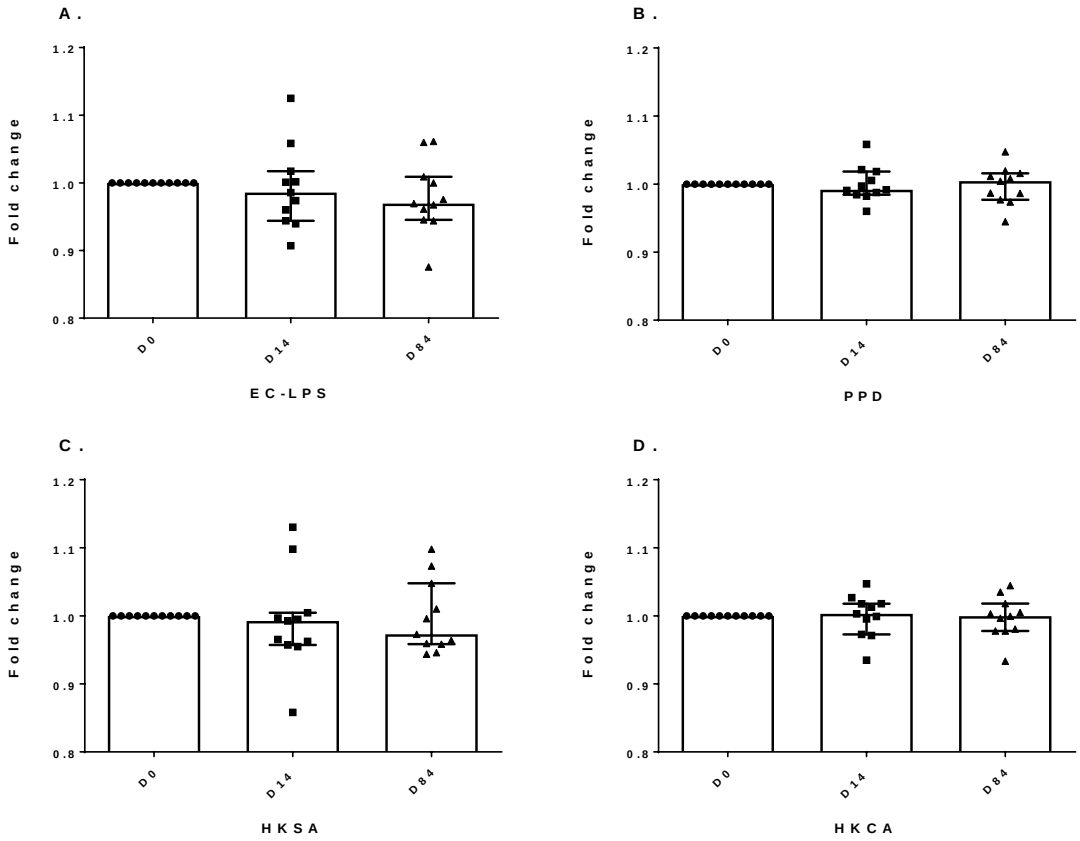


Figure 6.24 Fold change in IL-10 production following secondary *in-vitro* stimulation of volunteer PBMCs at D14 and D84 compared with baseline (D0). Secondary stimulant: A. EC-LPS, B. PPD, C. HKSA and D. HKCA. Bar represents median with interquartile range shown. Each dot corresponds with mean of duplicate values for each volunteer (n=12). One-way ANOVA and Dunn's multiple comparisons test performed.

6.7.7 IL-1ra

Fold change in IL-1ra production between baseline (D0) and D14 and D84 post BCG SSI vaccination is shown in Figure 6.25. Samples were processed in duplicate with mean result presented. Samples were adjusted for plate background and each individual value adjusted for their unstimulated response. Data presented is in OD_{450}.

There was no significant fold change in IL-1ra detection following 24 hour incubation of PBMCs with EC-LPS, PPD, HKSA and HKCA. Following stimulation with HKSA, 2 volunteers showed a 1.5-2 fold increase in IL-1ra production at D14 and three volunteers at D84. This did not result in a significant fold change for the group (Figure 6.21).

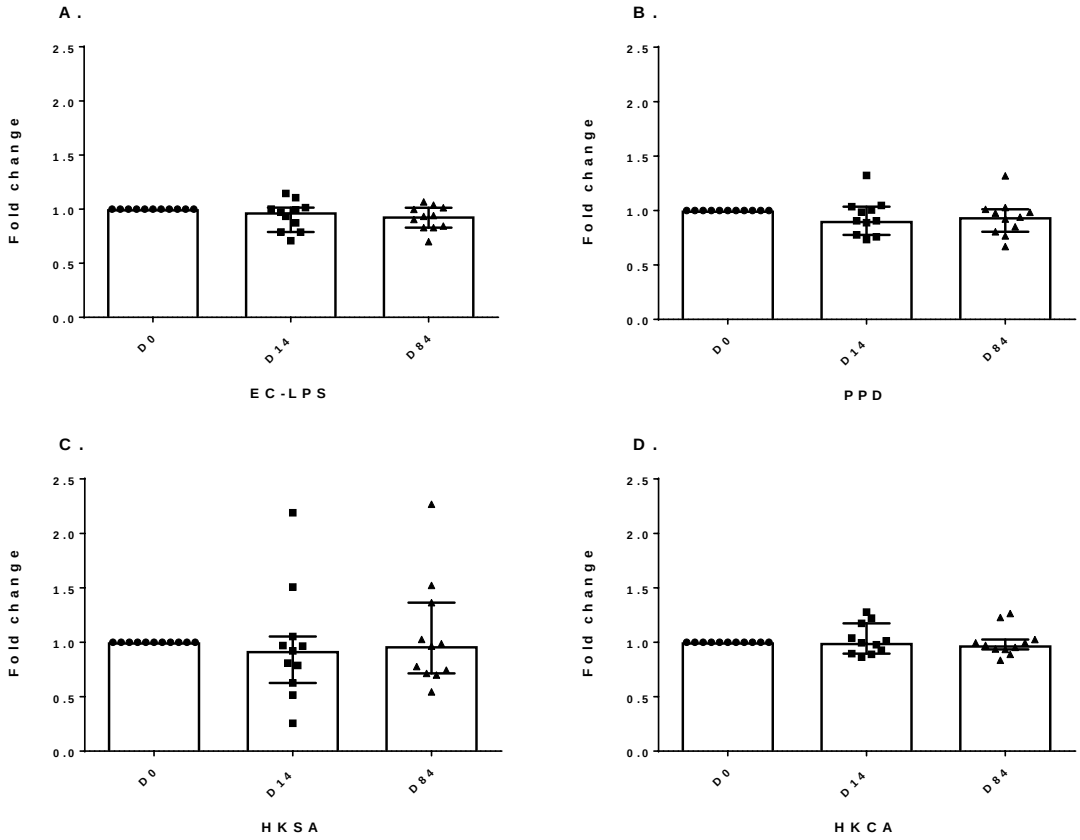


Figure 6.25 Fold change in IL-1ra production following secondary *in-vitro* stimulation of volunteer PBMCs at D14 and D84 compared with baseline (D0). Secondary stimulant: A. EC-LPS, B. PPD, C. HKSA and D. HKCA. Bar represents median with interquartile range shown. Each dot corresponds with mean of duplicate values for each volunteer (n=12). One-way ANOVA and Dunn's multiple comparisons test performed.

6.8 Discussion

It was important to know whether BCG vaccination induced the expected, antigen-specific immune response in the participants enrolled in this study. Clinical responses such as scar size and delayed-type hypersensitivity skin reaction were not collected during this study, as evidence suggests that there is no correlation between scar size/skin response and vaccine induced protection against TB[237-240]. Th1 cellular immune responses to mycobacterial infection is typified by the production of IFN-γ by antigen specific T cells[241] and development of the ELISpot assay has allowed reliable and reproducible measurement of PPD-specific IFN-γ producing T-cell responses following BCG vaccination[242]. Whilst measurement of this biomarker provides a quantifiable, cellular specific response in this cohort of volunteers, it is important to remember that to date, significant increases in levels of antigen-specific INF-γ producing T-cell responses following BCG vaccination have been found to be a poor correlate of protection against *M. tb* infection in TB vaccine research[243-245]. Therefore no interpretation can be made as to the level of specific protection provided to group 1.

In this study, the median PPD-specific response in group 1 (BCG vaccinated) peaked at D7 following vaccination and was sustained at the final visit on D84. As expected, PBMCs from Group 2, non-vaccinated volunteers had no increase in median response over time.

As discussed in Chapter 3: Clinical results, there was variability in the CFU/dose of BCG from the vials of vaccine used as quantified through plating of a sample of the vials onto 5H11 agar. Three of the five vials of the first batch of BCG (batch no. 114015A), were lower in CFU/dose than the expected range (2-8 x 10^5CFU/dose), and all three of the vials of the second batch (batch no. 114022A) were within range, but used during the six month extended expiry period. Overall this did not have a significant impact on the PPD-specific IFN-γ responses. Volunteers in group 1, phase 1 had higher and more sustained responses, however by directly comparing the volunteer responses for each phase at D0,

D7, D14 and D28, there was no significant difference overall. Whilst the numbers are small, the potential variability of dose, and use of BCG six months beyond the manufacturer expiry date, did not significantly impact on PPD-specific ELISpot responses.

At present, there is no universally accepted, validated, immune marker for BCG induced protection [243, 246-249], but the direct MGIA has been demonstrated to correlate with *in-vivo* protection across different species[186]. In this study there was a significant decrease in net growth in the group 1 volunteers between D0 and D84, and D28 and D84, not demonstrated in the non-vaccinated, group 2 volunteers, suggesting a vaccine specific protective response. When group 1 volunteer net growth results were split by batch of BCG SSI used, volunteers vaccinated from the first batch of BCG with the greatest variability in dose, though showing an decreasing trend in net growth, did not reach statistical significance, likely due to small numbers. The net growth of BCG following incubation with inoculated PBMCs from volunteers vaccinated from the second batch of BCG, which had a more consistent CFU/dose of BCG vaccine, demonstrated a more uniform response with a significant increase between D0 and D84.

Overall, PPD-specific IFN-γ ELISpot responses and the net growth results from the direct MGIA provide evidence that volunteers vaccinated in this study had an expected specific response to BCG. Additionally, it supports the use of the direct MGIA as a reliable tool for the detection of specific *ex-vivo* protection against mycobacteria following BCG vaccination.

As previously mentioned, there is growing interest in the possible role of antibodies in protection against TB, but as yet no consensus opinion[233, 250, 251]. A study by Lu et al found that individuals with LTBI and active *M. tb* infection had functionally distinct antibody profiles, with antibodies from individuals with LTBI driving an enhanced phagolysosomal maturation, inflammasome activation and macrophage killing of intracellular *M. tb*, suggesting a potential role of Fc-mediated Ab effector functions in *M. tb*

control[251]. Whilst B cell and antibody deficiencies have not been found to be a risk factor for progression of *M. tb* disease in humans [252], studies in mice deficient in B cells and secreted antibodies have an increased susceptibility to *M. tb* infection[253-255] and NHPs have an increased burden of infection[256]. Data from the immune correlates of risk analysis performed on the MVA85A infant efficacy trial samples found that increased levels of Ag85A-specific IgG at D28 correlated with lower *M. tb* risk. The Ag85A IgG levels were raised in both the MVA85A vaccinated group and the control group, implying that the neonatal BCG vaccination likely primed the antibody response and it was hypothesised that natural NTM exposure may have augmented the increase, especially in the control group[257]. Using an ELISA protocol developed by the TB vaccine group, total IgG responses to whole cell BCG (the same SSI strain used for vaccination), before, and 28 days following enrolment was undertaken on serum from both group 1 (BCG vaccinated) and group 2 (non-vaccinated control) volunteers. There was a significant increase in median OD_{405} between D0 (pre-vaccination) and D28 (post-vaccination) in group 1 which was not seen in group 2, and there was a significant difference between the fold change seen in group 1 and group 2. With such small numbers and disparity in size of the groups, one can only make an observation at this time. Unlike infants in Africa, in this cohort, NTM exposure is likely to be low, though cannot be excluded as a confounder for the high readings at baseline. However the significant increase in responses between D0 and D28 in group 1 is likely due to BCG vaccination, and undertaking the direct MGIA using autologous serum in this cohort, would provide valuable information as to the functionality of the antibodies.

The purpose of quantifying the antibody responses specific to the four pathogens used in the GIA, was to assess any possible confounding that antibodies, already present in the blood of the enrolled adult volunteers, may have on the primary outcome (bacterial recovery from the whole blood GIA). Total IgG to *S. aureus* whole bacteria and IgG1 subclass (4E3 clone) to ClfB were measured. The rational for choosing a total response to

whole *S. aureus*, was to measure generalised antibody responses to cell surface markers of *S. aureus*. ClfB, more specifically, is one of a family of microbial surface components recognizing adhesion matrix molecules first identified in 1998[258] and has been shown to be a major determining factor in *S. aureus* colonisation status of mice and humans[259], where in humans, it binds to human type 1 cytokeratin 10 expressed on epithelial cells[260].

Total IgG responses to whole *S. aureus* from stored serum obtained from volunteers enrolled in this study, overall, were lower than IgG1 responses to ClfB. Responses to ClfB exhibited a bimodal distribution as seen in different human volunteer cohorts studied by the *S. aureus* group (Wylie et al, unpublished data). When the results were divided according to paired nasal swab carrier status data (as defined by one or more positive nasal swab) there was no overall significant difference in the median responses between carriers and non-carriers to whole *S. aureus* or ClfB, however, the highest responders to both whole *S. aureus* and ClfB were in the nasal carrier cohort. This suggests a possible correlation at least between high IgG1 (4E3) responses and positive nasal swab carrier status, and as ClfB has been shown to play a role in the colonisation of human nares with *S. aureus*[259], it would not be unreasonable to expect to find a correlation between high levels of IgG1 to ClfB and a positive nasal swab carrier status.

Volunteer serum antibody responses to ClfB, were classified as either having high or low 4E3 binding affinity as defined by the bimodal distribution seen. In order to assess whether this high or low antibody binding affinity impacted on the recovery of bacteria from the whole blood GIA, bacterial recovery at D0 was divided according to binding affinity. Only following 24 hour incubation with a bacterial inoculum of approximately x 10^8CFU/mL was a significant difference seen – volunteers with a high binding affinity had a lower recovery of bacteria from the whole blood GIA. Subsequently, this was also explored with recovery of the other three pathogens from the whole blood GIA. No

difference was detected with *K. pneumoniae* or *S. agalactiae*, however there was a significant difference seen in bacterial recovery following 1 hour incubation with *E. coli* – volunteers with high binding affinity had a significantly lower recovery of bacteria than those with a low binding affinity. This suggests that there is potentially a functional role for these antibodies in improved bacterial killing, and potentially some limited antibody/antigenic cross-reactivity. Studies have shown that monoclonal antibodies specific to EC-LPS are able to cross-react with other gram negative species[261]. However, numbers were small and there was discordance between the number of volunteers with high binding and low binding affinity – volunteers with high binding affinity in phase 1 and phase 2 of enrolment were 5 and 4 respectively compared with 15 and 11 volunteers with low binding affinity. Whilst I used an optimised protocol developed by the *S. aureus* group; the limited time available restricted the opportunity to explore IgG isotypes further, perhaps providing a better characterisation of volunteer serum antibodies to whole *S. aureus* and *S. aureus* surface proteins. Further optimisation of the IgG responses in parallel with enrichment of nasal swab samples to identify low level carriers, could perhaps provide more valuable information in relation to the potential functional implication of antibodies in the recovery of bacteria from the whole blood GIA. Further work in order to optimise the ELISA assay to evaluate antibody responses to the other three pathogens is required, but unfortunately outside the scope of this thesis.

Analysis of key cytokines involved in the innate immune response was undertaken. The best evidence supporting the potential mechanism for the non-specific effects of BCG is from studies showing an increase in these cytokines in both human and animal models following BCG vaccination[134, 135, 139]. It was therefore important to demonstrate whether a similar response was possible from this cohort of volunteers for the following reason: if no effect was seen in either the whole blood or PBMC GIA, but there was a cytokine response to BCG vaccination suggesting innate immune 'training', then this

would support the non-specific effects but suggest that the assay was not a sensitive enough model to detect this effect and further optimisation required. A protocol was developed from work done in Professor Netea's group in Nijmegen[134, 138]. Cytokines chosen were IFN-γ, TNF-α, IL-6, IL-10, IL-1ra and IL-1β as they had all been demonstrated to increase in BCG primed PBMC's following a secondary *in-vitro* stimulation. No clear effect of any non-specific antigenic stimulation was seen for any of the cytokines measured, although there were a few volunteers who did seem to respond. This lack of a clear innate effect may have been because the assay was not fully optimised. Furthermore, in this work I used cryopreserved PBMC and it may be that fresh cells would have been better for the detection of this work. Given the PPD-specific ELISpot responses and the MGIA responses, it is clear that BCG vaccine 'take' has occurred and it is not clear why a non-specific innate immune response was not detectable.

6.9 Conclusions

- PPD specific IFN-γ ELISpot responses combined with net growth from the direct MGIA demonstrated that volunteers vaccinated with BCG in this study demonstrated an expected specific response
- Results from the direct MGIA support its use as a reliable tool for the detection of specific *ex-vivo* protection against mycobacteria following BCG vaccination and its ongoing use in TB vaccine research
- Group 1 volunteers (BCG vaccinated) demonstrated a significant increased fold change in IgG responses to whole cell BCG not demonstrated in group 2 of unclear significant
- Study volunteers demonstrate a bimodal distribution of IgG1 (4E3) responses to *S. aureus* ClfB with a possible correlation with *S. aureus* nasal swab carrier status, and an observation that volunteers with high 4E3 binding affinity potentially have better control

of bacteria growth under some conditions, however numbers are small and data inconsistent to draw conclusions. More work is needed.

- No clear effect of non-specific antigenic stimulation was demonstrated for IFN-γ, TNF-α, IL-6, IL-10, IL-1ra or IL-1β in a pilot study of 15 phase 2 volunteers. Whilst some volunteers did demonstrate an effect of BCG vaccination, further work is necessary to optimise the assay protocol in order to draw any conclusions

7 Conclusions and future work

The global burden of LTBI and TB disease, with the added complication of the evolving complex combinations of *M. tb* antimicrobial drug resistance means that the desperate need for a safe and effective TB vaccine is undisputable. Development of such a vaccine is a challenging field of research and despite over 15 different candidate vaccines being in various stages of development; BCG remains our only licenced vaccine with unfortunately extremely variable efficacy. The non-specific effects of vaccines is a contentious issue, not simply because of the potential non-specific beneficial effects (as in the case of BCG), but more so because of reported potential non-specific deleterious effects of some vaccines on infant mortality (as in the case of the DTP vaccine). The relative dearth of robust, reproducible, validated, multicentre, randomised, controlled, clinical trials in infants in TB high burden countries undoubtedly confounds the issue, and despite biological plausibility and emerging molecular and cellular data providing supporting evidence of 'training' of the innate immune system following BCG vaccination, there remains no definitive study demonstrating that the changes seen in the laboratory, translate into the clinically meaningful endpoint of improved infant morbidity and mortality.

The aim of this study was to bridge the divide in our understanding of the non-specific effects of BCG vaccination through the development of a functional *in-vitro* whole blood GIA as a laboratory tool which could act as a surrogate marker for an individual's ability to control growth of four common pathogenic bacteria, pre and post BCG vaccination. By measuring the 'sum of the parts' of the immune system in this way, it would potentially be possible to provide the link between the biology and the clinical picture.

Despite various challenges faced during the study such as the delay caused by the global shortage of BCG, I showed that it was possible to design, obtain ethical approval and enrol 35 volunteers into the study. It was more common to enrol female volunteers overall, but as expected due to the randomisation process, little difference in baseline demographics

of the volunteers between group 1 and group 2 and between phase 1 and phase 2 of enrolment was found. There were many logistical and assay related challenges faced during the study. One of the biggest hurdles was the fluctuations in bacterial inoculum following each culture period and the possible impact that this could have on the performance of the assay. The rapidity of the replication time of the bacteria meant that timing of blood collection and culture periods were very important with little room for flexibility. Importantly, therefore, the design of the study allowed for two controls. Whilst each volunteer in group 1 (BCG vaccinated) acted as their own control for any potential effect of BCG vaccination on control of bacterial growth as D0 was pre-vaccination, there was in addition a non-vaccinated volunteer (group 2) who acted as a control for inoculum variability. On reflection, in order to have a better comparison between the groups, the randomisation process should have been 1:1, however the reason for not doing this at the time was to ensure that the majority of volunteers were enrolled into the BCG vaccinated group as this was the primary intervention. Due to the exploratory nature of the study, the impact of the inoculum was not fully known and rather than having large numbers of non-vaccinated volunteers, it was the best use of valuable resources at the time to limit the number in this group to one per enrolment schedule. If time, money, and number of volunteers which could be enrolled were not limited, then I would enrol volunteers into group 1 and group 2 equally to allow for a more direct comparison between the groups and a better ability to control for batch effect. In addition I would also reduce the frequency of volunteer venepuncture, as this would considerably improve the logistical considerations in the laboratory and allow for a smoother optimisation process.

One of the factors considered to be a potential confounder was the colonisation status of the volunteers and what impact it may have on the performance of the assay. Due to time constraints and the required optimisation steps, it was not possible to fully explore this. A binary approach to faecal colonisation status was taken, culturing nasal swabs and faecal samples on bacteria specific selective agar and adapting a protocol used in the

microbiology department at the JR hospital for faecal samples and using a protocol for the nasal swabs from the *S. aureus* group. On reflection a more robust method would better allow for control of such factors, but with stored samples available, it is something which can be taken forward. Technology such as 16s rRNA sequencing would provide better a more accurate characterisation of different faecal bacteria and the individuals biofilm. Additionally, the identification of antibodies to all four pathogens in the blood of the volunteers was time-consuming and challenging. It was possible to explore the relevant antibodies to *S. aureus* as there was an optimised protocol available; however, again it was not possible in the limited time available to undertake this work fully and explore the other three pathogens. More time was required to explore this. In chapter 6, I showed a small but significant correlation between high 4E3 binding affinity to *S. aureus* ClfB and *S. aureus* net growth from the GIA following 24hour incubation and *E. coli* GIA following 1 hour incubation; those with higher antibody levels had lower net growth at baseline suggesting some specificity and cross-reactivity. However, this was not consistent across bacterial GIA's, incubation periods, or bacterial inoculum concentrations, and the overall number of volunteers was small, therefore it is impossible to make any conclusions regarding the clinical significance of this finding. Further work exploring the functionality of these antibodies and potential role in the GIA would need to be done in order to draw any conclusions.

BCG SSI vaccination as supplied by the manufacturer provides a dose of 2-8 x 10^5CFU in 100µL. An aliquot of each vial used during the study was cultured to ensure each vial was within the expected dose range particularly as the second batch was used during the approved six month expiry extension period. There was considerable variability in CFU/mL quantified using the standard laboratory method for culture from vials in the first batch, but much more consistency from the second batch. Overall, the numbers are too small to make any formal conclusion, but there did not appear to be any significant correlation between BCG dose and assay outcome. Importantly, all vaccinated volunteers

had an expected clinical skin reaction to BCG, and an expected significant PPD specific IFN-γ ELISpot response regardless of dose/batch of vaccine used. Additionally, group 1 (BCG vaccinated) volunteers had a significant reduction in net growth of BCG at D28 and D84 following BCG vaccination using the direct MGIA, and a significant fold change in IgG to whole cell BCG, not seen in group 2 volunteers (non-vaccinated).

Although I undertook significant optimisation of both the whole blood and PBMC GIA during this project, further work could still be done to optimise these assays further. Bacteria were found to be reliable and reproducible in terms of growth from frozen stocks, which was particularly important in view of the fact that they were wild type bacteria and could have behaved in an unpredictable manner. Several variabilities were evaluated such as impact of incubation equipment, bacterial growth media, time from volunteer bleed to inoculation with bacteria and blood collection tube additive, with some standardisation applied before enrolment. The most important variable – bacterial inoculum concentration – could not be fully explored before enrolment as only static measurements in the absence of an intervention (BCG vaccination). This was therefore explored and optimised during enrolment. The addition of a non-vaccinated control volunteer demonstrated that there was similarity in the pattern of recovery of bacteria from the GIA over time following enrolment between both group 1 and group 2 volunteers. This suggested a significant batch effect caused by the daily fluctuations in inoculum used. In order to get the right MOI, different inoculum concentrations were evaluated during the study. The higher concentrations overwhelmed the assay, and whilst better discrimination was seen between volunteers using the lower inoculum concentrations, there was no single inoculum concentration which stood out as optimal. Combined with the batch effect, ultimately the whole blood GIA was unable to detect a significant improvement in control of bacterial growth in the GIA over time following BCG vaccination. Using lower inoculum concentrations could well have allowed for a difference to be seen, but it was not possible within the time available and the logistical

consideration already imposed, to undertake this during the study. This would be a consideration for any future work.

Refining the assay and using stored PBMCs meant that it was possible to process all time-points for each volunteer together using the same batch of inoculum and therefore removing this as a variable. The *S. aureus*, *E. coli*, and *K. pneumoniae* GIA all demonstrated a significant improvement in control of growth of bacteria following BCG vaccination using a linear mixed model approach. The improvement seen in *S. aureus* net growth at D7 was no longer evident when we controlled for multiple variables including Hb and Hct. Using one-way ANOVA, there was no significant difference seen in *E. coli* and *K. pneumoniae* GIA. Whilst there are several advantages to using this method, it has some limitations when considering the biological relevance of such a refined technique as applied to these bacteria, particularly knowing the complex interactions of the human immune system in controlling infection from these extra-cellular pathogens, vital components of which are absent in the PBMCs. One needs a system by which to relate these potential findings to the clinical setting. One such approach would be to use a controlled human challenge model. Whilst there are optimised and validated human challenge studies used in the vaccine development field (Malaria and Typhoid), there are no models available for these bacteria, and there would be considerable ethical implications ultimately making this unfeasible in a human model setting, considering the high risks associated with these infections. However, there are murine challenge models which could be substituted and allow for a direct comparison between *in-vitro* and *in-vivo* bacterial challenge following BCG vaccination.

A significant limitation to the optimisation of the assay was the logistical requirements, in particular the time taken to process the samples. Working in collaboration with the microbiology department at the JR hospital, I was able to demonstrate that there was a significant correlation between the CFU/mL quantified from the blood lysis/plating step

and the TTP using the BACTEC FX system in both the *S. aureus* and *E. coli* GIA. Going forward with this work, I would choose to replace the blood/lysis plating method with the BACTEC FX liquid culture system as it would significantly reduce the laboratory time involved and allow for an improved standardised approach using a widely used validated system. Unfortunately, without access to our own machine, we were limited in this study in the number of research samples which our collaborators could process on a machine which was also used for clinical samples. This method would also need to be validated in another cohort and optimised for the other pathogens.

The strongest evidence for the non-specific effects of BCG vaccination is the data relating to cytokine responses and innate immune 'training'. I attempted to reproduce this work in my study population. Pilot work was done with stored PBMCs from the 15 volunteers from phase 2. This provided a starting point and a manageable number in order to undertake some optimisation. Of the six cytokines chosen, there was no clear effect seen following antigenic stimulation, however this was a small number of volunteers, and whilst some volunteers had a very clear response, others were too high at baseline to detect a fold change. More time was needed to undertake further optimisation of supernatant sample dilutions. This will be important in taking this work forward for the remaining phase 1 volunteers. Another possible reason why responses seen were not as strong as had been reported by other groups was that some work was performed on fresh samples, whereas this was performed on frozen samples. Freeze thaw action on the cells may have significantly blunted the responses. Additionally, the technique used, whilst cost effective, was perhaps limiting in its ability to look at large number of cytokine responses from the small volume of supernatant obtained following secondary antigenic stimulation of PBMCs. Another option would be to use techniques applied by other groups such as Luminex.

Paxgene tubes for mRNA transcriptomic work was obtained and stored from each time-point for each volunteer who took part in this study. The plan was that should the GIA demonstrate a time-point where net growth of bacteria was better controlled following BCG vaccination, then this could be explored. As no time-point was identified, this work was not undertaken, but moving forward this resource could provide some interesting data if a peak time-point for the non-specific effects of BCG could be identified.

In summary:

- Undertaking a study of this nature was feasible
- Volunteers enrolled into group 1 (BCG vaccinated) had an expected clinical and immunological response to BCG vaccination despite subtle variation in BCG dose given
- Using the whole blood *in-vitro* GIA as a functional measure of the 'sum of the parts' of the non-specific effect of BCG vaccination in a healthy UK adult population with *S. aureus*, *E. coli*, *K. pneumoniae*, and *S. agalactiae*, I was unable to demonstrate any effect of BCG vaccination on inhibition of bacterial growth
- The PBMC GIA method may be a more refined tool to detect improved control of growth of bacteria following BCG vaccination, but requires further optimisation and validation
- Volunteer Hb concentration had a positive correlation with some bacterial growth in the whole blood GIA likely to reflect the importance of iron in bacterial growth and metabolism
- *S. aureus* nasal swab carrier status and faecal culture status did not correlate with assay outcome
- Volunteers with a high 4E3 binding affinity for IgG1 to *S. aureus* ClfB may have better control of *S. aureus* net growth from the GIA at baseline and may cross-react with some other bacteria, but numbers are small and require further work and characterisation

- Pilot work looking at cytokine responses to BCG vaccination in this study population was limited. No clear response was detected, however some volunteers had a very clear significant response. Further work is necessary to explore this fully.

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Appendix: Study Protocol

RESEARCH STUDY PROTOCOL

Study reference: TB038

Evaluation of the heterologous effects of Bacille Calmette-Guérin (BCG) vaccination in healthy UK adults

Study Reference:	TB038
Protocol Version Number:	3.1
Date:	17 November 2015
REC Reference:	15/SC/0022
Sponsor:	University of Oxford
Chief Investigator:	Professor Helen McShane
Local Safety Committee Chair:	Dr Brian Angus
Funding body:	The Bill and Melinda Gates Foundation and The Wellcome Trust
Author:	Dr Morven Wilkie

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1. INVESTIGATOR AGREEMENT

"I have read this protocol and agree to abide by all provisions set forth therein. I agree to comply with the International Conference on Harmonisation Tripartite Guideline on Good Clinical Practice."

Chief Investigator	Investigator Signature	Date
Professor Helen McShane		18.11.15

Confidentiality Statement

This document contains confidential information that must not be disclosed to anyone other than the Sponsor, the Investigator Team, and members of the Independent Ethics Committee. This information cannot be used for any purpose other than the evaluation or conduct of the clinical investigation without the prior written consent of Professor Helen McShane.

Conflict of Interest

"According to the Declaration of Helsinki, 2008, I have read this protocol, and declare no conflict of interest with any investigators"

Chief Investigator	Investigator Signature	Date
Professor Helen McShane		18.11.15

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3. SYNOPSIS

Study Title	Evaluation of the heterologous effects of Bacille Calmette-Guérin (BCG) vaccination in healthy UK adults	
Study Number	TB038	
Sponsor	University of Oxford	
Chief Investigator	Professor Helen McShane	
Study Centres	Centre for Clinical Vaccinology and Tropical Medicine (CCVTM) Churchill Hospital, Old Road, Headington Oxford, OX3 7LE John Warin Ward, Oxford University Hospital NHS Foundation Trust Churchill Hospital, Old Road, Headington Oxford, OX3 7LE The National Institute for Health Research Wellcome Trust Clinical Research Facility (NIHR WTCRF) The Queen Elizabeth Hospital, Edgbaston, Birmingham, B15 2TH	
Study Design	Randomised controlled clinical study	
Study Participants	Healthy, BCG naïve, IGRA negative, HIV negative, adult volunteers aged 18-45 years	
Planned Sample Size	40-48	Growth inhibition Assay Bacterial Pathogen
Stage 1	Group 1: 8 volunteers to receive BCG vaccine 2-8x10^5 cfu ID Group 2: 2-4 non-vaccinated control volunteers	*S. aureus* *E. coli*
Stage 2	Group 1: 8 volunteers to receive BCG vaccine 2-8x10^5 cfu ID Group 2: 2-4 non-vaccinated control volunteers	*S. aureus* *E. coli*
Stage 3	Group 1: 8 volunteers to receive BCG vaccine 2-8x10^5 cfu ID Group 2: 2-4 non-vaccinated control volunteers	*K. pneumoniae* *Group B Strep*
Stage 4	Group 1: 8 volunteers to receive BCG vaccine 2-8x10^5 cfu ID Group 2: 2-4 non-vaccinated control volunteers	*K. pneumoniae* *Group B Strep*
Planned Study Period	Prospective start date of enrolment is February 2015. Estimated duration of study of 12-18 months	
	Objectives	**Endpoints**
Primary	Determine the heterologous effects of BCG vaccination in healthy UK adults using *in-vitro* Growth Inhibition Assays as a surrogate marker to assess the individuals' capacity to control growth of *S. aureus, K. pneumoniae*, Group B *streptococci* and *E.coli*.	Assessment of bacterial growth using *in-vitro* Growth Inhibition Assay.
Secondary	Evaluation of laboratory markers of the immune response that correlate with levels of bacterial growth inhibition identified using the *in-vitro* GIAs.	Established and exploratory markers of cell mediated immunity in blood samples, including transcriptomics.

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Tertiary	Determine background levels of *S. aureus, K. pneumoniae*, Group B *streptococci* and *E.coli* carriage in subjects in this study and evaluate any confounding on the growth in-vitro seen.	Antibody titres in blood serum for *S. aureus, K. pneumoniae*, Group B *streptococci* and *E.coli*, nasal swab culture and faecal culture.

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3.1. **Schedule of visits and procedures**

Visit number		1	2	3	4	5	6	7	8	9
Day of visit	Screening	Day 0 (Vaccination)	Day 2	Day 4	Day 7	Day 10	Day 14	Day 21	Day 28	Day 84
Time windows (days)*			+/-1	+/-1	+/-1	+/-2	+/-2	+/-3	+/-3	+/-7
Consent	X									
History & examination	X									
Urinalysis	X									
Blood test	X	X	X	X	X	X	X	X	X	X
ß-HCG urine test	X	X								
Nasal swab^	X^	X^					X^			
Vital signs	X	X								
Faecal sample		X^^			X^^		X^^			
BCG vaccination		X								
Blood for GIA (mL)		5	5	5	5	5	5	5	5	5
Blood for screening (mL)**	10									
Blood for immunology (mL)	10	50	50	50	50	50	50	50	50	50
Blood for Paxgene tube		2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5
Blood for FBC		2	2	2	2	2	2	2	2	2
HLA typing (mL)		4								
S. aureus, E. coli, K. pneumonia and Group B strep antibody titres (mL)		5								
Blood total for visit (mL)	20	68.5	59.5	59.5	59.5	59.5	59.5	59.5	59.5	59.5
Cumulative blood vol (mL)	20	88.5	148	207.5	267	326.5	386	445.5	505	564.5

Table 1a: Schedule of visits and procedures for Oxford volunteers – All visits will take place in Oxford

X Event scheduled to occur

* Timeline is approximate only, as exact timings (± time windows) of visits relate to the actual (not intended) date of the previous visit

** Includes full blood count (2 mL), biochemistry (3 mL), and viral serology (5 mL)

^ Nasal swab will be taken at a minimum of 2 of these visits and a maximum of 3

^^ Faecal samples will be collected at a minimum of 1 of these visits and a maximum of 3.

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Visit number		1	2	3	4	5	6	7
Day of visit	Screening	Day 0 (Vaccination)	Day 2	Day 7	Day 14	Day 21	Day 28	Day 84
Time windows (days)*			+/-1	+/-1	+/-2	+/-3	+/-3	+/-7
Consent	X							
History & examination	X							
Urinalysis	X							
Blood test	X	X	X	X	X	X	X	X
ß-HCG urine test	X	X						
Nasal swab^	X^	X^			X^			
Vital signs	X	X						
Faecal sample		X^^		X^^	X^^			
BCG vaccination		X						
Blood for GIA (mL)		5	5	5	5	5	5	5
Blood for screening (mL)**	10							
Blood for immunology (mL)	10	50	50	50	50	50	50	50
Blood for Paxgene tube		2.5	2.5	2.5	2.5	2.5	2.5	2.5
Blood for FBC		2	2	2	2	2	2	2
HLA typing (mL)		4						
S. aureus, E. coli, K. pneumoniae and Group B strep antibody titres (mL)		5						
Blood total for visit (mL)	20	68.5	59.5	59.5	59.5	59.5	59.5	59.5
Cumulative blood vol (mL)	20	88.5	148	207.5	267	326.5	386	445.5

Table 1b: Schedule of visits and procedures for Birmingham volunteers – Screening will take place in Birmingham, visits 1-7 will take place in Oxford

X Event scheduled to occur

* Timeline is approximate only, as exact timings (± time windows) of visits relate to the actual (not intended) date of the previous visit

** Includes full blood count (2 mL), biochemistry (3 mL), and viral serology (5 mL)

^ Nasal swab will be taken at a minimum of 2 of these visits and a maximum of 3

^^ Faecal samples will be collected at a minimum of 1 of these visits and a maximum of 3.

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4. ABBREVIATIONS

AE	Adverse Event
ALT	Alanine Aminotransferase
ALP	Alkaline Phosphatase
BCC	Basal cell carcinoma
BCG	Bacille Calmette-Guérin
CCVTM	Centre for Clinical Vaccinology and Tropical Medicine
CI	Chief Investigator
CIS	Carcinoma in situ
CFP-10	Culture filtrate protein 10kDa
CFU	Colony-forming unit
CRF	Case Report Form
EC	Ethics Committee
E. *coli*	Escherichia coli
ELISPOT	Enzyme-Linked Immunosorbent Spot
ESAT-6	Early-secreted antigenic target 6kDa protein
FBC	Full Blood Count
GCP	Good Clinical Practice
GIA	Growth Inhibition Assay
GLP	Good Laboratory Practice
GMP	Good Manufacturing Practice
GP	General Practitioner (Family Doctor)
Group B strep	Group B streptococcus
HBsAg	Hepatitis B Surface Antigen
HBV	Hepatitis B Virus
HCG	Human Chorionic Gonadotropin
HCV	Hepatitis C Virus
HIV	Human Immunodeficiency Virus
HLA	Human Leukocyte Antigen
ICH	International Committee on Harmonisation
ID	Intradermal
IFN-ɣ	Interferon Gamma
IGRA	Interferon Gamma Release Assay
K. *pneumoniae*	Klebsiella pneumoniae
LFT	Liver Function Test
LSC	Local Safety Committee
LSM	Local Safety Monitor
M. *tb*	Mycobacterium tuberculosis
NHS	National Health Service
PBMC	Peripheral Blood Mononuclear Cells
PCR	Polymerase Chain Reaction
SAE	Serious Adverse Event
Sii	**Serum Institute of India**
SOP	Standard Operating Procedure
S. *aureus*	Staphylococcus aureus
TB	Tuberculosis
NIHR WTCRF	National Institute for Health Research Wellcome Trust Clinical Research Facility

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5. KEY ROLES AND GENERAL INFORMATION

Study Centre:
Centre for Clinical Vaccinology and Tropical Medicine (CCVTM)
Churchill Hospital, University of Oxford
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Headington
Oxford, OX3 7LE

John Warin Ward
Oxford University Hospital NHS Foundation Trust
Churchill Hospital
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The National Institute for Health Research Wellcome Trust Clinical Research Facility (NIHR WTCRF)
The Queen Elizabeth Hospital, Edgbaston
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Prof Paul Moss
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Lead Investigator:
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CCVTM

Investigators
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6. BACKGROUND AND RATIONALE

6.1. Introduction; Tuberculosis as a global health problem

Tuberculosis (TB), caused by the pathogen *Mycobacterium tuberculosis* (*M. tb*) is second only to Human Immunodeficiency Virus (HIV) as the greatest killer worldwide due to an infectious disease (1). A third of the world's population is latently infected with *M. tb*, and these people carry a 10% lifetime risk of developing active life-threatening disease (2). In 2013, an estimated 9 million people developed TB and 1.5 million people died of TB (1). Co-infection with HIV greatly increases the risk of TB reactivation and death (3, 4). TB diagnosis is challenging, and drug treatment can be prolonged, harmful, costly and complex, especially in the increasingly common context of drug resistant TB. For these reasons an effective vaccine to prevent disease is a global public health priority.

6.2. Currently available vaccines

The Bacille Calmette-Guérin (BCG) vaccine, which is live attenuated *Mycobacterium bovis,* is the only licensed TB vaccine. It provides consistent and reliable protection against childhood disseminated TB, including TB meningitis, but confers poor efficacy against pulmonary disease in the developing world. Since pulmonary TB is associated with the highest morbidity and mortality, the need to develop an effective vaccine against this disease is paramount. Improved vaccine development strategies include boosting BCG with a subunit vaccine and/or replacing BCG with a more efficacious, recombinant BCG or other attenuated whole mycobacterial vaccine (5).

6.3. Heterologous effects of BCG

Since 1927, it has been observed that BCG-vaccinated neonates have lower all-cause mortality rates (6). This heterologous or non-specific effect within the first 6-12 months of life has been demonstrated in randomised and observational studies in low income countries with high childhood mortality rates (7-13). The most consistent effect is reduced neonatal mortality due to fewer cases of neonatal sepsis, respiratory infection and fever (11). The main limitation of these studies is the risk of confounding inherent in their cross sectional and observational designs. It is essential that we determine the cogency of this effect, as potential BCG replacement vaccines must be non-inferior to BCG in this regard.

There is a plausible rationale that BCG, a replicating mycobacterium, is capable of inducing non-specific innate immunity, which could induce protection against disease and death from non-mycobacterial infections early in life (14-16). For example, intravesical BCG is an effective treatment for bladder cancer, an effect presumed to be non-specific and innate (17, 18). However, our understanding of the immunological mechanisms involved is incomplete. Data is needed from robust experiments to quantify any causal relationship between infant survival and BCG vaccination. Demonstrating an effect of recent BCG vaccination on the growth of common bacterial pathogens involved in neonatal sepsis, using whole blood in an *in-vitro* human model, would provide evidence to support a randomised controlled trial in infants in TB high burden countries and would impact on public health vaccination scheduling. In addition it would provide us with an *in-vitro* model by which to assess future BCG replacement vaccines.

6.4. Growth Inhibition Assay

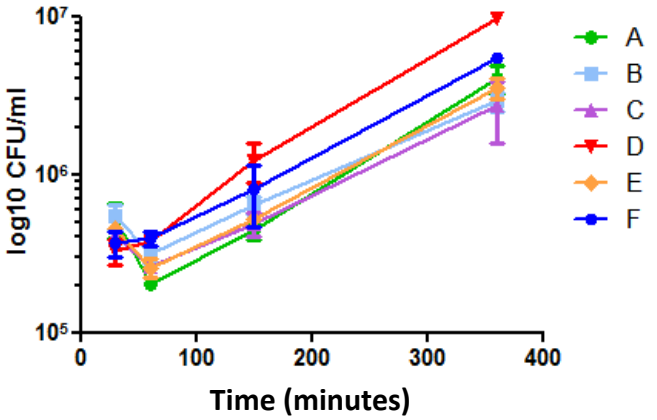
Mycobacterial growth inhibition assays (GIA) (BD MGIT BACTEC technology), a continuously monitored broth culture system, detects significant reduction in mycobacterial replication following BCG vaccination in animal models and UK adults (19, 20). The GIA approach may be generalizable to measuring the impact of BCG vaccination on other bacteria, including common causes of early neonatal sepsis in the developing world (21, 22): *S. aureus, E. coli, K. pneumoniae* and Group B *Strep*. The GIA could either use

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broth culture systems (e.g. BACTEC FX) or blood lysis – plating steps. We have proof of concept of this latter approach (Figure 1).

Pilot work has demonstrated that highly reproducible results can be obtained using repeated testing of blood from six healthy subjects and that these subjects differ markedly in their ability to control *S. aureus* growth in an *in-vitro S. aureus* GIA (Figure 1 and 2).

Figure 1: Differentiation in growth inhibition of *S.aureus* in blood taken from 6 healthy adult volunteers A-F over time in minutes



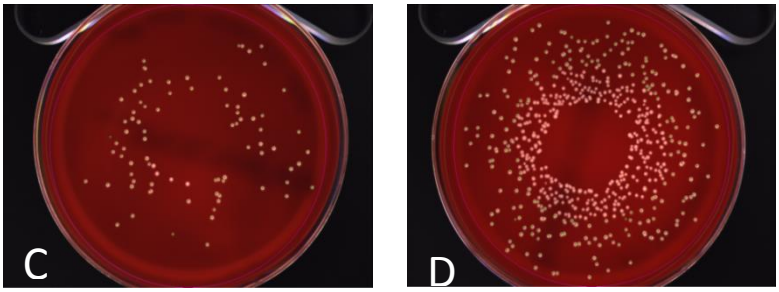
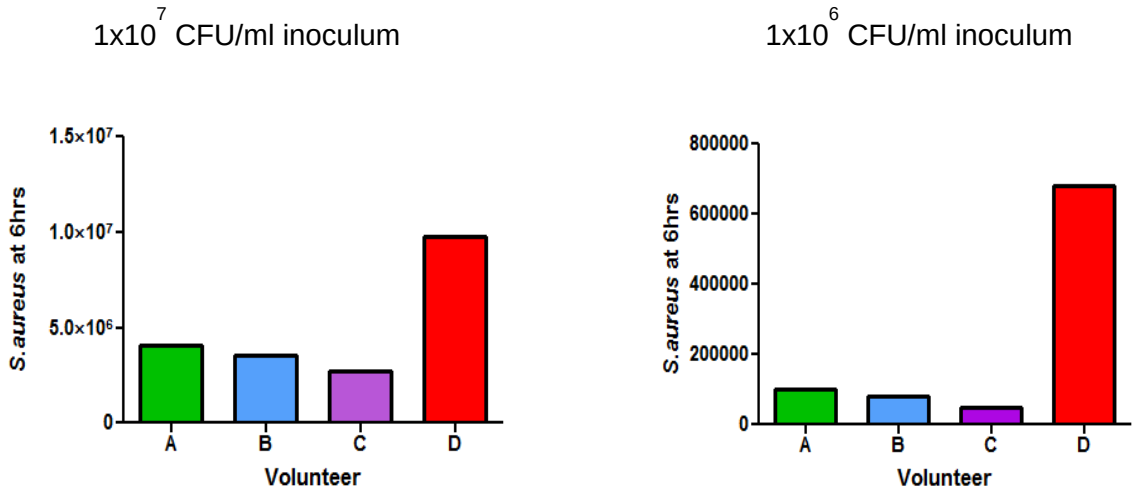


Figure 2: S. *aureus* growth in Volunteers A-D using S. *aureus* growth inhibition assay. Despite using a log difference in inoculum, the same pattern of inhibition is visible between volunteers, demonstrating that the assay is reproducible and robust.



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7. HYPOTHESIS

We hypothesise that an individual's ability to control growth of *S. aureus, K. pneumoniae*, Group B *streptococci* and *E.coli* in a whole blood *in-vitro* assay is improved following BCG vaccination, and is dependent on innate immunity.

8. OBJECTIVES AND OUTCOME MEASURES/ENDPOINTS

Objectives	**Outcome Measures/Endpoints**
Primary Objective Determine the heterologous effects of BCG vaccination in healthy UK adults using *in-vitro* GIAs as a surrogate marker to assess the individuals' capacity to control growth of *S. aureus, K. pneumonia*, Group B *streptococci* and *E.coli*.	Assessment of bacterial growth using *in-vitro* GIAs.
Secondary Objectives Evaluation of laboratory markers of the immune response that correlate with levels of bacterial growth inhibition identified using the *in-vitro* GIAs.	Established and exploratory markers of cell mediated immunity in blood samples, including transcriptomics.
Tertiary Objectives Determine background levels of *S. aureus, K. pneumoniae*, Group B *streptococci* and *E.coli* carriage and any confounding of bacterial growth.	Anti-body titres in blood serum for *S. aureus, K. pneumoniae*, Group B *streptococci* and *E.coli*, nasal swab culture for *S. aureus,* and faecal culture for E. *coli.*

Table 2: Objectives and endpoints

9. STUDY DESIGN

This is a randomised controlled clinical study using BCG vaccine at standard dose. Specific aspects of study design, and the rationale for each, are described below.

9.1. **Population**

Healthy BCG naïve adults in the UK have been selected for this study because of their low baseline level of anti-mycobacterial immunity and therefore reduced ability to suppress BCG growth. Whilst the target population for the heterologous effects of BCG vaccination is infants, the blood volume required in order to optimise the GIA would not be possible to collect from infants. Therefore by undertaking this work in healthy BCG naïve UK adults we can obtain the blood volumes required for this exploratory work in a population of individuals with a similar background mycobacterial exposure to infants in TB high burden, low income countries. The full inclusion and exclusion criteria are detailed in sections 10.2 and 10.3.

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9.2. **Study numbers**

40-48 volunteers will be recruited into the study. 8-16 of those volunteers will be non-BCG vaccinated controls. Our previous experience suggests that this sample size is a feasible number to recruit, screen, enrol, and follow up in practical terms, whilst also allowing the determination of any substantial differences in bacterial growth inhibition. Sample size calculation in this group is challenging as it is early in the assay development. It is not clear as yet how much of a reduction in bacterial growth will be significant whilst accounting for variability. We know that this assay is highly reproducible from pilot work done on 6 healthy volunteers, and sufficiently sensitive to clearly distinguish between each individual's ability to control growth of *S. aureus*. Work done in the TB vaccine group using a similar technique for mycobacterial growth inhibition demonstrated that a sample size of 15 was sufficiently powered to observe a 50% reduction in mycobacterial growth following BCG vaccination. Therefore it is expected that this sample size will be sufficient for this study.

9.3. **Groups**

Volunteers will be assigned to 1 of 2 groups as below.

Stage	Group	GIA bacterial pathogen
1	Group 1: 8 volunteers to receive BCG vaccine 2-8x10^5 cfu ID Group 2: 2-4 non-vaccinated control volunteers	S. *aureus* E. *coli*
2	Group 1: 8 volunteers to receive BCG vaccine 2-8x10^5 cfu ID Group 2: 2-4 non-vaccinated control volunteers	S. aureus E. coli
3	Group 1: 8 volunteers to receive BCG vaccine 2-8x10^5 cfu ID Group 2: 2-4 non-vaccinated control volunteers	K. *pneumoniae* Group B Strep
4	Group 1: 8 volunteers to receive BCG vaccine 2-8x10^5 cfu ID Group 2: 2-4 non-vaccinated control volunteers	K. *pneumoniae* Group B Strep

They will be enrolled in four stages, 10-12 volunteers per stage: 8 volunteers will receive BCG vaccination (group 1) and 2-4 volunteers will be non-vaccinated controls (group 2). The purpose for this is to accommodate for GIA logistics.

9.4. **Group allocation**

In order to reduce bias and the influence of any difference in the baseline characteristics in the BCG vaccinated individuals and the controls, we have chosen to randomise the allocation of the volunteers to receive either BCG vaccination or control. Three sets of envelopes will be prepared using block randomisation of 4:1, 3:1 or 2:1 of sequentially numbered sealed envelopes prepared by an independent colleague at the Centre for Statistics in Medicine, to ensure allocation concealment. Envelopes opened on day of vaccination will depend on the number of volunteers scheduled in for enrolment that day (i.e 5, 4 or 3). This will ensure that for each schedule of enrolled volunteers there is 1 control volunteer. Volunteers will not be blinded to their group allocation as there is not a suitable BCG vaccination placebo and we are not collecting data on BCG vaccine safety, therefore there is no potential for volunteer bias in reporting of adverse events. The laboratory team will be blinded to the group allocation to reduce any potential bias introduced at the sample processing stage and all samples will be anonymised.

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9.5. **BCG**

Volunteers will receive BCG vaccination at the standard dose of 2-8x10^5 cfu. BCG SSI containing *Mycobacterium bovis* strain Danish 1331 is preferred as it is licensed in the UK for vaccination. However BCG SSI can frequently go into short supply globally with impact on UK supply. In the event of this occurring, BCG vaccine supplied by the Sii will be used instead which contains *Mycobacterium bovis* BCG strain Moscow 361 I and is on the WHO list of prequalified vaccines. The same strain will be used for all volunteers.

9.6. **Choice of route**

BCG vaccination will be given using the standard intradermal technique according to site specific SOPs.

9.7. **Blood samples**

The blood volumes taken at each visit (see section 3.1) are based on the screening blood requirements, GIA, bacterial antibody titres, HLA, and planned exploratory immunological investigations.

9.8. **Study duration**

This is estimated at 12-18 months based on normal recruitment rates across the two clinical study sites.

10. PARTICIPANT IDENTIFICATION

10.1. **Study Participants**

Healthy BCG naïve, IGRA negative, HIV negative, UK adult volunteers, between the ages of 18 and 45.

10.2. **Inclusion Criteria**

Volunteers must meet all of the following criteria to enter the study:

- Healthy adult aged 18-45 years
- BCG naïve
- Resident in or near Oxford (for CCTVM and John Warin Ward) or Birmingham (for NIHR WTCRF) for the duration of the study period
- No relevant findings in medical history or on physical examination
- Allow the Investigators to discuss the volunteer's medical history with their GP
- Use effective contraception for the duration of the study period (females only)
- Agreement to refrain from blood donation during the duration of the study and for a period of 3 months after their last visit
- Give written informed consent
- Allow the Investigator to register volunteer details with a confidential database to prevent concurrent entry into clinical trials or studies
- Able and willing (in the Investigator's opinion) to comply with all the study requirements

10.3. **Exclusion criteria**

Volunteers must meet none of the following criteria to enter the study:

- Laboratory evidence at screening of latent *M. tb* infection as indicated by a positive IGRA response [a]
- Clinical, radiological, or laboratory evidence of current active TB disease [b]
- Previous vaccination with BCG, or any candidate TB vaccine

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- Within the last year had close household contact with an individual with smear positive pulmonary tuberculosis
- Clinically significant history of skin disorder, allergy, immunodeficiency (including HIV), cancer (except BCC or CIS), cardiovascular disease, respiratory disease, gastrointestinal disease, liver disease, renal disease, endocrine disorder, neurological illness, psychiatric disorder, drug or alcohol abuse
- History of serious psychiatric condition
- Concurrent oral or systemic steroid medication or the concurrent use of other immunosuppressive agents
- Concurrent use of long term antibiotic therapy
- History of anaphylaxis to vaccination or any allergy likely to be exacerbated by any component of the BCG vaccine
- Any abnormality of screening blood or urine tests that is deemed to be clinically significant or that may compromise the safety of the volunteer in the study[b]
- Positive HBsAg, HCV or HIV antibodies
- Female confirmed pregnant or intention to become pregnant during study period, or currently lactating
- Current involvement in another study or trial that involves regular blood tests or an investigational medicinal product[c]
- Use of an investigational medicinal product or non-registered drug, live vaccine, or investigational medical device for four weeks prior to dosing with the study vaccine
- Administration of immunoglobulins and/or any blood products within the three months preceding the planned challenge date
- Any other significant disease, disorder, or finding, which, in the opinion of the Investigator, may either put the volunteer at risk, or may influence the result of the study, or may affect the volunteer's ability to participate in the study

[a] Volunteers discovered to have evidence of latent M. *tb* infection as defined by a positive IGRA will be referred for a plain chest x-ray. If there is any evidence of active TB disease either on clinical or radiological grounds, further investigation and treatment will be offered under the supervision of a consultant physician in respiratory medicine or infectious diseases.

[b] Volunteers who are excluded from the study because they have been discovered to have a previously undiagnosed condition thought to require further medical attention will be referred appropriately to their GP or an NHS specialist service for further investigation and treatment.

[c] Volunteers will be excluded from the study if they are concurrently involved in another study or trial that involves regular blood tests or an investigational medicinal product. In order to check this, volunteers will be asked to provide their National Insurance or Passport number (if they are not entitled to a NI number) and will be registered on a national database of participants in clinical trials (www.tops.org.uk).

11. STUDY PROCEDURES

See the study schedule of visits and procedures in section 3.1.

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11.1. **Recruitment**

Volunteers may be recruited by use of an advertisement formally approved by the ethics committee and distributed or posted in the following places:

- In public places (including NHS hospitals and university buildings) with the agreement of the owner or proprietor
- In newspapers or other literature for circulation
- On radio via announcements
- On a website operated by our group or with the agreement of the owner or operator
- By e-mail distribution to a group or list only with the express agreement of the network administrator or with equivalent authorisation
- On stalls or stands at exhibitions or fairs
- Direct mail-out: this will involve obtaining names and addresses of adults via the most recent Electoral Roll. The contact details of individuals who have indicated that they do not wish to receive postal mail-shots would be removed prior to the Investigators being given this information. The company providing this service is registered under the Data Protection Act 1998. Investigators would not be given dates of birth or ages of individuals, but the list supplied would only contain names of those aged between 18-45 years (as per the inclusion criteria).
- Oxford Vaccine Centre databases: We may contact individuals from databases of groups within the CCVTM (including the Oxford Vaccine Centre database) of previous study and trial participants who have expressed an interest in receiving information about all future studies for which they may be eligible.

A copy of intended advertising will be submitted with the initial application for ethical approval and any significant changes to this advertisement will be submitted as an amendment to the ethics committee for approval before use.

11.2. **Informed Consent**

Written informed consent will be obtained at screening by a GCP trained clinician.

The volunteer must personally sign and date the latest approved version of the Informed Consent Form before any study specific procedures are performed.

Written and verbal versions of the volunteer information and informed consent will be presented to the volunteers detailing no less than:

- the exact nature of the study
- what it will involve for the volunteer
- the implications and constraints of the protocol
- the known side effects and any risks involved in taking part

It will be clearly stated that the volunteer is free to withdraw from the study at any time for any reason without prejudice to future care, and with no obligation to give the reason for withdrawal.

The volunteer will be allowed as much time as wished to consider the information, and the opportunity to question the Investigator, their GP or other independent parties to decide whether they will participate in the study. A copy of the signed Informed Consent will be given to the volunteer. The original signed form will be retained at the study site in the CRF.

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11.3. **Screening and Eligibility Assessment**

Volunteers will be offered a screening visit at their respective study site if they express an interest in participating in the study. They will be assigned a volunteer identifier number and provided with and asked to read the volunteer information sheet at least 24 hours prior to attending for screening. At the screening visit the general process of screening will be outlined, and all questions about the screening process and the study answered. The following general principles will be emphasised:

- Participation in the study is entirely voluntary
- Refusal to participate involves no penalty or loss of medical benefits
- The volunteer may withdraw from the study at any time
- The volunteer is free to ask questions at any time to allow him or her to understand the purpose of the study and the procedures involved
- There is no direct benefit from participating
- The volunteer's GP will be contacted to corroborate their medical history and confirm that the volunteer does not meet any of the exclusion criteria.
- The volunteer will be registered on the TOPS database (The Over-volunteering Prevention System)
- The volunteer's blood samples taken as part of the study will be stored indefinitely and samples may be sent outside of the UK and Europe to laboratories in collaboration with the University of Oxford. These will be anonymised.

The aims of the study and all tests to be carried out will be explained by the Investigator. The volunteer will be given the opportunity to ask about details of the study, and will then have time to consider whether or not to participate. If they do decide to participate, they will sign and personally date two copies of the Informed Consent Form, one for them to take away and keep, and one for the Investigator. These forms will also be signed and dated by the Investigator.

Having agreed to undergo screening, a baseline medical history (including concomitant medication) and physical examination will be performed. Inclusion and exclusion criteria will be checked. Demographic, occupational and travel information relating to risk of prior TB exposure will be collected. Vital signs will be checked (blood pressure, pulse and temperature). Volunteers will be counselled by one of the Investigators for HIV, Hepatitis B and Hepatitis C testing. Blood tests (as specified in the schedule of visits and procedures in section 3.1) will also be taken to assess renal function, liver function, full blood count and IGRA for identification of latent TB infection. Urinalysis will be performed. A pregnancy test will be performed on all female volunteers. A nasal swab to assess for S. *aureus* carriage will be performed. The total duration of the screening visit will be approximately two hours.

Volunteers will be informed that there may be leftover samples of their blood. With the volunteers' informed consent, any leftover cells and serum/plasma will be frozen indefinitely for future immunological analysis of BCG-specific or vaccine-related responses. This may include human DNA and RNA analysis to search for correlates of vaccine immunogenicity and efficacy. Samples may be shipped to other parties involved in this study in anonymised form for immunological analysis. Volunteers will be able to decide if they will permit such future use of any leftover samples. If they elect not to permit this, all of those leftover samples will be discarded after the required period of storage to meet GCP and regulatory requirements.

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After satisfactory screening, the volunteer's GP will be contacted (with the written permission of the volunteer) as notification that the volunteer has volunteered to take part in the study and to ascertain any significant medical history.

Enrolment will take place no longer than 120 days following the date of the screening appointment. If more than 120 days elapse, the screening visit will be repeated in full prior to enrolment in order to minimise the risk to volunteers of any new unidentified health problems having arisen during that period.

11.4. **Enrolment**

Volunteers who fulfil the screening criteria, satisfy all the inclusion criteria, and meet none of the exclusion criteria will be enrolled into the study and their visits scheduled in the calendar. The ongoing eligibility of the volunteer will be reviewed on the day of enrolment and any new events, medications, or changes to the screening documents recorded.

See section 3.1 for the visit schedule.

11.5. **Day of vaccination (Day 0)**

All volunteers (Oxford and Birmingham) will attend the CCVTM in Oxford for this and all follow-up visits. Any new medical issues or symptoms that have arisen will be assessed on Day 0, vaccination day. Urinary pregnancy test for female volunteers, a nasal swab for S. *aureus* carriage, and venepuncture for GIA, exploratory immunology, S. *aureus, E. coli, K. pneumoniae* and Group B strep antibody titres, HLA profile, FBC and Paxgene tube for transcriptomics will be undertaken according to the table in section 3.1. Volunteers will also be asked to submit a stool sample for *E.coli, K. pneumoniae* and Group B strep carriage assessment. The inclusion and exclusion criteria for the study will be reviewed. Provided that the volunteer still satisfies all inclusion criteria (and no exclusion criteria) and their consent remains valid, the volunteer will be randomised to either BCG or control group. BCG vaccination will be performed by the intradermal route according to the site specific SOP. Volunteers will be kept under observation for 15-20 minutes after vaccination as per SmPC. The vaccination visit will last approximately 90 minutes.

11.6. **Follow-up visits for venepuncture**

Volunteers will attend the clinic for venepuncture as outlined in section 3.1. Volunteers will be asked if they have experienced any illness since last visit and did this illness require antibiotic use (as this would affect their GIA results). Visits will take 20-30 minutes. Those who receive BCG will have appropriate review of vaccination site. As per section 3.1, volunteers may be asked to submit a faecal sample on Day 7 and 14 depending on the result of their first sample. All bloods must be taken in Oxford to prevent unnecessary delay and potential confounding incurred in transportation of the blood samples between sites. With a reduced travel time involved for Oxford volunteers they will have 2 extra follow-up visits at day 4 and 10. The rational for this is day 2, 7, 14, 21, 28, and 84 are the most important time-points to see the anticipated non-specific effects of BCG post vaccination whilst taking into consideration logistical aspects of undertaking the GIA, but also ensuring that travel time incurred for Birmingham volunteers to attend visits in Oxford is not unachievable. As this work is exploratory the additional follow-up visits would only need to be undertaken in a small number of volunteers and can be obtained from the Oxford volunteers to allow evaluation of any potential benefit of these more frequent early time-points.

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11.7. **Discontinuation/Withdrawal of volunteers from the study**

Each volunteer has the right to withdraw from the study at any time. In addition, the Investigator may discontinue a volunteer from the study at any time if the Investigator considers it necessary for any reason including:

- Confirmed pregnancy during the study
- Ineligibility (either arising during the study or retrospectively having been overlooked at screening)
- Significant protocol deviation
- Any adverse event which results in inability to comply with study procedures
- Significant non-compliance with treatment regimen or study requirements
- Withdrawal of consent
- Loss to follow up (applies to a volunteer who does not return for protocol study visits, is not reachable by telephone or other means of communication and/or is not able to be located)
- On the advice of the Local Safety Committee

The reason for withdrawal will be recorded in the CRF. Volunteers withdrawn from the study may be replaced if possible. The extent of follow up after premature discontinuation will be determined by the Investigator but will be at least for the whole study period, and if pregnant, until pregnancy outcome. Withdrawal from the study will not exclude the volunteer's data from analysis.

11.8. **Delay in BCG vaccination**

BCG vaccination will not proceed on the scheduled day in any of the following situations:

- The volunteer has a temperature > 37.5^{0}C
- The Investigator judges the volunteer to have an acute moderate or severe illness (whether febrile or not)
- The volunteer has received a live non-study vaccine within the preceding four weeks
- The volunteer has received a course of antibiotics within the preceding four weeks
- The Investigator has any other concern that vaccination with BCG may not be in the volunteer's best interests

In this case the volunteer may be vaccinated with BCG at a later date or withdrawn from the study at the discretion of the Investigator.

Every reasonable effort will be made to maintain protocol compliance and participation in the study. If a volunteer is prematurely withdrawn from the study for any reason (detailed in section 11.7), the reason for early study withdrawal will be recorded in the CRF. Reasons for a discontinuation of the study are detailed in sections 11.7.

11.9. **Definition of End of Study**

The Study will be completed when the last volunteer enrolled into the study has completed their final follow up visit.

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12. RISKS AND BENEFITS

12.1. **Potential risks**

The potential risks to participants in this study include risks associated with:

Phlebotomy

The total amount of blood collected over the study period will be a maximum of 565mL for Oxford volunteers and 446mL for Birmingham volunteers (due to fewer visits). Neither amount should compromise otherwise healthy volunteers, who can donate 470mL during a single blood donation for the National Blood transfusion Service over a 3-4 month period. Volunteers will be asked to refrain from blood donation for the duration of their involvement in the study and for a period of 3 months after their last visit. Risks occasionally associated with venepuncture include pain and bruising at the site of venepuncture, light-headedness, and syncope (rarely).

Nasal Swabs

Two to three nasal swabs will be taken from each volunteer to assess for the presence of S. *aureus* carriage. This is a painless and safe procedure.

Faecal sample

Volunteers will be provided with a specimen pot and gloves. Following appropriate instruction they will be asked to provide a faecal sample on 1 or all of Day 0, 7 and 14. This is a painless and safe specimen collection procedure. This will be used to detect for carriage status of *E. coli, K. pneumoniae* and Group B *Strep.*

Vaccination with BCG

BCG SSI is a licensed vaccine that has been given to over 3 billion people throughout the world since it was first developed in 1921. Full details are given in the SmPCs. BCG Sii is on the WHO list of pre-qualified vaccines and has a well-defined side effect profile as outlined in the SmPC that is similar to that of BCG SSI.

<u>Local reaction from intradermal BCG injection</u>

An inflammatory reaction manifested by redness and swelling is expected to occur at the site of injection, followed by a local lesion that may ulcerate. This heals over some weeks to months to leave a small flat scar. It is also possible to develop some swelling (<1cm diameter) of the axillary lymph nodes.

Uncommonly (less than 1 in 100 people) swelling of axillary lymph nodes to more than 1cm across, or an ulcer that discharges fluid at the injection site, may occur.

Rare side effects (less than 1 in 1000 people) include inflammation of lymph nodes leading to abscesses and discharge of fluid from the swellings.

<u>Systemic reactions</u>

Systemic reactions to BCG are rare (less than 1 in 1000 people) and include low-grade fever, headache, and allergic reactions. Disseminated complications of BCG, such as bone infections, have also been reported, but are extremely rare and have usually been reported in immunocompromised, not immunocompetent, individuals.

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Allergic reactions

Allergic reactions from mild to severe may occur in response to any constituent of a medicinal product. Anaphylaxis is extremely rare but can occur.

12.2. **Known potential benefits**

Volunteers are not expected to benefit directly from participation in this study. Volunteers will however gain some information about their general health as a result of the screening history, examination, blood tests and urine tests. It is hoped that their contribution will further develop our understanding of the heterologous effects of BCG, and in turn enable us to develop a model by which we can improve the design of replacement BCG vaccines

13. INTERVENTION – BCG Vaccination

13.1. **Formulation**

BCG SSI contains live attenuated Danish strain 1331 *Mycobacterium bovis* BCG. It is supplied as a powder and solvent for suspension. Each vial contains 2-8 x 10^5 cfu (see SmPC). BCG Sii contains live attenuated *Mycobaterium bovis* BCG Moscow strain 361I. It is supplied as a powder and solvent for suspension. Each vial contains 2-8 x 10^5 cfu (see SmPC).

13.2. **Vaccine supply**

BCG SSI will be supplied to the CCVTM in Oxford from the Churchill Hospital pharmacy, if available nationally. BCG Sii will be shipped to the CCVTM. It will be shipped at between +2°C and +8°C to ensure cold chain.

All movements of the study vaccine will be documented. Vaccine accountability, storage, shipment and handling will be in accordance with local SOPs.

13.3. **Vaccine storage**

Dispensed BCG vials are supplied in boxes containing multiple vials and each vial is clearly labelled with the market product (BCG SSI, or BCG Sii).

All vaccines to be administered to the volunteers will be stored in a safe and locked place with no access by unauthorised personnel. The BCG will be stored between +2°C and +8°C in a monitored fridge. The storage conditions will be the responsibility of the clinical site.

Any temperature deviation outside the range +2°C to 8°C will be reported to the CI as soon as detected. Following an exposure to such a temperature deviation, vaccines will not be used until CI approval has been given.

13.4. **Vaccine administration**

BCG will be administered as follows:

The correct volume will be drawn up and administered intradermally over the deltoid region of the non-dominant arm, according to the site specific SOP. During the administration of the vaccine, resuscitation medicines and equipment will be immediately available for the management of anaphylaxis.

After vaccination volunteers will be monitored for 15-20 minutes, as per SmPC, and will then be permitted to leave the site.

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13.5. **Vaccine accountability**

To ensure integrity and accountability for the BCG, the vaccine will be stored, checked and recorded, and disposed or returned according to the site specific SOP.

14. SAFETY REPORTING

The risks and benefits of participating in this study are detailed in section 12. The safety profile of BCG vaccination is well understood and therefore we will not be routinely collecting information regarding safety of the vaccine. Only information relating to SAEs will be collected.

14.1. **Definitions**

Adverse Event (AE)	Any untoward medical occurrence in a volunteer administered a medicinal product and which does not necessarily have a causal relationship with this treatment. An adverse event can therefore be any unfavourable and unintended sign (including an abnormal laboratory finding, for example), symptom or disease temporally associated with the use of a medicinal product, whether or not considered related to the medicinal product.
Serious Adverse Event (SAE)	A serious adverse event is any untoward medical occurrence that: • results in death • is life-threatening • requires inpatient hospitalisation or prolongation of existing hospitalisation • results in persistent or significant disability/incapacity • consists of a congenital anomaly or birth defect. Other 'important medical events' may also be considered serious if they jeopardise the participant or require an intervention to prevent one of the above consequences. NOTE: The term "life-threatening" in the definition of "serious" refers to an event in which the participant was at risk of death at the time of the event; it does not refer to an event which hypothetically might have caused death if it were more severe.

Table 4: Definitions

14.2. **Reporting Procedures for Serious Adverse Reactions**

As BCG SSI is a vaccine with Marketing Authorisation, the mechanism for reporting any SARs to the MHRA is via yellow card. Should BCG Sii be used in the event that BCG SSI is in short supply, SARs will be reported using the same mechanism and Sii will be notified of the event.

14.3. **Safety Monitoring Committee**

A Local Safety Committee (LSC) will be appointed to provide real-time safety oversight. The LSC will review SAEs deemed possibly, probably or definitely related to study interventions. The LSC will be notified within 24 hours of the Investigators' being aware of their occurrence. The LSC has the power to

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terminate the study if deemed necessary following a study intervention-related SAE. At the time of writing the LSC will be chaired by Dr Brian Angus, a Clinical Tutor in Medicine, Honorary Consultant Physician and Director, Centre for Tropical Medicine at the University of Oxford. There will be a minimum of two other appropriately qualified committee members.

The chair of the LSC may be contacted for advice and independent review by the Investigator or study Sponsor in the following situations:

- Following any SAE deemed to be possibly, probably, or definitely related to a study intervention
- Any other situation where the Investigator or study sponsor feels independent advice or review is important

14.4. **Pregnancy**

Volunteers who become pregnant during the study after the BCG vaccination may continue study procedures, including venepuncture, if appropriate, at the discretion of the Investigator. Volunteers who become pregnant before vaccination with BCG will be withdrawn and will not be vaccinated. The Investigator will collect pregnancy information on any volunteer who becomes pregnant while participating in this study. The volunteer will be followed up to determine the outcome of the pregnancy.

14.5. **Discontinuation of study**

The study will be discontinued in the event that we were unable to recruit the required number of volunteers in the specified timeframe.

15. STATISTICS AND ANALYSIS

15.1. **The Number of Volunteers**

This is primarily an exploratory study, with 40-48 volunteers being recruited. There will be 16 BCG vaccinated volunteers recruited for each pair of bacterial pathogens assessed using the GIA as highlighted in section 9.3. To allow for the logistics involved in undertaking the GIA this will be carried out in 4 stages. Our previous experience suggests that this sample size is a feasible number to recruit, screen, enrol, and follow up in practical terms, whilst also allowing the determination of any substantial differences in GIA. Our research groups experience with mycobacterial GIA (a similar technique) has shown that a sample size of 15 was sufficiently powered to show a statistically significant reduction in mycobacterial growth following BCG vaccination. As discussed in section 9.2 sample size calculation in this study is challenging as it is early in the assay development and it is not clear as yet how much of a reduction in bacterial growth will be significant whilst accounting for variability. We know that this assay is highly reproducible from pilot work done on 6 healthy volunteers, and sufficiently sensitive to clearly distinguish between each individual's ability to control growth of S. aureus. Therefore it is expected that a sample size of 16 BCG vaccinated volunteers per bacterial pathogen will be sufficient to see a statistically significant reduction in growth of the aforementioned bacteria in this study.

15.2. **Statistical methods for Analysis of Outcome Measures/Endpoints**

One-way analysis of variance (ANOVA) and Students *t* tests (or appropriate non-parametric tests) will be used to determine significant differences in growth inhibition. Analysis of paired data will allow the effect in an individual to control for confounding. Grouped immunological data will be assumed to be non-parametric and summarised by medians and inter-quartile ranges. Between-group comparisons will be made using the Mann-Whitney U test. The Wilcoxon Matched Pairs test will be used to make paired

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comparisons over time within groups. Pearson's test of correlation will be used to analyse associations between the primary endpoint and secondary immunological outcomes.

16. DATA MANAGEMENT

The Chief Investigator will be responsible for collecting, recording, analysing, and storing all the data accruing from the study. These tasks may be delegated to other Investigators. Study data will be captured and stored in CRFs held in a key-locked cabinet at the CCVTM or NIHR WTCRF (in the case of paper source data) and/or electronically on the OpenClinica database on secure servers which are outsourced by Openclinica™. All clinical sites will use OpenClinica, however Oxford is responsible for the data. Some data may be duplicated anonymously into an electronic Microsoft Excel™ file also stored on a secure server.

Access to the source data documents will be provided if necessary to the Ethics Committee and to the Sponsor for study-related monitoring and audit. All information relating to the study and its subjects will be held in strict confidence, and in accordance with GCP and institutional requirements.

Study records will be held by the Investigator for as long as required by legislation, initially in a key-locked cabinet at the CCVTM or NIHR WTCRF and subsequently in a secure archive facility, and in accordance with the Data Protection Act. Subjects will be assigned individual unique study numbers for identification on all study records, except where the use of identifiable information is unavoidable (including on GP correspondence, registration documents, and consent forms).

17. QUALITY ASSURANCE PROCEDURES

17.1. **Investigator Procedures**

Approved site-specific SOPs will be used at all clinical and laboratory sites.

17.2. **Modifications to Protocol**

No substantial amendments to this protocol will be made without consultation with, and agreement of, the Sponsor. Any amendments to the study that appear necessary during the course of the study will be discussed by the Investigator and Sponsor concurrently. If agreement is reached concerning the need for an amendment, it will be produced in writing by the Chief Investigator and will be made a formal part of the protocol following ethical and R&D approval. R&D will also be informed of any administrative changes of protocol.

17.3. **Protocol Deviations**

All deviations from the protocol will be documented in a protocol deviation form and filed in the trial master file.

17.4. **Monitoring**

The study will be conducted in accordance with procedures identified in the protocol. Regular monitoring will be performed according to principles of GCP by an internal independent monitor. According to applicable SOPs and the study monitoring plan, the Monitors will verify that the clinical study is initiated, conducted and completed, and data are generated, documented and reported in compliance with the protocol and GCP.

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18. SERIOUS BREACHES

A serious breach is defined as "A breach of GCP or the study protocol which is likely to affect to a significant degree:

- the safety or physical or mental integrity of the subjects of the study
- the scientific value of the study"

In the event that a serious breach is suspected, the Sponsor will be contacted as soon as possible.

19. ETHICAL AND REGULATORY CONSIDERATIONS

19.1. **Declaration of Helsinki**

This study will be conducted in accordance with the principles of the Declaration of Helsinki.

19.2. **Guidelines for Good Clinical Practice**

This study will be conducted in full conformity with relevant regulations and with the Guidelines for Good Clinical Practice.

19.3. **Approvals**

The protocol, informed consent form, volunteer information sheet, faecal sample collection instructions, GP letter, and proposed advertising material will be submitted to REC and R&D for written approval.

The Investigator will seek approval from the REC and R&D where required for all substantial amendments to the original approved documents.

19.4. **Reporting**

The CI shall submit once a year throughout the study, or on request, an Annual Progress report to the REC Committee, host organisation and Sponsor. In addition, an End of Study notification and final report will be submitted to the same parties.

19.5. **Participant Confidentiality**

The study staff will ensure that the volunteer' anonymity is maintained. The volunteers will be identified only by a volunteers ID number on the CRF and any electronic database unless the use of identifiable information is unavoidable (including on GP correspondence, registration documents, bank details request form and consent forms). Bank details will be requested using a bank details request form and stored on a secure electronic database and destroyed at the end of the study. All documents will be stored securely and only accessible by study staff and authorised personnel. The study will comply with the Data Protection Act, which requires data to be anonymised as soon as it is practical to do so.

19.6. **Expenses and Benefits**

Volunteers will be compensated pro rata for their time and travel and for study procedures while participating in the study, amounting to a total of approximately £320 for Oxford volunteers and £440 for Birmingham volunteers due to the additional time incurred due to travel. If extra visits are required, compensation will be adjusted accordingly. Volunteers will be compensated via BACS within 6 weeks of their completion of the study.

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20. FINANCE AND INSURANCE

20.1. **Funding**

This study will be financed by funding from the Bill and Melinda Gates Foundation and The Wellcome Trust.

20.2. **Insurance and Indemnity**

The University has a specialist insurance policy in place, which would operate in the event of any participant suffering harm as a result of their involvement in the research (Newline Underwriting Management Ltd, at Lloyd's of London). Oxford University Investigators participating in this study will receive insurance coverage from the University clinical trials insurance policy.

21. PUBLICATION POLICY

The Investigators will be involved in reviewing drafts of the manuscripts, abstracts, press releases and any other publications arising from the study. Authors will acknowledge that the study was funded by the Bill and Melinda Gates Foundation and The Wellcome Trust. Authorship will be determined in accordance with the International Committee of Medical Journal Editors guidelines and other contributors will be acknowledged.

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22. APPENDIX

APPENDIX A: Laboratory values for exclusion

The following reference ranges (based on Oxford University Hospitals NHS Foundation Trust laboratory reference ranges) are provided for the purpose of guidance only for Investigators during the study.

PARAMETER	**LOWER LIMIT OF EXCLUSION**	**UPPER LIMIT OF EXCLUSION**
BIOCHEMISTRY		
Potassium [mmol/L]	<3.2	>5.5
Sodium [mmol/L]	<132	>148
Urea [mmol/L]	N/A	>9
Creatinine [μmol/L]	N/A	>145
Albumin [g/L]	<30	N/A
Total bilirubin [μmol/L]	N/A	>19 when accompanied by elevated liver enzyme
ALT [IU/L]	N/A	>56
ALP [IU/L]	N/A	>131
HAEMATOLOGY		
Haemoglobin [g/L]	Male: < 110 Female: < 100	Male: > 175 Female: >170
White Cell Count [x 10^9/L] Neutrophil count [x 10^9/L]	<3.5 <1.5	>14.0
Platelet Count [x 10^9/L]	<136	>500
URINE ANALYSIS (using MULTISTIX * 10 SG Bayer Diagnostics)		
Protein		2+ or 0.5-1gm loss/day
Blood		1+ on 2 separate readings
Glucose		1+

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APPENDIX B: Laboratory values for exclusion

The following reference ranges (based on Birmingham University Hospitals NHS Trust laboratory reference ranges) are provided for the purpose of guidance only for Investigators during the study.

PARAMETER	**LOWER LIMIT OF EXCLUSION**	**UPPER LIMIT OF EXCLUSION**
BIOCHEMISTRY		
Potassium [mmol/L]	<3.1	>5.5
Sodium [mmol/L]	<131	>149
Urea [mmol/L]	N/A	>9.5
Creatinine [μmol/L]	N/A	>101
Albumin [g/L]	<29	N/A
Total bilirubin [μmol/L]	N/A	>25 when accompanied by elevated liver enzyme
ALT [IU/L]	N/A	>51
ALP [IU/L]	N/A	>350
HAEMATOLOGY		
Haemoglobin [g/L]	Male: < 115 Female: < 105	Male: > 180 Female: >175
White Cell Count [x 10^9/L] Neutrophil count [x 10^9/L]	<3.5 <1.5	>14.0
Platelet Count [x 10^9/L]	<136	>500
URINE ANALYSIS (using MULTISTIX * 10 SG Bayer Diagnostics)		
Protein		2+ or 0.5-1gm loss/day
Blood		1+ on 2 separate readings
Glucose		1+

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APPENDIX C: AMENDMENT HISTORY

Protocol Version No.	Author(s) of changes	Details of Changes made
2.0	M Wilkie	1. Throughout document – Spelling and grammatical changes and BCG SSI changed to BCG vaccine 2. Section 3 Synopsis – Addition of John Warin Ward OUH NHS Trust site, BCG SSI changed to BCG vaccine and dose corrected to 2-8x10^5 cfu 3. Section 3.1 Schedule of visits and procedures – Table 1 changed to Table 1a schedule of visits and procedures for Oxford volunteers and addition of Table 1b schedule of visits and procedures for Birmingham volunteers 4. Section 4 Abbreviations – updated to include Serum Institute India 5. Section 5 Key roles and general information – Addition of John Warin Ward OUH NHS Trust site 6. Section 9.3 Groups – Table updated, BCG SSI changed to BCG vaccine and dose corrected to 2-8x10^5 cfu 7. Section 9.5 BCG – Section amended to reflect addition of potential use of BCG Sii *Mycobacterium bovis* BCG Moscow strain 361 I in the event of BCG SSI UK shortage 8. Section 11.5 Day of vaccination (Day 0) – Additional explanation that all visits excluding screening will take place at the CCVTM Oxford for Oxford and Birmingham volunteers and additional of '*K. pneumoniae* and Group B strep' to list of bacteria to be assessed on faecal culture, accidental omission from initial protocol 9. Section 11.6 Follow-up visits for venepuncture – Additional section outlining reason that all volunteers need to attend the CCVTM for venepuncture and also explaining reason for difference in schedule of visits for Oxford and Birmingham volunteers 10. Section 12.1 Potential risks – Phlebotomy section updated re blood volumes, BCG vaccination section updated regarding BCG Moscow strain 11. Section 13.1 Formulation – updated to include BCG Moscow strain 12. Section 13.2 Vaccine supply – Addition of supply of BCG Moscow strain and removal of storage of BCG in NIHR WTCRF 13. Section 14.2 Reporting procedures for serious adverse reactions – Updated section to outline plan for reporting of SARs for BCG Sii 14. Section 19.5 Participant confidentiality – Updated section to include use

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		of bank details request form 15. Section 19.5 Expenses and benefits – Section updated to reflect new Birmingham volunteer reimbursement and addition of BACS payment method 16. Appendix A and Appendix B – updated exclusion range for urinalysis from 2+ to 1+ on 2 separate readings and updated unit of measurement for haemoglobin specifically in Appendix B
2.1	M Wilkie	Section 10.3 Exclusion criteria - Non-substantial amendment to incorporate the change of screening test for Latent TB infection in Birmingham from ELISpot to ELISA using the term IGRA which encompasses both assays, this will not affect scientific outcomes.
3.0	M Wilkie	1. Section 3 Synopsis – Section updated to reflect new number of volunteers to be recruited 2. Section 3.1 Schedule of visits and procedures – Section updated to reflect addition of FBC at each visit and new blood volumes 3. Section 9.2 Study numbers – Section updated to reflect new number of volunteers to be recruited 4. Section 9.3 Groups – Section updated to reflect new number of volunteers to be recruited 5. Section 9.4 Group Allocation – Section updated to reflect change to number of volunteers to be recruited and subsequent change to block randomisation to accommodate enrolling smaller numbers on a single schedule 6. Section 11.5 Day of vaccination (day 0) – Section updated with removal of block randomisation 4:1 to reflect potential for either 2:1 or 3:1 block randomisation depending on number of volunteers enrolled on that particular schedule, and updated to reflect addition of FBC on that visit 7. Section 12.1 Potential risks (phlebotomy) – Section updated to reflect updated total blood volumes 8. Section 15.1 The number of volunteers – Section updated to reflect new number of volunteers to be recruited and the clarification of number of volunteers allocated to bacterial pathogens 9. Section 16 Data Management – Section updated to reflect upgrade to the OpenClinica electronic database. 10. Throughout document – minor spelling changes
3.1	H. Johnson	Throughout document – Oxford University Hospital NHS Trust changed to Oxford University Hospital NHS Foundation Trust

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