Natural and vaccine mediated correlates of protection against enteric fever

Elizabeth Jones

St Edmund's Hall

University of Oxford

A thesis submitted for the degree of

Doctor of Philosophy

Paediatrics

Hilary Term 2024

Supervisors: Professor Sir Andrew Pollard, Dr Elizabeth Clutterbuck, and Dr Adriana Tomic

Table of Contents

Acknowledgements

Firstly, I would like to thank all the amazing volunteers who have dedicated their time to participate in the clinical trials included in this thesis. This research would not be possible without their commitment.

I am grateful to my supervisor, Professor Sir Andrew Pollard, for the opportunity to work on this project and I would like to thank him for supporting me while I am research assistant in the OVG laboratory. I am also grateful for the support and supervision from Dr Elizabeth Clutterbuck. Her immunology knowledge is second to none! I would like to thank her for her valuable contributions to this thesis and for her support in navigating the balance between RA work, management work, and research all while going through a pandemic. I would also like to thank my co-supervisor, Dr Adriana Tomic, she has provided a valuable perspective to this project and I am grateful for her guidance.

I would also like share my thanks to Dr Jennifer Hill and Dr Lisa Stockdale, my initial supervisors. They are two incredible scientists who provided much support at the beginning of this project and gave my research confidence a significant boost by supporting me in publishing my first paper. I would simultaneously like to express my gratitude for their support during the COVID-19 pandemic.

I am incredibly lucky to work with so many gifted individuals at the Oxford vaccine group. Due to the complex nature of the *Salmonella* challenge trials, they would not be possible without the dedication and hard work from many individuals. I would like to thank the clinical and admin teams, (past and present – of whom there are far too many to name), for their incredible hard work and delivery of the clinical trials.

This work would also not be possible without the wonderful lab team (past and present) who put in exceptional effort processing all the samples and generally make the lab a great place to be. In particular, I would like to thank Dr Susana Camara, Sonu Shrestha, Dr Sagida Bibi, Dr Jennifer Hill, Dr Lisa Stockdale, Dr Helena Thomaides-Brears, Dr Helena Juel, Harri Hughes, Soumya Parinparajah,

Alex Maytum, Jaclyn Bowman, Kat Sanders, Sally Felle, Sarthak Sahoo, Nicole Day, Tanya Dinesh, Amy Flaxman, Florence McLean, and Spyridoula Marianou.

It has been a privilege to work with Dr Sagida Bibi. We started one week apart, over 9 years ago and her support has never been far from my side. I have learnt a lot from her during these years, her honesty and sense of humour put any problem into perspective.

I would also like to thank my *Salmonella* sister, Dr Mari Johnson. Our mutual love of science and dogs created a bond like no other. I feel lucky that our time at OVG overlapped for as long as it did, hopefully it was long enough for me to absorb some of her wisdom.

I would like to thank my family; my Dad (Hywel), Stepmum (Steph), Mum (Dawn), and my Grandparents (Hazel and Gordon, and Mansel and Rose). One of my biggest thank yous is reserved for my wonderful sister, Louise, she is the best sister that anyone could wish for.

The list of those to whom I owe my gratitude would not be complete without sharing my thanks to my wonderful friends, and of course, my Berry Mugfins and Cardi-gang; Lucy Cornell, Giannhs Spanos, Aleksei Malyshev, and Marie-Claire Jalaguier. I am so glad that Teddy Hall brought us together, you made life during these unprecedented times bearable. What's the most important thing we've learnt during this time? Potatoes are delicious in any form.

Lastly, I would like to thank my dear friend Daisy. Our friendship is one of the best things to come out of my time at OVG, she is a kind and intelligent person and without her support over the last few years there would be many more blank pages in this thesis.

Declaration of contribution

The *Salmonella* challenge trials are complex and required the input from many people at Oxford vaccine group. In particular, the paratyphoid trial (P1 1013/07) was set up by Dr Hazel Dobinson, and the paratyphoid challenge – rechallenge study (PATCH 2014/01) was lead by Dr Malick Gibani. The VAST study (2014/08) was lead by Dr Celina jin. Oversight of the challenge trials was provided by Professor Sir Andrew Pollard and Professor Brian Angus.

The phase I study investigating the safety and immunogenicity of the typhoid-paratyphoid bivalent vaccine (Sii-PTCV) was conducted by Serum Institute of India Private Limited. The delivery of the immunogenicity part of this study was completed with collaboration between OVG and SIIPL, co-ordinated by the author, Dr Florence Mclean, Dr Young Kim, Dr Amy Flaxman and Professor Sir Andrew Pollard from OVG, and from Anirudha Vyankatesh Potey, Sandesh Bharati, Vinay Gavade, and Chandrashekhar D. Kamat from SIIPL.

Management of laboratory sample processing was carried out by Sonu Shrestha (P1 and PATCH studies), by the author (VAST study), and by Nicole Day and Susana Camara (VASP study). Optimisation of the assays was provided by OVG staff at laboratory meetings. In particular, assay optimisation feedback was provided by Dr Elizabeth Clutterbuck, Dr Jennifer Hill, Dr Lisa Stockdale, Dr Celina Jin, and Dr Christina Dold. Original luminescence SBA concept was developed and shared by Francesca Necchi and Francesca Micoli employed GlaxoSmithKline. Generation of the green fluorescent protein expressing *Salmonella* Paratyphi A strain was performed by Dr Jennifer Hill, the plasmid and protocol was provided by Dr Nancy Wang and Professor Dick Struggnell from the University of Melbourne. Optimisation of the flow cytometry panels and training on the flow cytometer was provided by Dr Mari Johnson and Dr Elizabeth Clutterbuck. The colony counting SBA protocol was developed and shared by SIIPL, further optimisation work by the author was performed with assistance from Tanya Dinesh and Sarthak Sahoo, with oversight and input from Vinay Gavade.

Sarthak Sahoo, Tanya Dinesh, Dr Amy Flaxman, Dr Eirini Pantazi also contributed in collecting the SIIPL SBA data presented in chapter 4.

The P1/PATCH ELISA data presented in chapters 4-6 was collected by Helena Thomiades-Brears and Sonu Shrestha. The SIIPL ELISA data presented in chapters 4-6 was collected by JuYeon Park and Nicole Day. Symptom severity and diagnosis data were collated by Dr Celina Jin (VAST), Dr Hazel Dobinson (P1), and Dr Malick Gibani (PATCH). Blood and stool cultures were processed by the Microbiology Laboratory at the John Radcliffe Hospital, Oxford University NHS Foundation Trust.. Haematology and biochemistry samples were processed by the Clinical Biochemistry and Haematology laboratories, at the John Radcliffe Hospital, Oxford University NHS Foundation Trust.

The data and analyses presented herein have not been submitted for any degree elsewhere and, unless otherwise stated, all work presented in this thesis is my own.

Impact of the COVID-19 pandemic

The emergence of the COVID-19 pandemic in 2020 lead to unprecedented challenges across various facets of life. As a doctoral candidate during the UK lockdowns my ability to perform experiments and progress my research was significantly impacted.

My role as a full time research assistant and CL3 deputy manager in OVG during this time meant that I was actively working on the delivery of SARs-COV-2 infection and vaccine related clinical trials during this time. Moreover, in the face of post-pandemic career progression and staff departures I was assigned to cover the CL3 management and biological safety for approximately 1 year which imposed additional demands and constraints on my research endeavors.

As someone who has enjoyed working in the field of translatable medicine witnessing the real-time impact of medical research on public health was an invaluable experience. I learnt a lot during this time and I am grateful that I could participate in the global effort to tackle the COVID-19 pandemic, however this undoubtedly impacted the progression and quality of my thesis work.

Abstract

Enteric fever is a febrile illness caused by systemic infection with *Salmonella* Typhi or Paratyphi. It causes approximately 136,000 deaths a year. Enteric fever has largely been eradicated in developed countries but there remains a high incidence in lower-middle income countries. Emergence of antimicrobial resistant strains poses a significant health problem. Vaccine intervention can help reduce the burden of disease and is recommended in regions of enteric fever endemicity. However, current enteric fever vaccines are not effective against paratyphoid fever and recent data show that in some regions the incidence and proportion of disease attributable to *S*. Paratyphi infection is increasing. Comprehensive control of enteric fever requires effective vaccines against both etiological agents. Currently there are no licensed vaccines against paratyphoid fever. Licensure of novel vaccines is partially hindered by our limited understanding of protective immunity and lack of immunological correlates of protection.

Development and application of immunoassays to characterise the immune response to *S*. Paratyphi infection or vaccination can further our understanding of protective immunity and help support licensure of new vaccines to help control enteric fever.

I have developed multiple robust immunoassays to reliably measure the functional humoral immune responses against *S*. Typhi and *S*. Paratyphi A. Using these standardised methods I have measured serum bactericidal activity (SBA), antibody dependent monocyte phagocytosis (ADMP), antibody dependent neutrophil phagocytosis (ADNP) responses to enteric fever vaccines and experimental challenge in a series of clinical trials.

I measured pre-existing, and post-challenge functional immunity in two oral *S*. Paratyphi A experimental challenge studies. Although there was variability levels of pre-exisiting immunity at baseline, none of the features significantly correlated with protection from acute paratyphoid fever after challenge. Ninety days after challenge there were significant increases in SBA, ADNP, ADMP.

For the SBA, challenge induced increases were dependant on systemic infection whereas increases in ADMP and ADNP occurred regardless of systemic infection. A subset of *S*. Paratyphi A challenged individuals undertook a second challenge. Significant waning of functional immunity occurred by 17 months, at the point of rechallenge and no features measured at the rechallenge baseline correlated with diagnostic outcome of the second challenge.

In another study, healthy volunteers received either two doses two weeks apart of oral paratyphoid vaccine, CVD 1902, or sodium bicarbonate placebo control approximately one month before oral *S*. Paratyphi A challenge. SBA and ADMP titres were significantly increased 42 days after receiving vaccine or placebo. However, ADNP titres were significantly elevated after vaccine/placebo in those who were protected from infection. A similar trend was observed for ADMP, but it did not reach statistical significance. Analysis of this data while the study is still blinded may impact these findings and further analysis should be conducted once the study is unblinded. Integrative analysis approaches were unable to identify any immune signatures that correlated with protection in any of the three challenge studies.

Functional immunity against *S*. Paratyphi A was also measured in participants in a phase 1 clinical trial evaluating the safety and immunogenicity of a novel *S*. Typhi – Paratyphi A bivalent conjugate vaccine, Sii-PTCV. A single dose of Sii-PTCV elicited significant increases in SBA, ADMP, ADNP suggesting polyfunctional immune stimulation.

In conclusion, I have developed robust immunoassays that can reliably measure antibody effector functions. In paratyphoid naïve adults or adults who have experience a single exposure to *S*. Paratyphi A there are no baseline immune functions that correlate with protection in an experimental challenge model. I have used these assays to demonstrate that functional immunity can be stimulated by oral *S*. Paratyphi A challenge, a single dose of Sii-PTCV, and potentially by CVD1902. However further work is required to determine whether vaccine induced responses correlate with protection from paratyphoid fever. While no correlates of protection could be

identified in these studies, this work adds to our growing understanding of host immune responses to typhoidal *Salmonella*. Furthermore, this work serves a 'proof of principal' demonstrating that these assays can be used, in addition to other assays, to assess immunogenicity and correlates of protection against paratyphoid fever future studies.

List of tables

List of figures

Publications that include data from this thesis

1. Jones, E. *et al.* A Salmonella Typhi Controlled Human Infection Study for Assessing Correlation between Bactericidal Antibodies and Protection against Infection Induced by Typhoid Vaccination. *Microorganisms* **9**, (2021).
2. Bentley, T. *et al.* Persistence of Antibody After a Vi-Tetanus Toxoid Conjugate Vaccine and Effect of Boosting With a Plain Polysaccharide Vaccine on Vi Antibody and Antigen-Specific B Cells. *Front. Trop. Dis.* **0**, 24 (2021).
3. Kulkarni, P. S. *et al.* The safety and immunogenicity of a bivalent conjugate vaccine against Salmonella enterica Typhi and Paratyphi A in healthy Indian adults: a phase 1, randomised, active-controlled, double-blind trial. *Lancet* **403**, 1554–1562 (2024).

Abbreviatons

AC	Active complement
ADMP	Antibody dependant monocyte phagocytosis
ADNP	Antibody dependant neutrophil phagocytosis
APC	Antigen presenting cell
ASC	Antibody secreting cell
ATCC	American Type culture collection
BCR	B cell receptor
BSA	Bovine serum albumin
CAMP	Cationic antimicrobial membrane proteins
CD	Cluster of differentiation
CFU	Colony forming units
CHIM	Controlled human infeciton model
CoP	Correlates of protection
CPS	Capsular polysaccharide
CRM_{197}	Cross-reactive material 197
CV	Coefficient of variation
CVD	Center for Vaccine Development
DC	Dendritic cell
DC-SIGN	Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin
DNA	Dioxyribonucealic acid
DMF	Dimethylformamide
E. *coli*	Escherichia coli
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
Fc	Fraction crystalizable
FC	Fold change
FcR	Fc Receptor
FSC	Forward scatter
GALT	Gut associated lymphoid tissue
GBD	Global burden of disease
GFP	Green fluorescent protein
GMT	Geometric mean titre
H	Flagellar antigen
HBSS	Hank's balanced salt solution
HIC	Heat inactivated complement
HIV	Human Immunodeficency Virus
HLA	Human leukocyte antigen
IFN	Interferon
IL	Interleukin
IMDM	Iscove's Modified Dulbecco's Medium
iNTS	Invasive Non-typhoidal Salmonella Disease
LB	Luria–Bertani broth
LPS	Lipopolysaccharide

M	Molar
MAC	membrance attack complex
MAIT cell	Mucosal associated invariant T cell
MAPS	Multiple Antigen Presenting System
MDR	Multi-drug resistant
MFI	Median fluorescence intensity
MHC	Major histocompatibility complex
mL	Millilitres
MLN	Mesenteric lymph node
MOI	Multiplicity of infection
NIBSC	National Institute for Biological Standards and Controls
NK cell	Natural killer cell
NO	Nitric oxide
nPD	Not paratyphoid diagnosed
nTD	Not typhoid diagnosed
NTS	Non-typhoidal *Salmonella*
NVGH	Novartis Vaccines Institute for Global Health
OD	Optical density
OVG	Oxford Vaccine Group
PAMPs	Pathogen associated molecular patterns
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline
PCA	Principal componant analysis
PCR	Polymerase chain reaction
PD	Paratyphoid diagnosis
PhoP-PhoQ	Pleiotropic Two-Component Regulatory System
PRR	Pattern recognition receptors
RLU	Relative light units
ROS	Reactive oxygen species
RpoS	RNA polymerase, sigma S
rpm	Rotations per minute
RPMI	Roswell Park Memorial Institute cell culture media
r	Spearman's correlation coefficient
SBA	Serum bactericidal activity
SCV	Salmonella-containing vacuole
SD	Standard deviation
SEAP	Surveillance for Enteric Fever in Asia Project
SED	Sub-epithelial dome
SIIPLTD	Serum Institute India Private Limited
Sii-PTCV	Serum Institute India Private Limited - *S*. Paratyphi *S*. Typhi bivalent conjugate vaccine
SPI	Salmonella Pathogenicity Island
SPtA	S. Paratyphi A exposed positive pool of serum
SSC	Side scatter
STRATAA	Strategic Typhoid Alliance Across Africa and Asia
TAB	Vaccine against Salmonella *S*. Typhi and *S*. Paratyphi A & B
TCR	T cell receptor

TD	Typhoid diagnosis
T_H1	Type 1 T helper cell
TLR	Toll like receptor
TNF	Tumor necrosis factor
TMB	Tetramethylbenzidine
TSAP	Typhoid surveillance in Africa Program
T3SS	Type 3 secretion system
VASP	Vaccines Against *Salmonella* Paratyphi A
VAST	Vaccines against *Salmonella* Typhi
Vi capsule	Virulence capsule
Vi-PS	Vi capsular polysaccharide vaccine
Vi-TCV	Vi Polysaccharide typhoid conjugate vaccine
Vi -TT	Vi polysaccaride tetanus toxoid conjugate vaccine
WHO	World Health Organisation
WT	Wild-type
XDR	Extensively drug resistant
xg	Relative centrifugal force

Chapter 1. Introduction

1.1. Enteric fever clinical manifestation

Typhoid and paratyphoid fever, collectively termed enteric fever, are systemic diseases caused by infection with *Salmonella enterica* subspecies *enterica* serovars Typhi and Paratyphi A, B, and C. Infection with either *S*. Typhi or *S*. Paratyphi causes a clinically indistinguishable, febrile illness associated with, non-specific symptoms of malaise, headache, anorexia, abdominal discomfort, arthralgia, dry cough, and nausea [1,2]. Onset of symptoms typically occurs 8-14 days after exposure [2]. Clinical presentation varies based on geography, patient age, and the presence of comorbidities, such as HIV [3]. Disease severity can range from mild to life threatening, with complications, such as intestinal perforation, gastrointestinal bleeding, and neurological complications occurring in 10-15% of cases.

1.2. Burden of disease

Enteric fever predominantly effects low and middle income countries (**Figure 1-1** shows the distribution of disease). The true burden of disease is difficult to accurately measure due to its non-specific presentation, lack of availability and affordability of sensitive and specific point of care diagnostics, and health care seeking behaviour e.g. the use of over the counter antibiotics to treat non-specific febrile illness [4]. The WHO recommends the use of facility-based surveillance in endemic countries to get a better estimate of true burden of disease [5].

An estimated 14.3 million cases of enteric fever occurred globally in 2017, the majority of which were attributable to typhoid fever (76·3% , 10.9 million) while 3.4 million cases were caused by paratyphoid fever [6]. The global fatality rate was 0.95% (135,900 deaths in 2017) and notably the case fatality was 1.89 times higher for typhoid compared with paratyphoid.

There are no standardized methods for collecting epidemiological data, most studies use data collected from various sources such as hospital records, health care centre reporting, and

community based household visits from surveillance studies [6–8]. Heterogeneity in reporting also exists due adjustments made using different risk factors associated with disease and diagnostics, meta analyses by Mogasale *et al*, Antilion *et a*l, and Buckle *et al* estimate the global burden to be 11.9, 17.8, or 26.9 million cases per annum [9–11].

Outbreaks of enteric fever also contribute to the global disease burden, between 1990 and 2018 303 outbreaks were identified [12].

1.2.1.Geographical variation

The number of enteric fever cases varies geographically, the highest burden of disease is reported in Africa and Asia. Stanaway *et al* report the burden of disease in the south Asia super-region to be 10.3 million cases per year, accounting for 71.8% of the global disease burden, the burden in the proximally located southeast Asia, east Asia, and Oceania accounting for 14·1% of global cases (**Figure 1-1**) [6].

Data collated from sub-Saharan Africa reports 1.73 million yearly cases, wide levels of variation are seen throughout Africa as noted in the TSAP (Typhoid surveillance in Africa Program) study which reported an adjusted incidence rate ranging 0 in Sudan to 383 per 100 000 person-years of observation in Burkina Faso [6,13]. The same global pattern was observed in the STRATAA (Strategic Typhoid Alliance Across Africa and Asia) study. Data collected from STRATAA showed that the incidence of enteric fever was highest in Asia (adjusted incidence rate of 1062 and 1135 per 100 000 person-years of observation in Kathmandu and Dhaka, respectively) with a lower burden of disease being reported in Africa, as measured in Blantyre, Malawi which reported an adjusted incidence rate of 444 cases per 100 000 person-years of observation [14,15].

The global burden of enteric fever attributable to *S*. Paratyphi infection is lower compared with that caused by *S*. Typhi infection [6,9–11]. However, this varies regionally and recently, reports of increasing paratyphoid fever incidence have been emerging. Studies in Nepal and India have reported an increase in the proportion of enteric fever cases caused by *S*. Paratyphi, while a study in Cambodia

found the incidence of paratyphoid fever increased 44 fold between 2007 and 2010 [16–18]. The number of paratyphoid fever reports are highest in China, where four studies claimed an incidence of 150 cases per 100,000 person years of observation, and in the Guangxi more than 90% of isolates collected from enteric fever patients were positive for *S*. Paratyphi [19,20].

Interestingly, there are only a few reports indicating the presence of *S*. Paratyphi A in Africa. Studies in Kenya and Malawi found that *S*. Paratyphi was not isolated from any cultures, despite both areas being endemic for typhoid fever [14,21]. However, 2 hospital based studies conducted in Nigeria isolated *S*. Paratyphi in 16.7 – 34.3% of cultures that were *Salmonella* positive [22,23].

The majority of isolates from paratyphoid cases belong to the *S.* Paratpyhi A subtype, followed by the B and C subtypes [24].

In developed countries, enteric fever is mostly eliminated and only remains a problem in travellers returning from regions where enteric fever is endemic [25]. The same geographical and subtype distribution described above is reflected in cases in returning travellers, the highest number of cases in UK are from people returning from South-Central Asia, followed by South-East Asia then Africa. Another study conducted in England between 2014-19 identified 1088 cases of *S*. Typhi, 729 *S*. Paratyphi A, 93 *S*. Paratyphi B, and one *S*. Paratyphi C [25,26].

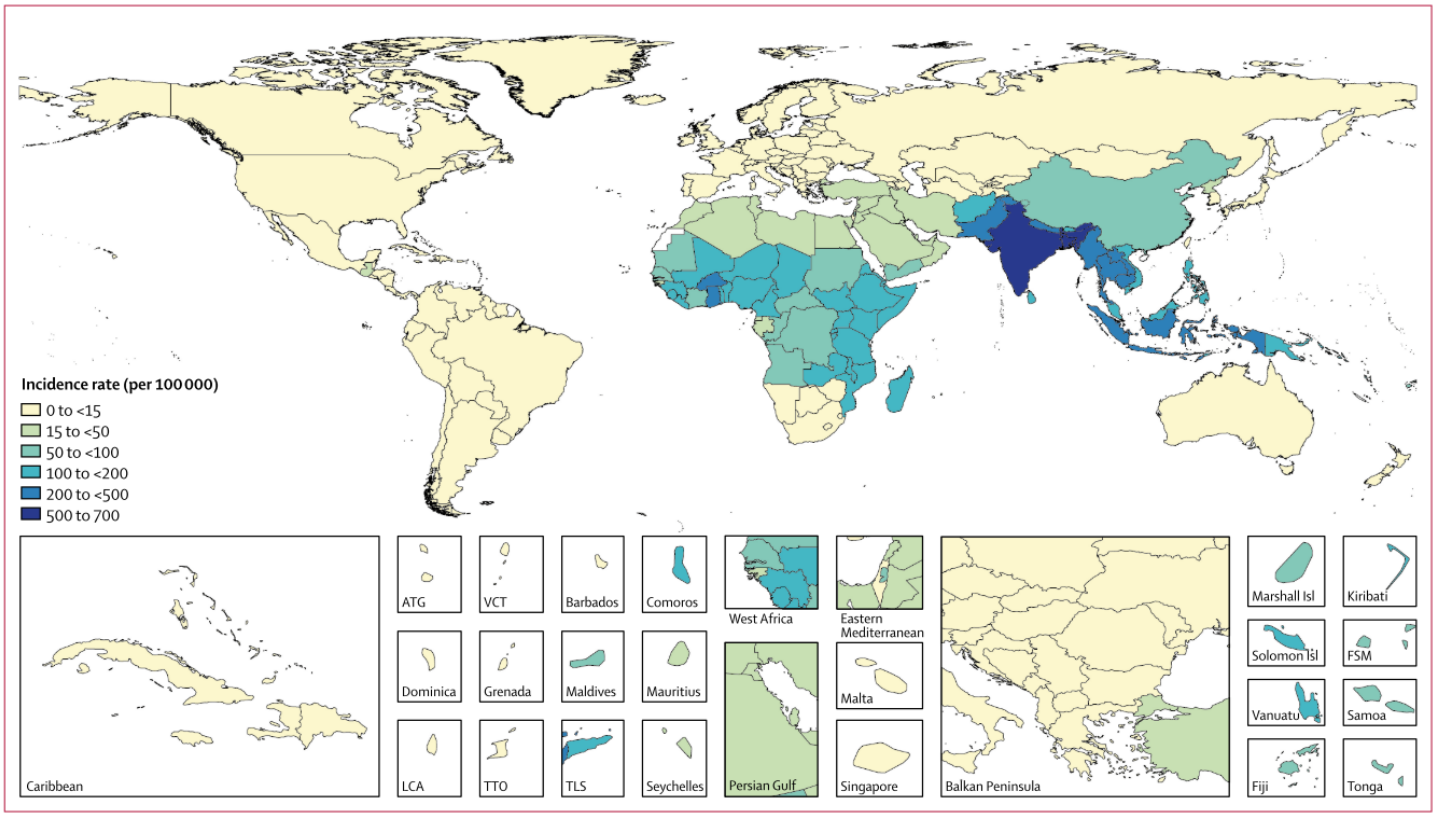


Figure 1-1 Estimated incidence of typhoid and paratyphoid fever (per 100,000) in 2017.

Global enteric fever incidence data presented by GBD 2017 Typhoid and Paratyphoid Collaborators collected from a combination of vital registration data and natural history data. Figure reproduced under the CC BY license, from Stanaway, J. D. *et al.* The global burden of typhoid and paratyphoid fevers: a systematic analysis for the Global Burden of Disease Study 2017. *Lancet Infect. Dis.* 19, 369–381 (2019).

1.2.2. Age distribution

Collecting corresponding demographic data identifies at risk groups and helps inform policy makers of the best intervention strategies. Most studies corroborate that the highest disease burden lies in school aged children, with the peak infection frequency occurring at either 2-4, or 5-15 years of age [6,9,10]. Interestingly, the SEAP study reported that the median age a laboratory confirmed typhoid diagnosis was 7 years of age where as the median age for paratyphoid was 12 years of age [24]. Although it's worth noting that burden data are likely underestimated in younger children due to the atypical symptomology and difficulties in obtaining a large enough sample volume for accurate laboratory based diagnostics.

1.3. Transmission

Typhoidal *Salmonella* serovars are human restricted pathogens and therefore there is no animal reservoir harbouring bacteria. Transmission of enteric fever occurs primarily through consuming contaminated food and water. Transmission of typhoidal *Salmonella* is divided into two categories. Short cycle transmission occurs by exposure through household contacts and food preppers. Long cycle transmission occurs by exposure through environmental contamination e.g. water sources [27]. Enteric fever has mostly been eliminated in developed countries owing to improvements in water and sanitation [28]. In endemic regions, transmission dynamics vary based on location, burden of disease, and serovar/clade [29]. Interestingly, transmission patterns appear to differ between typhoidal serovars, where the primary risk factor associated with *S*. Paratyphi infection is consumption of contaminated food, while for *S*. Typhi infection, poor water sanitation and socioeconomic status are the main risk factors [30]. Understanding transmission is paramount for elimination of enteric fever and interrupting transmission though infrastructure changes (clean water supply) and policy (direct and indirect vaccine effects) can greatly reduce the burden of disease [31].

1.4. Diagnostics

Diagnosis of typhoid and paratyphoid fever is problematic because the non-specific symptoms make it hard to distinguish from other acute febrile illnesses. Without additional laboratory based diagnostics this can lead to use of unsuitable treatments such as ineffective antibiotics. Diagnostic tests are traditionally based on either isolating the pathogen, or a fragment of the pathogen in a patient sample, or on host responses that are indicative of a recent infection[32] . For enteric fever the gold standard test is bone marrow culture, although blood culture is more commonly used owing to the relative ease and comfort of sample collection. However, the number of viable bacteria in the blood is low (estimated at about 1 CFU/mL) and accuracy of blood culture ranges from 51 – 65 % when compared with bone marrow culture, although this depends on sample volume [33,34,35]. In areas with frequent antimicrobial use, the accuracy can even be as low as 40% [36]. Stool culture is sometimes used as part of a composite diagnostic, but as shedding occurs intermittently it cannot distinguish between current infection and chronic carriage.

The need for accurate, rapid, and affordable diagnostics for these bacterial pathogens is urgent, particularly as increases in rates of antimicrobial resistance are exacerbated by inappropriate use of antibiotics.

Serological diagnostic tests rely on detection of *Salmonella* specific antibodies in the blood, usually targeted towards specific surface molecules such as the LPS, H, or Vi antigens. The historical Widal test has been replaced by commercial kits like TUBEX® and Typhidot [32]. Such diagnostics platforms perform well compared with blood culture, with medium to high levels of sensitivity and specificity. However, timing of the patient sample can compromise the sensitivity of the technique, as can the presence of antibodies generated from recent infection, vaccination, or exposure in the endemic environment, thus one study concluded these were valuable tools for assessing outbreaks, but less useful for individual patient diagnosis [32,37].

Recent innovations in genomic and transcriptomic approaches are underway. A recent study identified 5 host genes that were able to identify enteric fever from other febrile infections with greater than 96% accuracy. However, the comparator febrile diseases included in this study were limited and more work is needed to assess the feasibility of using host transcriptomics in endemic settings [38]. Diagnostics based on detecting bacterial DNA offer an advantage in that they can detect unculturable bacteria, however in practice blood based PCR assays have mixed sensitivity and specificity ranging from <50-90% [39]. While these molecular diagnostic approaches show promise, these techniques are costly and more research is needed before they could become viable point of care diagnostics [32,39].

1.5. Treatment of disease and antimicrobial resistance

Acute enteric fever can be treated with the use of appropriate antibiotics. Introduction of first-line antimicrobials such as chloramphenicol and ampicillin reduced the mortality rate due to enteric fever, however resistance to these antimicrobials has been emerging since 1900s [40]. Since then emergence of multi-drug resistant clones of *S*. Typhi, in particular the H58 clade, have been reported in Asia, Africa and Oceania displacing other strains which are sensitive to antimicrobial treatment [41]. Up to 90% of cases are treated at home with over the counter antibiotics [3]. The ready availability of antibiotics and the treatment of non-laboratory confirmed febrile disease with improper antibiotics puts selective pressure on the organism giving advantages to antimicrobial resistant strains, causing outbreaks [42]. Recently, extensively drug resistant strains (strains resistant to first, second, and third generation antibiotics such as chloramphenicol and ampicillin, fluoroquinolones, and cephalosporin) of H58 lineage have been reported [40,43,44].

MDR strains cause more severe disease in children (in adults it's indistinguishable), possibly due to delay to treatment or use of incorrect treatments. In combination with higher rates of prolonged asymptomatic carriage by MDR *S*. Typhi strains, this highlights the importance of treatment by vaccine intervention [45,46]

The pattern of antibiotic resistant *S*. Paratyphi is complex, while globally the frequency of antimicrobial resistant paratyphoid strains is increasing, variations are seen between regions. A study in Bangladesh observed that *S*. Paratyphi isolates remain sensitive to most antibiotics [47,48]. Interestingly, a study in India found *S*. Paratyphi isolates were sensitive to first generation antibiotics, but had increased resistance to fluoroquinolones, the current drug of choice for treating enteric fever [49]. These observations emphasise the essential role of adequate diagnostics and antimicrobial sensitivity testing in the treatment of paratyphoid fever.

1.6. Vaccine development

Persistent high disease burden and the rise of antibiotic resistant strains is a major obstacle in the elimination of enteric fever. Long-term strategies to eliminate enteric fever require improvements in sanitation, hygiene, and accessibility to clean water, however in the short to medium term vaccination is an effective preventative measure.

1.6.1.Historical enteric fever vaccines

The first typhoid vaccines were developed in England and Germany over 100 years ago, consisting of heat killed whole *S*. Typhi. An oral, heat killed *S*. Typhi vaccine caused severe reactogenicity and caused typhoid fever due to incomplete inactivation and ingestion of live bacteria. Subsequently, multiple preparations of injectable vaccines were developed consisting of heat killed, phenol preserved or acetone killed, lyophilized whole cell *S*. Typhi. Vaccine efficacy studies conducted in the 1960s and showed efficacies of 51-67% for the phenol inactivated vaccine or 79-88% for the lyophilized acetone inactivated vaccine. Despite the promising protection levels, these vaccines elicited adverse reactions and have since been largely discontinued [50]. Heat killed, phenol and alcohol preserved vaccine against *S*. Typhi and Paratyphi A and B (TAB) was developed, these combined vaccines were later discontinued in favour of other typhoid vaccines such as the Vi polysaccharide subunit vaccine, due to adverse reactions and limited coverage against paratyphoid fever [51,52].

1.6.2.Oral vaccines

The first licensed oral typhoid vaccine was Ty21a, a live attenuated vaccine created by non-specific chemical mutagenesis of the parent strain Ty2. The non-specific nature of the mutations resulted in several genetic changes, most immunologically notable is the mutation of *gal*E gene, and the lack of Vi antigen [53]. Loss of UDP-galactose-4-epimerase, the conventional product of *gal*E transcription causes a loss of galactose, an important component of LPS, as a result the bacteria do not express smooth LPS and are less immunopotent. Initial field trials in Egypt demonstrated an efficacy of 96% in children after 3 doses after 3 years [54]. Additional studies in Chile calculated an efficacy of 66% at 5 years , or 62% efficacy after 7 years in children who received 3 doses [55,56]. While Ty21a is an *S*. Typhi monoculture, evidence shows some cross protection against *S*. Paratyphi B, although evidence of cross-protection against *S*. Paratyphi A is inconclusive. Ty21a is licensed in 56 countries but is mainly used by travellers due the requirement for multiple doses, cold chain storage, requirement for gastric acid pre-treatment buffer, and the fact that it is not recommended for use in children <5 years of age because of the capsule formula [57].

1.6.2.1. M01ZH09

Other oral vaccine candidates are in development and have been tested in clinical trials but have not reached licensure. M01ZH09 is a live attenuated vaccine derived from the wild type Ty2 strain, it harbours independent, targeted deletions in the *aroC* and *ssaV* genes. Deletions in the aroC gene have auxotrophic effects hindering biosynthesis of aromatic amino acids, resulting in decreased replication without exogenous amino acid supplement [58]. Attempts to create a vaccine strain with *aro* gene mutations proved to be immunogenic but still caused bacteraemia [58]. The ssaV gene is part of the SPI-2, a cluster of virulence related genes that encode a needle like structure that injects effector proteins in to host cells to help avoid oxidative burst mediated killing. Mutations effecting this structure limit the survival of bacteria within phagocytes and prevent systemic spread [59]. M01ZH09 is safe and well tolerated and can stimulate *S*. Typhi specific IgG, IgA antibody and cellular

responses after a single dose in adults and children [58–61]. In a human challenge study where participants received either a single dose of M01ZH09 or 3 doses of Ty21a 1 month before oral challenge with live *Salmonella* Typhi bacteria, the vaccine efficacies were low at 13% for M01ZH09 or 35% for Ty21a [62].

1.6.2.2. CVD908, CVD908-htrA, and CVD909

The family of vaccines developed at CVD (centre for vaccine development) in the 1990's also derived from wild type Ty2 strain. CVD908 contains double deletions of aroC and aroD genes, phase 1 clinical trials investigating safety and immunogenicity of this vaccine showed varied immunogenicity at low doses, and reported undesirable bacteraemia at higher doses [63]. CVD908-*htrA* strain includes mutations in the *htrA* gene which encodes heat shock proteins, deletion of this gene impairs the intracellular survival of the bacteria, therefore limiting systemic spread and risk of bacteraemia. No bacteraemia was detected in vaccine recipients, and the vaccine elicited strong serum and cellular immune responses against flagellar (H) and LPS antigens.

Vi specific immune responses are thought to be an important target due to expression of the Vi capsule at key points in the infection process. Stimulation of Vi specific responses by oral vaccines would give them a potential advantage over Vi polysaccharide vaccines because of the potential to induce immunological memory, which polysaccharide vaccines do not stimulate. A vaccine strain with constitutive Vi expression, CVD909, was developed by replacing the promotor of the capsule expression from the regulated P_{tvia} to the consituative P_{tac}. Vi specific serum responses were low in CVD909 recipients, however a single dose of $10^{8\text{-}9}$ CFU elicited Vi specific IgA antigen secreting cells (ASCs) [63,64]. Despite the induction of immune responses the efficacies of these oral vaccines have not been assessed.

1.6.2.3. CVD1902

While it's been reported that Ty21a confers cross protection against *S*. Paratyphi B and CVD909 induces cross reactive immune responses against *S*. Paratyphi A and B, there are currently no

licenced *S*. Paratyphi specific vaccines. Many candidate vaccines exist, including attenuated oral vaccine candidate CVD1902. CVD1902 contains two independent mutations, one responsible for virulence attenuation in the *guaBA* locus (disrupts the biosynthesis of guanine nucleotides), and the other intended to increase immunogenicity in *clpPX* locus (genetic code for a chaperone ATPase, *clpPX* mutants have increased flagellar expression) [65].

Phase I studies have shown CVD 1902 to be safe and immunogenic in human volunteers. After a single dose (up to 10^{10} CFU) significant increases in LPS specific antibodies, B memory, and T cells were observed. A phase II controlled human infection study investigating vaccine efficacy and immunogenicity of a two-dose schedule of CVD 1902 is ongoing at the University of Oxford at the time of writing this thesis [66]. Some preliminary humoral immunogenicity findings from this study will be discussed in chapters 4-7 of this thesis.

1.6.3.Polysaccharide vaccine

Vi polysaccharide vaccines contain purified Vi capsular polysaccharide (CPS), the outer capsule sugar expressed on *S*. Typhi and sometimes *S*. Paratyphi C. Clinical studies reported moderate protection from Vi-PS vaccines, with a 69% efficacy after 1 year and 59 % efficacy after 2 years [67]. However, by the third year the efficacy drops to 50%, possibly attributable to the absence of immunological memory due to lack of engagement of T cells [68]. Vi CPS vaccines also demonstrate a lower efficacy in younger children, the T-cell independent stimulation by polysaccharide antigens is less potent in children because marginal zone B cells do not mature until approximately 18 months of age [69,70]. Vi CPS vaccines are not recommended for use in children under 2 years and revaccination is advised every 2-3 years [71].

1.6.4.Conjugate vaccine

In an effort to induce immunological memory and increase efficacy, especially in infants, Vi polysaccharide typhoid conjugate vaccines (TCV) were developed. Initial vaccines used Vi CPS conjugated to non-toxic recombinant protein that is antigenically identical to *Pseudomonas*

aeruginosa exotoxin A, Vi-rEPA [72]. Vi-rEPA was immunogenic and efficacious in young children, as well as adults and induced long lasting protection with an efficacy of 82% was reported in children aged 2-4 years 46 months post vaccination [57,73,74]. More recently, typhoid conjugate vaccines have been developed with other carrier proteins such as tetanus toxoid, diptheria toxoid, or CRM_{197}. Early studies demonstrated safety and predicted vaccine efficacy using seroincidence models and controlled human infection models provided evidence for the WHO to support of typhoid conjugate vaccines [75,76]. A single dose of Typbar-TCV showed a vaccine efficacy of 79% after 2 years in a study conducted in Nepal and Malawi [77,78]. Evidence to determine the duration of protection of newer conjugate vaccines is currently being collected, one study demonstrates that Vi specific IgG persists up to 7 years in children who receive a single dose of Vi-TCV.

Conjugate vaccines against *S*. Paratyphi A have been developed linking O-antigen (O:2) to carrier proteins. Phase I and II trials demonstrated that O:2 conjugated to tetanus toxoid ($SPA\text{-}TT_2$)was safe and immunogenic in adults and children, inducing both binding and bactericidal LPS specific antibodies [79]. Another study of a typhoid-paratyphoid bivalent conjugate vaccine (Sii-PTCV) was safe and immunogenic in adults, and after a single dose, reported elevated binding and functional *S*. Paratyphi A specific antibodies 29 days post vaccine, that remain elevated at 181 days post vaccine [80]. However, the efficacy of these vaccines have not yet been evaluated, future challenge studies will be required to determine the protective effect of *S*. Paratyphi A conjugate vaccines.

Due to the overlapping epidemiology, future enteric fever vaccines will likely target a combination of serovars. Combined bivalent vaccines targeting typhoid and paratyphoid antigens would be advantageous in Asia where the two serovars colocalise, whereas a combined vaccine targeting typhoid and non-typhoidal *Salmonella* (NTS) would be beneficial to Africa where there is a high burden of NTS, but little evidence of paratyphoid. A trivalent vaccine, which provides protection and typhoidal and non-typhoidal *Salmonella* serovars is a desirable goal if it can provide global coverage [81].

1.7. The *Salmonella* infection lifecycle and evasion of the innate immune system

S. Typhi and *S*. Paratyphi are highly adapted for survival within the human host and have evolved many virulence mechanisms to evade the human immune system.

Salmonella enterica subspecies diverged from a common *E. coli* ancestor 100-150 million years ago years ago by obtaining genes that allowed the bacteria to invade cells, becoming facultative intracellular pathogens [82]. The subspecies of *S. enterica* are classified into serogroups based on the O (somatic; *S*. Typhi O:9,12, *S*. Paratyphi O:2, 12) and H (flagellar; *S*. Typhi Hd, *S*. Paratyphi Ha) antigen expression, according to the Kauffman-White classification system [83]. Typhoidal *Salmonella* serovars share 90% of their genes with non-typhoidal serovars. The distinct differences in pathogenesis are caused by genetic variation introduced through reductive evolution by mutation induced gene inactivation (pseudogenes) and by the incorporation of genetic elements from plasmids, prophages, and phages [82]. Interestingly, while *S*. Typhi and *S*. Paratyphi cause indistinguishable disease and both serovars contain ~5% pseudogenes, the overlap of pseudogenes is less than 20% between typhoidal serovars and the comparable disease phenotype by these bacteria is thought to have evolved independently [84,85].

Most genes encoding *Salmonella* related virulence factors are encoded by clusters of genes termed *Salmonella* pathogenicity islands (SPIs), in the next section I will describe the pathogenesis of typhoidal *Salmonella* serovars and highlight the role of some important virulent factors.

1.7.1. Ingestion and the gastric acid barrier

Once ingested, *Salmonella* enter the stomach where they resist the effects of low pH by utilising the arginine decarboxylase acid resistance system. Production of shock proteins (RpoS, PhoP, PhoQ) helps mitigate proton damage to the bacteria [86]. Vaccine strains that contain mutations in the acid shock response (e.g. Ty2 RpoS mutant: Ty21a) are more sensitive to lower pH levels, thus they are often prepared in protective capsules or co-administered with an acid neutraliser (e.g. sodium

bicarbonate) to help protect the bacteria from the low pH in stomach so they can reach the small intestine.

1.7.2. Invasion of the gastrointestinal epithelium (Figure 1-2)

Transient exposure to low pH levels in the stomach triggers upregulation of genes that aid in the bacterial survival in the intestine. Acid induced upregulation of RpoS genes increases bacterial resistance to the action of short chain fatty acids present in the small intestine, while upregulation of the OmpR/EnvZ two component system in response to organic stomach acid increases expression of SPI-1 genes, which mediate invasion of the epithelial cells [87].

In order to cause systemic illness, *Salmonella* must first cross the intestinal epithelium. It was thought that *Salmonella* preferentially traverse the epithelium in the terminal ilium by targeting M cells, the specialised epithelial cells associated with the Peyers patches. However, it is now thought that *Salmonella* also targets enterocytes and is able to cross the epithelium through direct capture by underlying phagocytes:

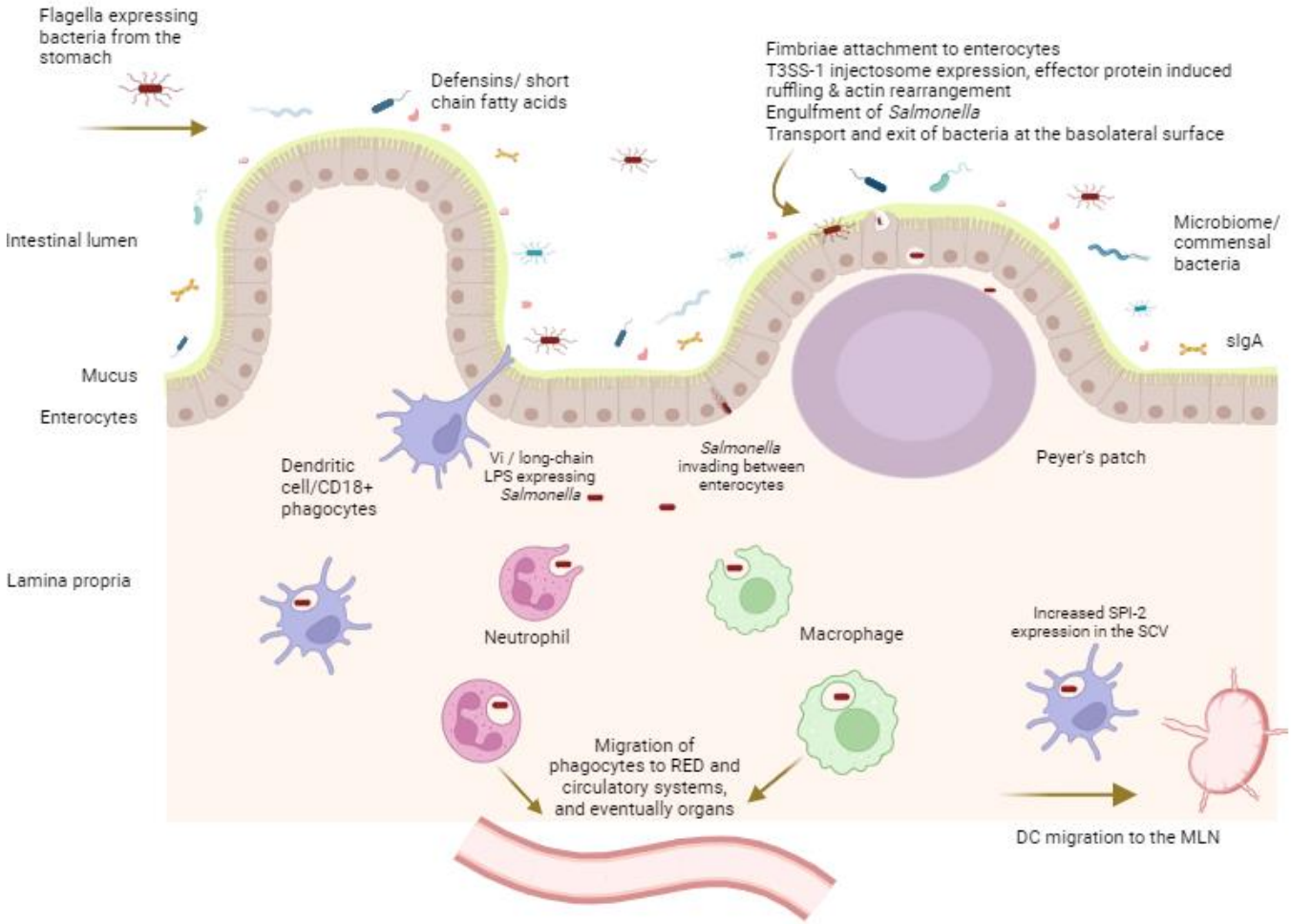


Figure 1-2 Schematic of epithelial invasion by Salmonella

Invasion of the gut epithelium by typhoidal *Salmonella* is primarily through:
1. T3SS-1 mediated entry through enterocytes
2. Sampling of the gut lumen by CD18+ phagocytes
3. Directly traversing through gaps between enterocytes

Followed by migration to the mesenteric lymph nodes and dissemination to secondary organs through the reticuloendothelial and circulatory system.
Figure made using BioRender

1.7.2.1. T3SS-1 mediated entry to epithelium

Primarily, *Salmonella* adhere to epithelial cells via fimbrae, *S*. Typhi fimH binds to terminal mannose residues on surface glycoproteins of human cells. From this proximal location, *Salmonella* utilise the needle like, type 3 secretion system 1 (T3SS-1) encoded on SPI-1 to inject effector proteins into the cytosol of the attached epithelial cell, resulting in actin cytoskeleton rearrangement and engulfment of the bacteria [88]. These effector proteins belong to the Rho GTPase family, activation of NF-κB transcription factor by NTS effector proteins triggers a proinflammatory response, and manifests in the conventional gastroenteritis symptoms of intestinal inflammation and diarrhoea [89]. Many T3SS-1 related effector proteins are pseudogenes in typhoidal serovars, epithelial uptake of *S*. Typhi and *S*. Paratyphi does not result in NF-κB activation and downstream inflammation. Interestingly, replacement of NTS effector proteins with typhoidal equivalents does not attenuate the inflammatory profile in a murine model, suggesting a potential redundant function of some effector proteins and an important role for other virulence factors in traversing the epithelium [90].

Responses to the high osmolality conditions in the ileum are mediated by the *viaB* locus, an operon located on SPI-7, which encodes genes related to regulation (tviA), biosynthesis (tviBCDE), and export (vexABCDE) of the Vi capsule. Osmoregulation in the ileum prevents TviA expression which prompts T3SS-1 and flagellar expression, aiding bacterial invasion. Upon entry into the cells and exit via the basolateral surface, the decrease in osmolality triggers TviA expression resulting in Vi capsule synthesis and export while simultaneously downregulating of flagellar expression[90]. Expression of the Vi capsule at the basolateral surface of the epithelium attenuates inflammation by preventing complement deposition, oxidative burst mediated killing and TLR-4 activation, furthermore reduced expression of flagellar reduces TLR-5 mediated inflammation [91]. This well co-ordinated, self-regulating system enables the bacteria to stealthily invade the intestinal epithelium while evading the immune system.

S. Paratyphi A and B do not express the Vi capsule, but infection with these serovars is characterised by a similar pathophysiology to *S*. Typhi and does not conventionally cause gastroenteritis in adults. Immune evasion by *S*. Paratyphi A is thought to have arisen by convergent evolution. Production of very long chain LPS (>100 repeating O side chains) regulated by FepE, a copolymerase that regulates LPS chain length, which is a pseudogene in *S*. Typhi [92,93]. *In vitro* expression of very long chain LPS has been shown to reduce IgM binding, complement deposition, reactive oxygen species (ROS) production, and pryoptosis, comparable to the effects of *S*. Typhi Vi CPS (**Figure 1-3**) [92].

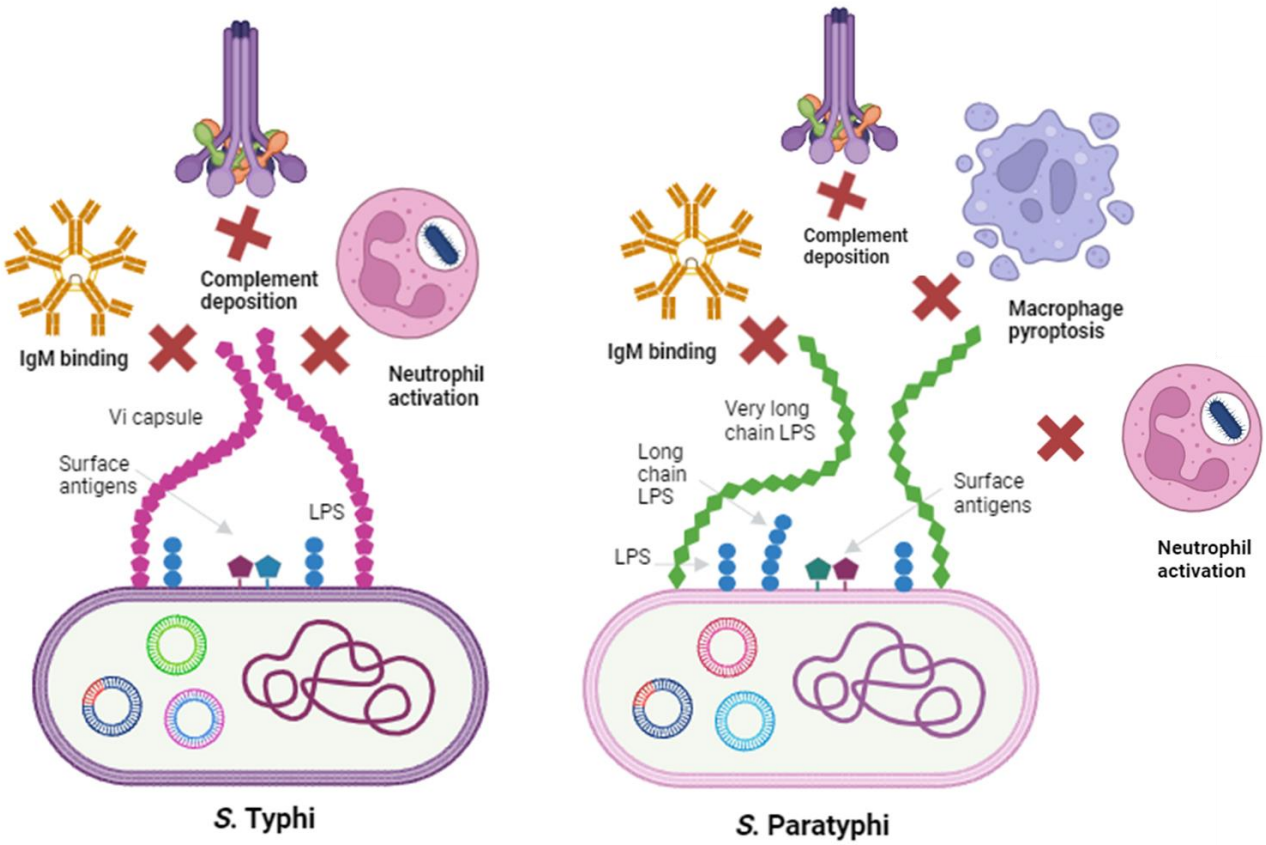


Figure 1-3 Surface antigen expression by typhoidal Salmonella serovars and immune evasion

Adapted from Hiyoshi *et al* 2018 [92].
Immune evasion by typhoidal *Salmonella* serovars
Vi CPS expression by *S*. Typhi reduces binding by IgM, complement deposition, and neutrophil mediated killing [91].

Very long chain LPS expression by *S*. Paratyphi, hypothesised to mediate immune evasion in a similar fashion to the Vi capsule. Very long chain LPS reduces IgM binding, complement deposition, neutrophil mediated killing, and macrophage pryoptosis [92,93]. Figure made using BioRender.

1.7.2.2. Uptake by phagocytes

The Peyer's patches contain professional CD18+ phagocytes that can directly sample the intestinal lumen. To cross the epithelium, *Salmonella* can also be taken up by these phagocytes translocating the bacteria to the basolateral surface [94].

1.7.3. *Salmonella* containing vacuole

Once inside the host cell *Salmonella* reside in the *Salmonella* containing vacuole (SCV), SPI-1 is downregulated and SPI-2 is simultaneously upregulated in response low pH and limited nutrients in the SCV [95]. SPI-2 contains genes encoding a second injectosome, T3SS-2, which translocates effector proteins from the bacterial cytosol to the host cell cytosol. SPI-2 related effector proteins have a myriad of functions, they aid in the maturation of the SCV to support bacterial replication, position the SCV near the golgi apparatus to obtain nutrients, and help prevent SCV-lysosome fusion [96,97]. The low pH of the vacuole also triggers the PhoP/Q two component system, downstream actions of the PhoPQ system cause changes in the membrane structure and contents increasing resistance to antimicrobial peptides such as cationic antimicrobial membrane proteins (CAMP) [98]. These are important virulence mechanisms that allow intracellular bacteria to replicate and evade killing within the phagocytes.

1.7.4.Systemic dissemination

Salmonella bacteria that have successfully traversed the epithelium travel within CD18+ phagocytes to the draining mesenteric lymph nodes (MLN). Systemic *Salmonella* infection occurs when invading bacteria from the gut associated lymphoid tissues (GALT, e.g. Peyers patches) and the MLNs disseminate to secondary, extra intestinal, organs through the reticuloendothelial and circulatory system [99]. Some evidence suggests this occurs rapidly after exposure, as early as 6 hours [100].

Vi expression by *S*. Typhi promotes phagocytic uptake by macrophages via binding to the human C-type lectin receptor DC-SIGN [101]. Successful binding to DC-SIGN is associated with dampened proinflammatory cytokine responses, noting another Vi capsule mediated immune evasion tactic. Neutrophils do not express DC-SIGN, and thus Vi expression is associated with reduced bacterial uptake by neutrophils, but not macrophages [102]. Expression of very long chain LPS by *S*. Paratyphi A serves as stealthy sheath to the immune system, as very long chain LPS dampens the proinflammatory response by macrophages by disrupting pryoptosis (**Figure 1-3**) [93]. In a murine model for typhoid fever, large numbers of *S*. Typhimurium can be recovered from the spleen and liver, since there are relatively few neutrophils in the intestines during typhoidal *Salmonella* infection, and macrophages reside within tissues it's thought that monocytes are the primary phagocyte involved in systemic dissemination [99].

The role of dendritic cells (DCs) in dissemination to secondary organs is under debate, however they do seem to play an important role in colonization of the MLNs as the majority of *S*. Typhimurium containing cells in the MLN express CD11, a phenotypic marker of DCs [103]. SPI-1 invasion knockout mice can also seed the spleen directly from the gastrointestinal tract through migration of CD18+ phagocytes (monocytes and DCs), suggesting that *Salmonella* has evolved many covert routes of infection [104]. Bacterial growth is restricted within DCs and in the MLN, potentially putting selective pressure on the bacteria to find more surreptitious methods of dissemination.

The exact mechanism of secondary organ colonization is not fully understood, within the spleen, the majority of *Salmonella* bacteria reside inside neutrophils at early stages of infection and in macrophages at later stages [99]. Within the tissues, *Salmonella* infected cells form foci, within which the bacteria rapidly replicate. The exact intracellular burden of bacteria varies, but replication rates are dependent on the number of bacteria present (quorum sensing) and the availability of nutrients [105]. New host cells are continually recruited to these foci and spread of bacteria from cell to cell provides fresh nutrients allowing continued replication and host colonisation [106]. The mechanism of

cell to cell spread is not fully understood but likely involves infected cell death, release of bacteria, and uptake by new cells. It possibly arises from a myriad of pathways influenced by host and bacterial cells including pryoptosis, apoptosis, and necrosis mediated cell death. The bacterial population grows rapidly and eventually spills out resulting in a secondary bacteraemia.

1.7.5.Shedding

During the course of infection, bacteria can reseed the intestine causing shedding into the external environment. Shedding of this human restricted pathogen plays a vital role in the transmission of disease. Data from CHIM studies suggest there is an early, transient, phase of shedding. Shedding at day 1 or 2 post exposure is significantly more likely to occur in individuals who later develop acute enteric fever after experimental challenge [107]. Shedding is observed twice as often in individuals exposed to *S*. Typhi compared with *S*. Paratyphi A in CHIM studies, although this could be a reflection of the higher exposure dose (10^4 for *S*. Typhi vs 10^3 for *S*. Paratyphi A colony-forming units). Serovar specific transmission patterns have also been observed in surveillance studies in endemic countries.

1.7.6.Chronic carriage

S. Typhi and *S*. Paratyphi A can also be found in the gall bladder. Infected host cells migrate to the gall bladder either directly from the intestine or from the liver during the systemic phase of the infection [108,109]. It's estimated that around 3-5% of *S*. Typhi infected individuals become chronic carriers who remain asymptomatic, but can transiently shed bacteria in their stool. Due, in part, to the asymptomatic nature of chronic carriage the contribution of carriers to the epidemiology and incidence of enteric fever is hard to determine, and requires further surveillance studies [108]. The gall bladder is thought to be the primary reservoir harbouring bacteria during carriage. Gall bladder pathology is present in 60% of acute enteric fever patients, however only a small proportion of individuals go on to become chronic carriers. The mechanisms that *Salmonella* utilise in the development of chronic carriage are poorly defined but *in vitro* studies show that *S*. Typhi

upregulates T3SS-1 mediated invasion of epithelial cells in the presence of bile allowing *Salmonella* to infect the gall bladder epithelium [84]. Biofilm formation also contributes to long-term carriage, where gall stones provide a favourable surface for biofilm formation and the presence of gall stones is a significant risk factor, present in 90% of carriers [109].

The ability of *Salmonella* to form persister cells to avoid antimicrobial action and death is also hypothesised to contribute to chronic carriage. Induced by various stresses, such as presence of antimicrobials, acidity, or osmotic stress, "persister" bacteria enter a metabolically dormant state. Formation of persister cells, coupled with biofilm mediated protection means that the bacteria can effectively avoid killing by host defences and antimicrobial intervention. Once these threats have lapsed they can emerge from their dormant state, repopulate and disseminate [110]. Development of persister cells is entirely stochastic, which may help explain why so few individuals become carriers.

Models of enteric fever transmission suggest that chronic carriage has a minor role in transmission in areas where enteric fever is highly endemic but plays a more prominent role in disease transmission when the incidence decreases [108].

1.8. Adaptive immune system responses

Typhoidal Salmonella have evolved effective mechanisms to evade host defences and innate immune stimulation. As a facultative, intracellular bacteria, successful immunity to Salmonella comprises of both cellular and humoral responses. Professional antigen presenting cells (APCs: DCs, and to a lesser extent macrophages and B cells) are able to prime the adaptive immune system by presenting bacterial antigens in the context of major histocompatibility complexes (MHCs) to T cells.

1.8.1. T cell mediated responses

The T cell receptor (TCR) on CD4+ T cells recognises intracellular antigens presented in the context of MHC-class II molecules, while the TCR on CD8+ T cells recognises cytosolic bacterial proteins

presented in a complex with MHC-class I molecules. APCs that can overcome *Salmonella*'s immune evasion tactics, process intracellular bacteria and present their antigens to T cells.

Host susceptibility associations provide somewhat contradictory evidence for a role of CD4+ T cells in enteric fever. While there are no strong links between reduced CD4+ cell counts in HIV patients and increased susceptibility to typhoidal *Salmonella* infection, there are links between some genetic variations in MHC class II genes (such as HLA-DRB1*0301/6/8, HLA-DQB1*0201-3 alleles) and increased susceptibility to disease [111,112].

Naturally acquired acute typhoid fever is associated with a T_h1 response, characterised by elevated IFNγ, TNFα and IL-17 levels, while experimental *S*. Typhi infection is associated by IFNγ dominated transcriptomic signature [113–115]. Cellular immunity to typhoid can be modelled using live attenuated vaccine strains in humans, for instance *S*. Typhi specific stimulation of PBMC isolated from Ty21a vaccinees stimulates multifunctional IL-17, IL-2, IFNγ and TNFα producing CD8+ T cells, and IFNγ expressing CD4+ T cells [116,117]. However, in a typhoid challenge model, higher circulating levels of *S*. Typhi specific CD8+ T cells correlated with protection from developing typhoid fever, and were associated with a delay to disease onset, but only when participants were challenged with 10^3 CFU, not 10^4 CFU. When participants were challenged with a higher dose of 10^4 CFU, higher frequencies of CD8+ T cells at baseline significantly correlated with an increased risk of developing disease [118,119]. In both challenge studies, the frequency of α4β7 (a gut homing marker) expressing *S*. Typhi responsive CD8+ T cells was reduced at the time of diagnosis in individuals who developed systemic disease, indicating migration of this cell subset to the intestine and associated tissues. Furthermore, pre-challenge circulating proportions of α4β7 CD4+ FoxP3+ T_{reg} cells were higher in individuals who went on to develop clinical disease, suggesting that the regulatory effects of gut homing T_{reg} cells suppressed the effector and memory T cell function, perpetuating systemic disease [120].

A similar pattern was occured in MAIT (mucosal associated invariant T). A sharp decline in circulating MAIT cells was observed post challenge in CHIM study volunteers who developed acute infection [109].

In vitro presentation of *S*. Typhi stimulated MAIT cells to secrete either IFN-γ, TNF-α, or IL-17A, or a combination of those three cytokines. Each phenotypic cell subsets presents distinct behavioural properties in individuals who develop typhoid fever versus those who do not, indicating a potential complex role of MAIT cells in enteric fever. Interestingly, polyfunctional (IFN-γ− TNF-α+IL-17A+, and IFN-γ+TNF-α+ IL-17A−) MAIT cell subsets discriminate between individuals who are susceptible to typhoid infection versus those who are not[123].

1.8.2. B cells

B cells play a crucial role in the immune system's response to enteric pathogens, they provide stimulus to T cells, form memory B cells, and produce antibodies to help fight infection. The exact role of B cells in immunity to enteric fever is unknown. Studies have shown that oral challenge with *S*. Typhi in human volunteers leads to reduced numbers of circulating B cells in individuals who developed acute typhoid disease, followed by an increase in frequency of B cells with an activated, gut homing plasmablast phenotype (CD40+, CD21+, and α4β7+), indicating they are sequestered the site of infection and employed to help fight infection [124]. Increases in LPS (O:9 or O:2) specific IgA, IgG, IgM, and Hd specific IgG antibody secreting cells are seen after challenge with *S*. Typhi. Similarly, a significant increase in LPS (O:2) IgG ASCs around diagnosis in *S*. Paratyphi A challenge recipients is also observed [125]. However, a single, homologous re-challenge does not seem to significantly boost ASC responses [125].

B cell responses to enteric fever vaccines have been described. Inoculation with Ty21a induces IgG and IgA ASCs to LPS purified from *S*. Typhi, *S*. Paratyphi A and B, which has been linked to the finding that Ty21a confers partial cross protection against paratyphoid serovars [126]. The majority of LPS specific IgG and IgA ASCs had a B memory phenotype (CD19+ CD27+) and coexpressed α4β7, or α4β7 and CD62L (secondary lymphoid tissue homing marker) [126].

Vaccination with either a Vi polysaccharide (Vi-PS) or Vi conjugate vaccine induced Vi specific IgG and IgM producing ASCs and further phenotyping analysis via mass spectrometry showed that a

significant proportion of these cells were IgA positive and co-expressed α4β7. Interestingly, increases in Vi specific IgA plasma cells did not correlate with protection in a Vi vaccine and typhoid challenge study, but induction of IgA negative plasma cells did [127]. Head to head comparisons of plasmablast responses in Vi-PS or Ty21a recipients showed that vaccines induced significant increases in antigen specific plasmablasts (Vi; Vi-PS only, O:9, 12; both vaccines), but those induced by Vi-PS were systemic homing and those induced by Ty21a were gut homing [128]. Responses to flagellar (H antigen) are low after either experimental challenge or Ty21a vaccine, indicating that this is not an immunodominant antigen for mucosal immunity, and supports the theory that flagellar is primarily present when the bacteria are in the gut lumen, but expression is promptly downregulated after *Salmonella* traverse the epithelium [125,128].

The relative heterogeneity of B cell responses to enteric fever infection or vaccine demonstrates the plasticity of B cell responses in immunity. Moderate protection provided by Vi-PS vaccines, which are purely T independent antigens shows that B cell responses can be an important mediator of protection against enteric fever. However, their role in infection-derived immunity is more complex, and to do date no studies have investigated the role of B cells in paratyphoid infection or *S*. Paratyphi A inoculation.

1.8.3.Humoral response and antibody effector functions

One major, and easily measurable, function of B cells is production of antigen specific antibodies by plasmablasts. Different antibody isotypes and subclasses engage in different functions, which can be further modulated depending on the glycosylation status. It's long been thought that induction of high levels of antigen specific antibodies by vaccination is a good indicator of immunity, and possibly protection [129]. For some pathogens other antibody biophysical properties (e.g. avidity) and function are better indicators of protection [130,131].

Oral challenge with *S*. Typhi or *S*. Paratyphi A induces strong antibody responses to O:9 and O:2 respectively, moderate responses to Hd and Ha, and no responses to Vi. Although increases in

binding antibody quantity are only significant in those who developed systemic disease [132,133]. In a human challenge model, no demonstrable booster effect is observed following homologous re-challenge, and despite the reduced risk of infection upon re-exposure, baseline levels at the time of re-challenge did not correlate with protection [125,134]. In partial contrast, seroepidemiology and modelling studies hypothesise a link between increasing seropositivity through repeated exposure and decreased disease incidence, and suggest antibody boosting can occur through subclinical infection [29,135]. Furthermore responses to alternative antigens, such as HlyE, which have not been routinely measured in challenge recipients, are promising markers of enteric fever infection in endemic settings [136].

Oral vaccines Ty21a and M01ZH09 both elicit strong anti-LPS and anti-H antigen responses. The magnitude of O-specific ASCs and proportion of O-antigen responders after Ty21a has been linked to the reported vaccine efficacy [43,54,137]. Inoculation with M01ZH09 induces strong ASC responses and produces multifunctional humoral response characterised by bactericidal and phagocytic enhancing antibodies [138]. Despite these promising observations the vaccine efficacy of M01ZH09 is estimated at 13% in a human challenge model [59,61].

Comprehensive studies have been conducted investigating Vi specific serological correlates of protection. Broad analysis suggests that anti-Vi IgA titres correlated with protection, but higher anti-Vi IgG was associated with reduced disease severity indicating distinct but complementary roles for humoral immunity against typhoid. Comparative analysis shows distinct protective signatures for each vaccine. Total anti-Vi IgA titre and fold change from baseline was associated with protection after Vi-PS in a human challenge model, whereas Vi specific IgA fold change and Vi specific IgG1 avidity more closely linked with protection after Vi-TT [139,140]. Furthermore, Vi-PS and Vi-TT vaccines elicited strong functional humoral responses (functional features measured: complement deposition, neutrophil and monocyte phagocytosis, neutrophil oxidative burst, and NK cell

acitivation). Polyfunctionality was associated with protection in both groups. Although, protective functional responses in Vi-TT recipients was primarily associated with neutrophil phagocytosis [141].

It is not possible to define a definitive antibody titre above which 100% of individuals are protected from disease after Vi vaccination, studies are underway to define a relative correlate by measuring antibody titres that correlate with 50% and 80% protection.

Few studies have looked at protective antibody activity against paratyphoid fever in humans. Typhoid vaccines, Ty21a, CVD909 and surprisingly Vi-PS, induce cross-reactive binding antibody responses to *S*. Paratyphi [126,142]. Inoculation with Ty21a and CVD909 have also been associated with enhanced phagocytic antibodies against *S*. Paratyphi A and B [143]. Although, no formal analysis has linked humoral responses to the cross protection observed by Ty21a. A single dose of *S*. Typhi – Paratyphi A bivalent conjugate vaccine induces strong O:2 specific and functional antibody responses [80]. The efficacy and related immunogenicity of an oral paratyphoid, and bivalent *S*. Typhi – Paratyphi A vaccine is being assessed in current and ongoing studies.

1.9. Correlates of protection

Identification of infection or vaccine induced correlates is a desirable goal, such knowledge helps our understanding of underlying protective immune mechanisms, provides informed targets for vaccine design, and can help identify protected individuals within a population. Correlates of protection can be absolute (an immune marker with a defined protective threshold) or relative (associated with protection above a certain level but not definitive), and due to the redundancy of the immune system multiple co-correlates can exist [144]. Establishing a correlate of protection can help expediate vaccine development and licensing vaccines through established correlates can be more economical than large-scale field trials, this is of particular importance for pathogens where disease incidence is low.

Correlates of protection for multiple bacterial infections have been identified; anti- *Haemophilius influenza* type B antibody titre for Hib infection (>1mcg/mL for polysaccharide vaccines, and >

0.15mcg/mL for conjugate vaccines), and a serum bactericidal activity assay titre of >1/8 after conjugate vaccine is sufficient for protection against infection with *Neisseria meningitidis* serogroup A, C, W, Y [145–147].

There are currently no licensed vaccines against paratyphoid fever. Comprehensive control of enteric fever requires successful implementation of paratyphoid vaccines as well as typhoid vaccines. Identification of immune correlates will help our understanding of how protection from these related pathogens differ, and will also help our understanding of which immune processes are crucial for vaccine mediated protection.

1.10. Aims and Objectives

The overall aim of this thesis is to address the knowledge gap surrounding functional humoral immunity to enteric fever.

Aim 1: Develop robust and reproducible assays to measure three key antibody effector functions: serum bactericidal activity (SBA), monocyte phagocytosis, and neutrophil phagocytosis against *S*. Typhi and *S*. Paratyphi A.

Aim 2: Does experimental typhoid, paratyphoid challenge, or enteric fever vaccine induce changes in antibody effector functions?

Aim 3. Do any of the measured effector functions correlate with protection in typhoid or paratyphoid challenge models? Do any changes in antibody effector functions induced by oral vaccine CVD1902 correlate with protection in an experimental paratyphoid model?

Aim 4: Do functional antibody characteristics correlate with each other or any other biophysical properties (e.g. isotype or antigen specificity) or disease severity after challenge (e.g. symptom severity and bacteraemia) in challenged individuals?

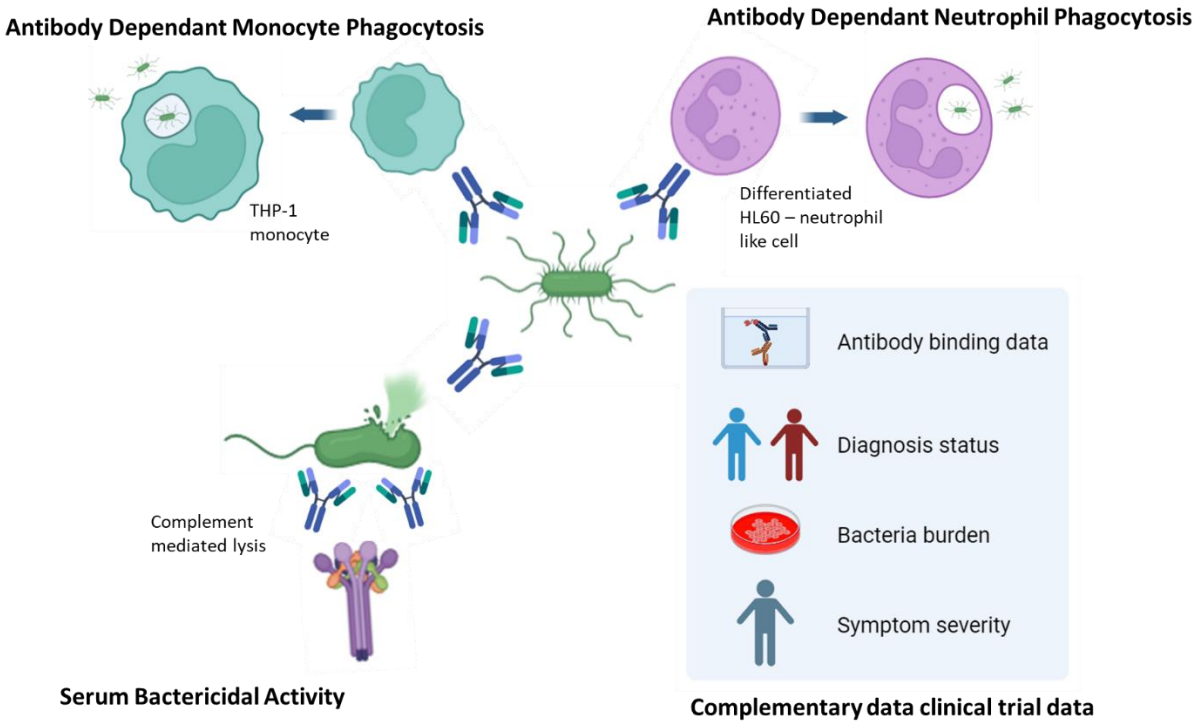


Figure 1-4 Overview of data collected and included in this analysis

Schematic overview of the three assays developed to measure antibody effector functions in the various clinical trial populations. Combined with antibody and symptom metadata from typhoid and paratyphoid with the aim of investigating the relationship between antibody function, diagnosis outcome, and disease severity in CHIM participants.
Figure made using BioRender.

Chapter 2. Materials and methods

Details for each of the methodologies used in this thesis have been compiled into a single chapter. Each subsequent chapter refers to the appropriate methods sections. In the methods development chapter (chapter 3) relevant deviations from the methods are mentioned within the text.

2.1. Clinical trial sample overview

Table 2-1 Details of the clinical trials and samples used

Study name	Study	Description	Study Location	Samples tested	Sample details	Common challenge study features
VAST	Vaccines against *Salmonella* Typhi (VAST, 2014/08)	Healthy volunteers were enrolled and randomised 1:1:1 to receive a single dose of either Vi – tetanus toxoid conjugate vaccine (Vi-TT, Tybar TCV), Vi polysaccharide vaccine (Vi-PS, Typhim-Vi), or MenACWY conjugate vaccine. Approximately 28 days after vaccination participants drank sodium bicarbonate buffer to neutralise stomach acid followed by oral challenge with 1-5 x 10^4 CFUs *S*. Typhi Quailes strain re-suspended in sodium bicarbonate buffer. Participants attended daily follow up visits for 14 days consecutively for health assessment and blood collection for culture [148].	UK	Vi-TT: 37 (TD: 13, nTD: 24) Vi-PS: 35 (TD: 13, nTD:22)	Serum for serological assays was isolated from blood samples collected immediately before vaccination (D-28) and challenge (D0).	Individuals were diagnosed with typhoid fever if they had a positive blood culture (at least 72 hours post-challenge), or a temperature >38°C for longer than 12 hours. Upon diagnosis participants started antibiotics, individuals who did not meet this pre-determined diagnosis criteria commenced antibiotics after 14 days. Participants completed an e-diary daily during the two week post challenge period where they recorded temperate and symptom data. Symptom data were collected in a graded fashion (0:not present, 1:mild, 2:moderate, 3:severe) on headache, malaise, myalgia, constipation, diarrhoea, abdominal pain, nausea, loss of appetite, cough, and arthralgia.
P1	*Salmonella* Paratyphi A challenge model dose finding study (P1, 2013/07)	The first *S*. Paratyphi challenge study was a dose finding study. Forty healthy volunteers were enrolled and challenged 1:1 with either 100-500 (low dose), or 1-5 x 10^3 (high dose) CFUs of *S*. Paratyphi A NVGH308 strain suspended in sodium bicarbonate buffer, following sodium bicarbonate buffer pretreatment [149].	UK	Low dose: 17 (PD: 8, nPD:9) High dose: 19 (PD: 11, nPD:8)	Serum for serological assays was isolated from blood samples collected immediately prior to challenge (D0) and 90 days later (D90)	
PATCH	*Salmonella* Typhi and Paratyphi A challenge re-challenge study (PATCH, 2014/01)	A challenge, rechallenge study comparing the attack rate in naïve individuals or those who had previously been challenged with *S*. typhi or Paratyphi A. Healthy volunteers received 1-5 x 10^3 CFUs of *S*. Paratyphi A NVGH308 strain suspended in sodium bicarbonate buffer, following sodium bicarbonate buffer pretreatment. Only naive or homologous *S*. Paratyphi A challenged individuals were analysed in this thesis [134].	UK	Naïve: 15 (PD: 8, nPD:7) Rech: 13 (PD: 4, nPD:9)	Serum for serological assays was isolated from blood samples collected immediately prior to challenge (D0) and 90 days later (D90)	
VASP	Vaccines against *Salmonella*	Healthy volunteers were enrolled and randomised 1:1 to receive either a *S*. Paratyphi A live attenuated oral vaccine, CVD1902 (2 x 10^{10} CFU), or a sodium	UK	Total samples tested: 27	Serum for serological assays was isolated from blood samples	

	Paratyphi A (VASP, 2018/07)	bicarbonate placebo, following a sodium bicarbonate pretreatment. Two doses were given, two weeks apart. Approximately one month after the second dose participants received 1-5 x 10^3 CFUs of *S*. Paratyphi A NVGH308 strain suspended in sodium bicarbonate buffer, following sodium bicarbonate buffer pretreatment. This study is actively recruiting at the point of writing this thesis, a subset of samples have been analysed.		(blinded to vaccine arm) PD: 13 nPD: 14	collected immediately prior to prime vaccination (D-42) and 42 days later, immediately prior to challenge (D0)	
SIIPL Phase 1	Serum Institute India *S*. Typhi - *S*. Paratyphi Bivalent Conjugate vaccine Phase I	A phase I study investigating the safety and immunogenicity profile of a novel *S*. Typhi - Paratyphi A bivalent conjugate vaccine (Sii-PTCV). Sixty healthy adults were enrolled and randomised to receive a single intra-muscular dose of Sii-PTCV or Tybar-TCV, a licensed Vi-conjugate vaccine [80]. The novel bivalent vaccine (Sii-PTCV) consists of *S*. Typhi Vi polysaccharide conjugated to tetanus toxoid and *S*. Paratyphi O-specific polysaccharide conjugated to diphtheria toxoid.	India	Sii-PTCV: 30 Tybar-TCV:30	Serum for serological assays was isolated from blood samples collected immediately prior to vaccination (D0) and 29 days later (D29)	NA

2.2. Bacterial strains used

The content of this thesis includes data from many clinical trials and projects, utilising multiple typhoidal *Salmonella* strains. **Table 2-2** summarises the strains used for each of the datasets presented.

Table 2-2 List of bacterial strains used

Project	Chapter	Serovar	Strain	Details	Assay specific culture methods
VAST L-SBA	Chapter 3	*Salmonella* Typhi	Quailes strain	Wild type *S*. Typhi strain isolated from the gall bladder of typhoid fever patient [133]	Using an inoculation loop, scrapes were taken from a vial of the GMP batch of each strain and dipped in LB broth. This was cultured until log phase: OD_{600} > 0.2. The bacteria were pelleted by centrifuging at 3200xg and a stock was made by resuspending in LB containing 20% glycerol. The day before the L-SBA assay 5mL LB broth was inoculated with the bacteria and grown overnight at 37C, 220rpm. On the day of the assay the cultured bacteria were diluted to an OD_{600} of 0.05 and cultured until the OD_{600} reached 0.2.
VAST L-SBA	Chapter 3	*Salmonella* Typhi	CVD908	Oral vaccine strain: Ty2 parent strain with aroC/aroD gene deletion impacting the biosynthesis of aromatic amino acids [63,150]	
VAST L-SBA	Chapter 3	*Salmonella* Typhi	CVD909	Oral vaccine strain: Ty2 parent strain with *aro*C/*aro*D deletion, *htr*A deletion (gene in the heat shock protein synthesis pathway), replacement of Vi expression promotor from P_{viA} to constitutive promotor P_{tac} [63,150]	
VAST L-SBA	Chapter 3	*Salmonella* Typhi	M01ZH09	Oral vaccine strain: Ty2 parent strain with *aro*C and *ssa*V deletion (inner membrane component of the T3SS encoded by SPI-2) [151]	
VAST L-SBA	Chapter 3	*Salmonella* Typhi	Ty21a	Oral vaccine strain: Ty2 parent strain with multiple mutations, including: *galE* mutation causing downstream galatose metabolism disruption [58,152]	
P1 L-SBA	Chapter 3	*Salmonella* Paratyphi A	NVGH308	Wild type strain of *S*. Paratyphi A isolated from a paratyphoid fever patient at the Oxford University Clinical Research Unit at Patan Hospital, Kathmandu, Nepal[149]	
PATCH L-SBA	Chapter 3	*Salmonella* Paratyphi A	NVGH308	See above	

SIIPL Phase 1 SBA	Chapter 3	*Salmonella* Paratyphi A	ATCC9120	Commercially available wild type *S*. Paratyphi A strain, used by SIIPL for the manufacture of the *S*. Typhi-Paratyphi A bivalent conjugate vaccine.	Bacteria were streaked from the stock vials onto TSA agar, cultured overnight at 37C, 5% CO_2 and inspected for contamination. A sweep was taken of the bacteria and used to inoculate 50mL LB broth. The culture was incubated at 37°C, 150rpm until stationary phase. 10mL of the bacterial culture was mixed with 14mL LB broth and glycerol with a final amount of 13-15%. Stocks were frozen and then thawed on the day of use.
SIIPL Phase 1 SBA	Chapter 3	*Salmonella* Paratyphi A	NVGH308	See above	
ADMP – all samples	Chapter 4	*Salmonella* Paratyphi A (GFP transfected)	NVGH308	See above	Bacteria were transfected using a plasmid gifted by Dr Nancy Wang and Professor Dick Strugnell from the University of Melbourne [153]. The plasmid encodes green fluorescent protein under a constitutive promoter. The transfection process is described in section 2.2.1. Stocks for the functional assays were made of the transfected bacteria by initially grown on tryptic soy agar (TSA) to confirm no contamination. A sweep was taken of the plate using an inoculation loop, bacteria were cultured in 50mL LB broth 150rpm 37°C, and grown to an OD_{600} = 0.2. Bacteria were centrifuged at 3200*xg* and resuspended in 50mL 2x BD cell fix (BD biosciences) for 20 minutes to fix the bacteria. The bacteria were centrifuged at 3200*xg* and resuspended in LB broth containing 20% glycerol.
ADNP - all samples	Chapter 5	*Salmonella* Paratyphi A (GFP transfected)	NVGH308	See above	

2.2.1.Transfection of *S*. Paratyphi A NVGH308 with a GFP containing plasmid

Transfection of *Salmonella* Paratyphi A, NVGH308 strain was performed by Dr Jennifer Hill using a protocol and plasmid provided by Dr Nancy Wang and Professor Dick Struggnell. The pTETtac4 plasmid contains GFP (GFPmutb3 variant) expressed under a constitutive promoter and an ampicillin antibiotic resistant gene. *S*. Paratyphi A was grown in 10mL Luria Bertani (LB) broth for 16-22 hours (overnight) at 37°C, 220rpm. 100µL of the overnight culture was added to 10mL of fresh LB broth and was then incubated for 3 hours at 37°C, 220rpm until the OD_{600} reached 0.6-0.8. The culture was placed on ice for 10 mins before centrifuging 4000rpm for 10 mins at 4°C. The supernatant was discarded and the pellet resuspended in 800µL ice-cold transformation and storage solution (TSS) buffer. Then 200µL of this bacterial suspension was added to 80µL of TSS enhancement buffer combined with 20-200ng of plasmid DNA. The resultant mixture was incubated on ice for 20 mins, followed by a further 20 min incubation at room temperature. 1mL of super optimal broth with catabolite repression (S.O.C. media) was added to transformed bacteria and incubated at 37°C, 220rpm for 60-90min. Bacteria were pelleted by centrifuging at 10,000rpm for 3 mins and resuspended in 700µL of LB broth.

Bacteria were streaked on to tryptic soya agar (TSA), containing ampicillin and incubated at 37°C, 5% CO_2 overnight. A single colony was picked and used to inoculate 10mL LB broth, containing 100 µg/mL ampicillin, and incubated overnight at 37°C, 220rpm. A subculture was made by adding 100µL of overnight culture to 10mL of fresh LB broth and was incubating it for 3 hours at 37°C, 220rpm. 300µL of culture was added to 200µL of 50% glycerol and the stocks were stored at -80°C. Successful plasmid transformation and GFP expression was confirmed by flow cytometry (**Figure 2-1**). Using an inoculation loop, scrapes were taken from the frozen stocks, mixed with 5mL LB broth and incubated overnight 37°C, 220rpm. A fresh subculture was then made with an OD_{600} of 0.05 which was incubated for 3 hours at 37°C, 220rpm until the OD_{600} reached 0.2. These bacteria were then diluted to 1 x 10^6 CFU/mL and 20µL was added to 220µL of 2 x BD cell fix and incubated in the dark for 10 mins. Bacteria were pelleted by centrifuging at 300xg for 5 mins and resuspended in 200µL of 1x PBS

for running on the cytometer (BD Fortessa). While the wild type bacteria have small amounts of autofluoresence, there is a significant shift in fluorescence at the 530nm wavelength in the transformed bacteria.

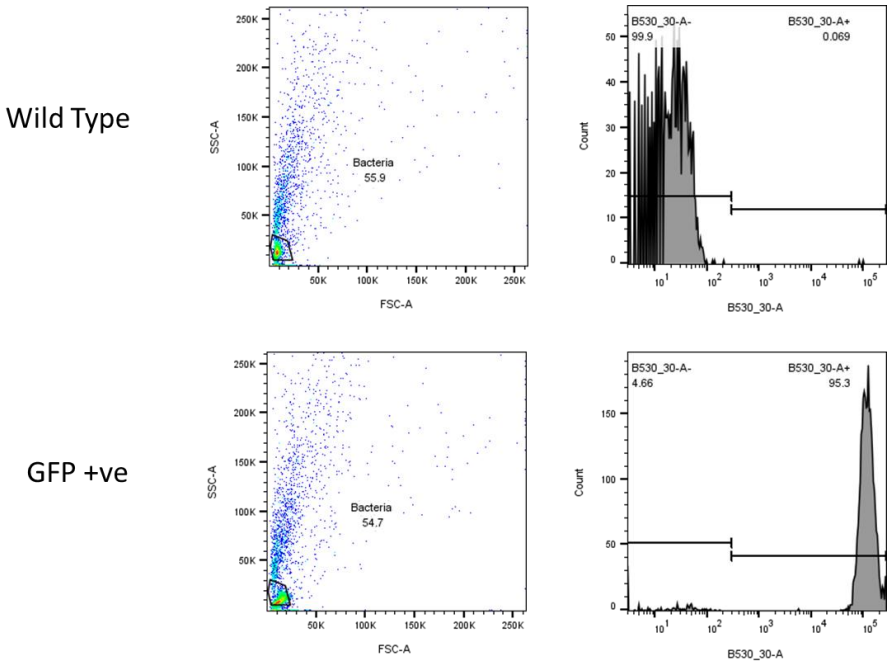


Figure 2-1 Transfection of S. Paratyphi A bacteria with GFP expressing plasmid

Salmonella Paratyphi A NVGH308 strain transfected with GFP containing plasmid. Single positive colony selected, grown, fixed and frozen to make a stock of GFP bacteria.

2.3. Cell Lines and culture methods

Table 2-3 Cell lines and culture condition details

Cell line	Supplier	Description	Culture methods
THP-1 (TIB-202)	ATCC	Monocyte cell line isolated from an acute monocytic leukemia patient	THP-1 monocytes were cultured in RPMI medium (Gibco) supplemented with 10% FBS (Merck) and 2mM L-Glutamine. The cell culture concentration was kept between 0.2 – 1 x 10^6 cells/mL, as recommended by ATCC
HL60 (CCL-240)	ATCC	Promyeoloblast cell line isolated from an acute promyelocytic leukemia patient	HL60 cells were cultured in IMDM containing L-glutamine and HEPES without phenol (Gibco) , supplemented with 20% FBS. The cell culture concentration was kept between 0.2 – 1 x 10^6 cells/mL, as recommended by ATCC

Table 2-4 Antibodies used for cell line phenotyping and phagocytosis assays

Surface marker	Clone	Fluorochrome	Filter	Manufacturer
CD64	10.1	PE	586/15	BD Life Sciences
CD32	FUN-2	AF647	660/20	BioLegend
CD16	3G8	AF700	730/45	BD Life Sciences
CD11b	ICRF44	Superbright702	710/50	BD Life Sciences
CD35	E11	BV786	780/60	BD Life Sciences
CD89	A59	BV605	610/20	BD Life Sciences
CD14	MϕP9	BV450	450/50	BD Life Sciences
CD71	M-A712	FITC	530/30	BD Life Sciences
Live dead (for phenotyping)	NA	NA	450/50	BD Life Sciences
Annexin V	NA	PE-CF594	610/20	BD Life Sciences
Live dead stain (for phagocytosis assays)	NA	NA	525/50	BD Life Sciences
GFP expressing bacteria	NA	GFP	530/30	NA

2.3.1.THP-1 surface marker expression

Expression of surface monocyte markers was assessed on thawed, overnight rested pre-banked THP-1 cells, and cells in fresh culture. Both culture types had positive expression of CD64 (FcγRI), CD32 (FcγRII), CD16 (FcγRIII), along with CD11b (leukocyte marker), and CD35 (complement receptor 1) expressed slightly higher than baseline. Little expression of CD14 (macrophage marker) and CD89 (FcαR) were present [154].

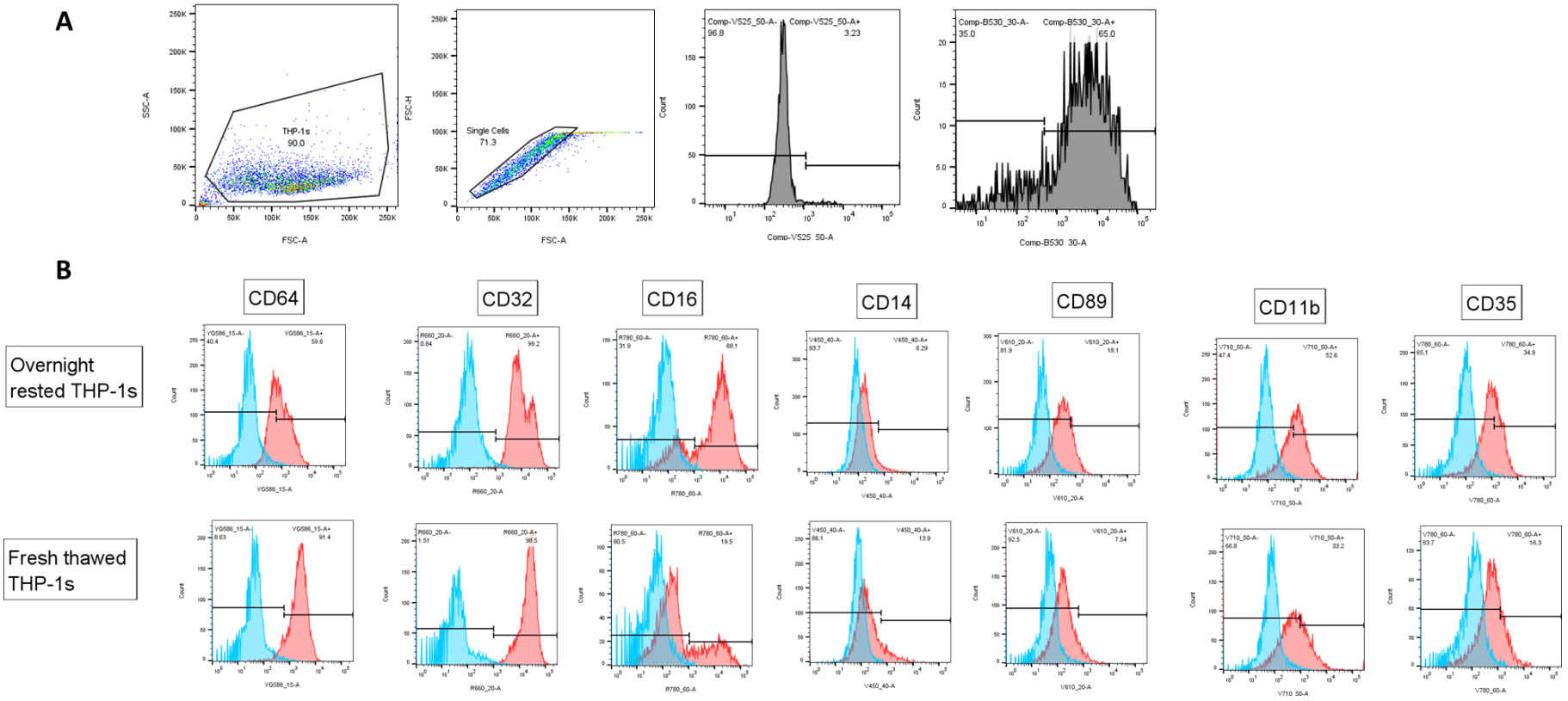


Figure 2-2 Gating strategy and phenotypic marker profiling of THP-1 cells in fresh culture compared with thawed and rested pre-banked THP-1 cells

(A) Representative example of the gating strategy of THP-1 cells in the ADMP assay: selection of THP-1 cells > selection of single cell population > gating on live cells (live/dead stain negative) > Selection of THP-1 cells that are also GFP positive.
(B)THP-1 cells were cultured in RPMI media with L-Glutamine, Hepes, and 10% FBS. Cells were stained with antibodies against, CD64, CD32, CD16, CD14, CD89, CD11b, and CD35.
Top panels represent surface marker expression on thawed pre-banked THP-1 cells after overnight resting, bottom panels represent THP-1 cells in fresh culture.
Blue population is the fluorescence intensity of the unstained population, red population is the stained population.

2.3.2.HL60 differentiation and Phenotyping

HL60 cells were cultured as per ATCC recommendations, detailed in **Table 2-3**. To differentiate the cells from a general granulocyte cell to neutrophil-like cells for use in the ADNP, cells were suspended IMDM media with 20% FBS and 0.08% dimethylformamide (DMF, Merck) at a concentration of 0.2 x 10^6 cells/mL and incubated for 5 days [155,156].

Flow cytometry was used to determine the relative level of expression of various surface differentiation markers, for details of antibodies used see **Table 2-4**. Successful differentiation was assessed by demonstrating the relative increase in expression of CD11b (leukocyte marker), CD16 (FcγRIII), CD64 (FcγRI) and CD32 (FcγRII) following the differentiation step, along with a relative decrease in CD71 expression [157,158]. Levels of CD35 (complement receptor 1), and CD89 (FcαR) were constant in both conditions.

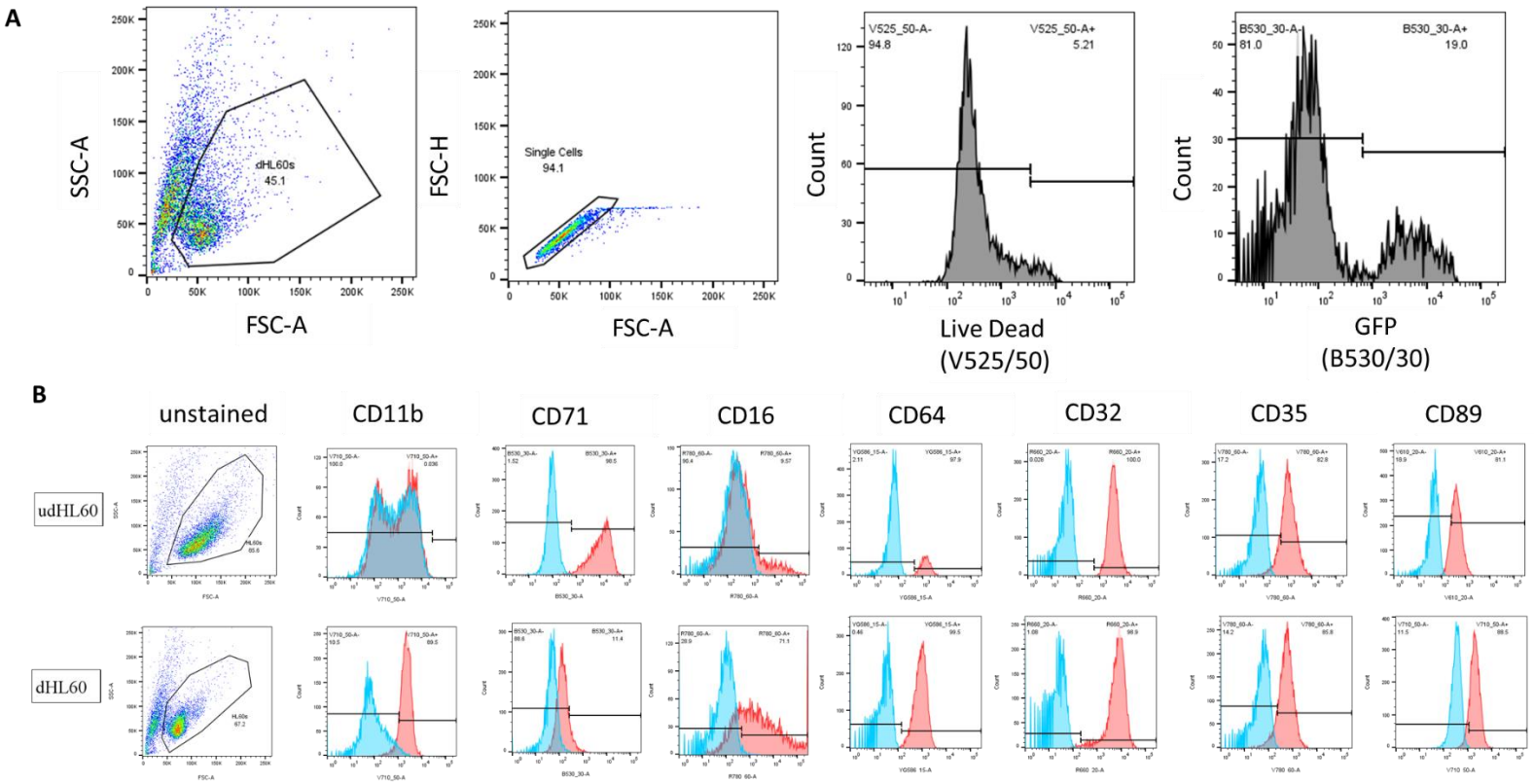


Figure 2-3 Phenotypic marker profiling of HL60 and differentiated HL60 cells

(A) Representative example of the gating strategy of dHL60 cells in the ADNP assay: selection of dHL60 cells > selection of single cell population > gating on live cells (live/dead stain negative) > Selection of dHL60 cells that are also GFP positive.
(B)HL60 cells were cultured in IMDM with L-Glutamine, Hepes, and 20% FBS. Cells stained with antibodies against, CD11b, CD66b, CD71, CD16, CD64, CD32, CD35, and CD89.
Top panels represent surface marker expression on undifferentiated cells, bottom panels represent differentiated cells, after a 5-day incubation with media containing 0.8% DMF.
Blue population is the fluorescence intensity of the unstained population, red population is the stained population

2.4. In house positive pool

Standardised assays require a consistent source of reference samples to facilitate intra-lab and inter-lab comparisons. For the *S*. Typhi specific protocols the commercially available international standard developed by national institute of biological standards and controls was used (NIBSC, sample 16/138). However, no *S*. Paratyphi A specific standard has been developed. Therefore, I developed an in house pool using serum collected from individuals enrolled in two experimental human challenge models (P1/PATCH). A subset of 15 serum samples taken 90 days after oral challenge with *S*. Paratyphi A were selected.

2.5. Serum bactericidal activity assay

2.5.1. *Salmonella* Typhi (Quailes) luminescence based (L-) SBA

Serum samples were heat inactivated, to remove endogenous complement, by incubating at 56^{o}C for 30 minutes. Serum samples were serially diluted in 1 x PBS (Gibco) in a 96 well u-bottom plate. 10µL of diluted test sera was added to 70uL LB broth (LB, Merck). 5mL of *Salmonella* Typhi, Quailes strain was grown overnight at 37^{o}C 220rpm to stationary phase. A subculture with an OD_{600} of 0.05 was incubated for approximately 2 hours until it reached log phase defined as having an OD_{600} of 0.20-0.22. Then 10µL of log phase bacteria were added to the diluted test sera along with 10µL of rabbit serum (Cedarlane). The resultant mixture was incubated for 3 hours at 37 °C, shaking at 220 rpm, before centrifuging at 3220×g for 10 minutes. The supernatant was discarded and the pelleted bacteria were resuspended in 100µL PBS. From this, 50µL was transferred to a white, flat-bottomed plate along with 50µL BacTiter-Glo. BacTiter-Glo is a buffer containing a bacteria lysis enzyme and a recombinant luciferase enzyme. It catalyzes ATP (from lysed bacteria) into a luminescent signal which is directly proportional to the number of surviving bacteria. The luminescence (relative light units, RLU) was quantified using the LUMIstar (BMG Labtech).

Quality control, acceptance criteria and analysis methods

Luminescence data were collected and processed using BMGLabTech Omega software version 5.11. The read outs from each test well were blank corrected and normalised to a no serum, complement only control and were fitted to a 4-parameter sigmoidal curve in GraphPad Prism version 10. The L-SBA titre was calculated as the serum dilution required to achieve 50% killing compared with the no serum, complement only control.

Samples were only included in the final analysis if they passed the following quality control criteria:

- The R^2 of the dilution curve was greater than 0.7
- The sera had been diluted sufficiently, determined as a <30% difference in RLU between the most dilute serum condition and the complement only control.
- The positive control (NIBSC 16/138) on the same plate was within 1 standard deviation of the average (taken from all plates where the standard curve passed QC).

2.5.2. *Salmonella* Paratyphi A (NVGH308) luminescence based (L-)SBA

L-SBA Serum samples were heat inactivated by incubating at 56^{o}C for 30 minutes. Serum samples were serially diluted 1/1.5 in 1 x PBS (Gibco), starting from neat serum to a final dilution of 1/87, in a 96 well u-bottom plate. 10µL of diluted test sera were added to 70µL LB broth (Merck). 5mL of Salmonella Typhi, Quailes strain was grown overnight at 37^{o}C 220rpm to stationary phase. Then a subculture with an OD_{600} of 0.05 was incubated for approximately 2 hours until it reached log phase, defined as having an OD_{600} of 0.20-0.22. From this, 10µL of log phase bacteria were added to the diluted test sera along with 10µL of rabbit serum (Cedarlane). The resultant mixture was incubated for 3 hours at 37 °C, with shaking at 220 rpm before centrifuging at 3220×g for 10 minutes. The supernatant was discarded and the pelleted bacteria were resuspended in 100µL PBS, 50µL was transferred to a white, flat-bottomed plate along with 50µL BacTiter-glo. The luminescence (relative light units, RLU) was quantified using the LUMIstar (BMG Labtech) and is directly proportional to the number of surviving bacteria.

Quality control and acceptance criteria

Luminescence data were collected and processed using BMGLabTech Omega software version 5.11. The read outs from each test well were blank corrected, normalised to a no serum, complement only control and were fitted to a 4-parameter sigmoidal curve. The L-SBA titre was calculated as the serum dilution required to achieve 50% killing compared with the no serum, complement only control.

Samples were only included in the final analysis if they passed the following quality control criteria:

- The R^2 of the dilution curve was greater than 0.7
- The sera had been diluted sufficiently, determined as a <30% difference in RLU between the most dilute serum condition and the complement only control.
- The positive control (in house pool, SPtA, see section **2.4** for details) on the same plate was within 1 standard deviation of the average (taken from all plates where the standard curve passed QC).

2.5.3.Salmonella Paratyphi A (ATCC9150) conventional colony counting based SBA

These methods were initially developed in collaboration with Serum Institute India Private Limited to test the immunogenicity of a bivalent *Salmonella* Typhi – *Salmonella* Paratyphi A conjugate vaccine. The paratyphoid component of this vaccine uses LPS purified from *S*. Paratyphi A ATCC9150 strain conjugated to diptheria toxoid.

Serum samples were thawed and heated inactivated for 30 minutes at 56^{o}C. Samples were pre-diluted 1/32 in Hanks Balanced Salt Solution (HBSS, Gibco) containing 0.5% FBS. Samples were then serially diluted 1:2 from 1/32 to 1/32 768 in HBSS in a 96 well plate. *S*. Paratyphi A, ATCC9150 strain bacteria were thawed and diluted 1/6000 in HBSS 0.5% FBS. 20µL of the bacterial suspension was added to each well of the 96 well plate. 10µL of baby rabbit complement (Pel-Freez) was added to all test wells and to the active complement control wells, 10µL of heat inactivated complement was

also added to the heat inactivated control (HIC) wells (no serum). The contents of the plate were mixed thoroughly by shaking the plate side to side, in all directions before incubating for 37^{o}C for 1 hour. After 1 hour, using the tilt plating technique, 10µL from each well were spotted onto LB agar plates, and then tilted to allow the formation of neat streaks down the plate. Plates were dried and incubated at 37^{o}C, 5% CO_2, after 16-22 hours the number of colonies were quantified using a semi-automated colony counter.

Quality control and acceptance criteria

The SBA titre was calculated as the highest serum dilution where the colony count is higher than or equal to 50% of the heat inactivated complement control (HIC).

Samples were included in the analysis if the duplicates of the same sample gave titres within a 2-fold dilution. The final SBA titre is the average of the duplicates.

Plates were accepted if the % difference between the active complement control and the HIC was ≤30% in 50% of the rows. The most dilute serum condition, or the bacteria only control could be substituted for the active complement control to meet these criteria.

2.5.4.*Salmonella* Paratyphi A (NVGH308) conventional colony counting based SBA

To determine if antibodies generated after bivalent vaccination were cross protective against other *S*. Paratyphi strains, the SBA protocol was performed with the NVGH308 strain. This protocol was identical to the one detailed in section **2.5.3** but with the ATCC9150 strain swapped for NVGH308 strain.

2.6. Antibody dependant monocyte phagocytosis

Test samples were heat inactivated for 30 minutes at 56°C before pre-diluting 1/8, 50µL of the diluted samples were added to u-bottom plate in duplicate. Frozen *S*. Paratyphi A NVGH308 containing a GFP expressing plasmid were thawed, centrifuged for 5 minutes at 13,000 *xg* and

resuspended in 1 x PBS at 6.25 x 10^7 CFU / mL. 20µL of the bacteria suspension was added to each well of the 96 well plate containing the diluted test sera and the plates were incubated for 2 hours at 37°C, shaking 50 rpm for pre-opsonisation. THP-1 cells were freshly cultured as described in section 2.4, cells were spun at 220 *xg* and resuspended in RPMI at a concentration of 2.5 x 10^5 cells/mL. The plates containing the pre-opsonised bacteria were spun at 3200 xg for 10 minutes and the supernatant was discarded, followed by addition of 100µL of THP-1 cells to the pelleted bacteria plus a further 100µL of RPMI. This was incubated for 30 minutes at 37°C, shaking 50 rpm. Then the plate was spun at 500*xg* for 5 minutes and 100µL of live dead stain (BD fixable viability dye 510) was added at a 1/1000 dilution to all the wells apart from the live dead compensation control well to which 1x PBS was added. The cells were left to stain for 15 minutes at 37°C, shaking 50 rpm before spinning again at 500*xg* for 5 minutes. The cells were then resuspended in 2% paraformaldehyde and incubated at room temperature, in the dark, for 15 minutes to fix. After fixing, the plate was then spun at 500*xg* for 5 minutes and the pelleted cells were resuspended in PBS. Cells were analysed on the BD fortessa flow cytometer. Phagocytosis was measured using a composite score, termed phagocytic score, defined as the number of THP-1 cells that are GFP positive multiplied by the median fluorescence intensity.

A positive pool, SPtA (see section **2.4** for details) was used as a reference standard. The standard was diluted ¼, and subsequently serially diluted ½ to generate a standard curve. The phagocytic score of the top standard, ¼ dilution, was assigned the arbitrary titre of 1000 phagocytic units and subsequent assigned titres were halved in line with the ½ serial dilution (see **Table 2-5** for dilution series). The titre of test samples were calculated by interpolating from the plate matched standard curve.

Table 2-5 ADMP standard dilution and corresponding phagocytic units

Standard dilution	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512
Arbitrary phagocytic units	1000.0	500.0	250.0	125.0	62.5	31.3	15.6	7.8

Quality control and acceptance criteria

Flow cytometry data were analysed using FlowJo software version 10.8.0.

The standard curve was plotted using a 4-parameter sigmoidal curve and which was used to interpolate the titres of the test samples. The curve was passed if the R^2 value was greater than 0.9.

Two QC samples were included on each plate, the plates were passed if the QC samples fell within range, which was determined as the average titres from all plates ± 1 standard deviation (see section 3.5, **Figure 3-18** F for plotted QC samples). Samples from failed plates were repeated.

If the titre of the samples was out of the range of the linear range of 62.5-500 phagocytic units (see standard curves examples, section 3.5, **Figure 3-18** A-C) when run at 1/8, samples were repeated at a higher dilution and the final titre was calculated taking that into account, e.g. if samples were test at 1/16 the final titre was double to account for this. Samples that consistently read below the lower part of the standard curve (15.63 phagocytic units) were assigned a titre of half the lower limit, 7.8.

2.7. Antibody dependant neutrophil phagocytosis

Test samples were heat inactivated for 30 minutes at 56°C before pre-diluting 1/8 in 1 x PBS, and double diluted 3 times, 50µL of the diluted samples were added to duplicate wells of a u-bottom. 20µL of *S*. Paratyphi A NVGH308 bacteria were added at 6.25 x 10^7 CFU / mL to each well and the plates were incubated for 2 hours at 37°C, shaking 50 rpm for pre-opsonisation.

dHL60 cells were freshly cultured as described in section 2.4.2, cells were spun at 100 *xg* and resuspended in RPMI at a concentration of 2.5 x 10^5 cells/mL. The plate containing the pre-opsonised bacteria was spun at 3200 xg for 10 minutes, 100µL of cells were added to the pelleted

bacteria along with 100µL of IMDM and incubated for 30 minutes at 37°C, 50 rpm. The plate was spun at 500*xg* for 5 minutes, 100 µL of live dead stain (BD fixable viability dye 510) was added at a 1/1000 dilution to appropriate wells and PBS was added to control wells. Cells were left to stain for 15 mins at 37°C, 50 rpm, before centrifuging again at 500*xg* for 5 minutes. Cells were resuspended in 2% paraformaldehyde and incubated at room temperature for 15 minutes to fix. After fixing, the plate was spun at 500*xg* for 5 minutes and the cells were resuspended in PBS. Cells were analysed on the BD fortessa flow cytometer, before downstream assessment cells were gated on live single cells.

Phagocytosis was measured using a composite score, termed phagocytic score, defined as the number of dHL60 cells that are GFP positive multiplied by the median fluorescence intensity.

A serum sample collected 90 days post-exposure to *S*. Paratyphi A from individual enrolled in a controlled human infection model at Oxford was used as a standard (ID 8949, time point D90). The standard was diluted 1/16, and subsequently serially diluted ½ to generate a standard curve. The phagocytic score of the top standard, 1/16, was assigned the arbitrary titre of 2000 phagocytic units and the titres of subsequent dilutions were halved in line with the ½ serial dilution (see **Table 2-6** for dilution series. The titre of test samples were calculated by interpolating from the plate matched standard curve.

Table 2-6 ADNP standard dilution and corresponding phagocytic units

Standard dilution	1/16	1/32	1/64	1/128	1/256	1/512	1/1024	1/2048
Arbitrary phagocytic units	2000.0	1000.0	500.0	250.0	125.0	62.5	31.3	15.6

Quality control and acceptance criteria

Flow cytometry data were analysed using FlowJo software version 10.8.0.

The standard curve was plotted using a 4-parameter sigmoidal curve using GraphPad Prism version 10 which was used to interpolate the titres of the test samples. The curve was passed if the R^2 value was greater than 0.9.

Two QC samples were included on each plate, the plates were passed if the QC samples fell within range, which was determined as the average titres from all plates ± 1 standard deviation (see section **3.6.2**, **Figure 3-20** for plotted QC samples). Samples from failed plates were repeated.

Four dilutions of each sample were run on the plate, samples were included if they read from the linear part of the standard curve, if the they fell above this the next dilution was taken and the dilution factor was taken into account. Samples that consistently read below the lower part of the standard curve (62.5 phagocytic units) were assigned a titre of 31.25.

2.8. Enzyme linked immunosorbent assay (ELISA)

S. Typhi Vi ELISA

Quantification of Vi specific IgG antibodies was performed using the Binding Site Vacczyme ELISA kit as per the manufacturers guidelines and has been described extensively [76,141,159]. Measurement of other subclasses (IgG1, IgG2, and IgG3) and isotypes (IgA and IgM) were performed using an adaption of the kit, optimisation of this was performed by Anna Nebykova and Dr Helene Juel. These data are not novel to this thesis and are included as part of the correlation analysis[76] .

S. Paratyphi LPS ELISA for CHIM samples

Quantification of LPS specific antibodies (IgG, IgM, and IgA) was completed using an in-house ELISA detailed by Dobinson *et al*[132]. Flat bottomed plates are coated with 1µg/mL of O:2 antigen, supplied by NVGH, and incubated overnight at 4°C. Plates were washed 5 times with 300µL 1xPBS + 0.05% Tween (PBST, Sigma), and blocked with 1xPBS +1% bovine serum albumin (BSA, Sigma) at room temperature for 60 mins. Plates were washed 5 times and test samples were added at a 1/200 dilution (diluted in 1xPBS+1% BSA), samples were serially diluted 1/3 for 5 iterations until a final dilution of 1/5400 and incubated for 90 mins at room temperature. Plates were washed 5 times in

PBST and 100µL/well of diluted anti-human IgG/IgA/IgM-HRP-conjugate antibody (ABD Serotec) was added and incubated for 60 mins at room temperature (conjugate dilutions: IgG 1/20,000, IgA 1/6,000, IgM 1/6,000). Plates were washed 5 times with PBST and 60µL of 3,3',5,5'-Tetramethylbenzidine (TMB) was added. When the top standard reached an OD 0.5-0.55 reading at 630nm wavelength the reaction was stopped by addition of 30µL 2.5M sulphuric acid. The final OD readings were taken at 450nm.

A standard and 3 QC samples, made from pooled sera from MOIZH09 vaccinated individuals, supplied by Emergent BioSolutions, were run on each plate.

Quality control and acceptance criteria

The standard curve was plotted using a 4-parameter sigmoidal curve which was used to interpolate the titres of the test samples. The curve was passed if the R^2 value was greater than 0.98. The plate passed if the titre of the QC samples were within the assigned range and %CV of duplicates was <10%. Quality control and acceptance criteria. Samples were accepted if the %CV of the duplicates was <20%. The assigned value was the value taken from the highest dilution that could be interpolated from the standard curve and met the QC criteria.

These assays were developed and performed by Dr Helena Thomaides-Briers and are included in this thesis as part of the correlation analysis.

S. Paratyphi LPS ELISA for SIIPL Phase I study samples

Quantification of LPS specific IgG was done using an ELISA based on the one above with some minor changes, namely:

- Plates were coated with 20µg/mL of purified LPS antigen supplied by SIIPL
- Blocking was achieved by incubation with PBS 0.05% tween and 5% non fat milk powder at 25°C for 2 hours
- Development of plates was stopped when the OD_{630} of the top standard reached 0.3-0.6

These assays were developed and performed by Dr Florence Mclean, JuYeon Park, Nicole Day, and Dr Amy Flaxman, and are included in this thesis as part of the correlation analysis

2.9. Vi capsular polysaccharide expression assay

Monoclonal antibody binding was used as a proxy for measuring Vi capsule expression. The bacterial strains were grown to early log phase (OD_{600} = 0.2) as described in table 2.2. 1mL of bacteria culture was pelleted by centrifuging for 5 mins at 13,000xg, they were washed in 1mL autoMACs running buffer and then pelleted by centrifuging for 5 mins at 13,000xg. Bacteria were resuspended in 200µL of autoMACs running buffer. An anti-Vi monoclonal antibody (in house, generated as part of the VAST trial) was added to the bacteria and incubated for 30 mins at 4°C. Bacteria were then washed twice in autoMACs running buffer, spinning at 13000xg inbetween. 100µL of add anti IgG-Alexa 488 (Invitrogen) was added to the pelleted bacteria and incubated for 30 mins at 4°C. Bacteria were pelleted by centrifuging for 5 mins at 13,000xg and fixed using cytofix/perm before running on the BD Fortessa flow cytometer.

2.10. Statistical analysis

Statistical analysis and plotting was completed using GraphPad Prism version 10 and R version 4.3.1.

Comparison of functional antibodies titres across timepoints was carried out using a Wilcoxon paired signed rank test. Analysis of unpaired samples (to compare between groups with different diagnosis outcomes) and fold-changes were assessed using a Mann Whitney-U test.

Correlations were assessed using a Spearmans Rank order correlation.

The relationship between the various immune markers and the diagnosis outcome from challenged was assessed using a logistic regression and odds ratio.

Principal Component Analysis (PCA) presented in chapter 7 was performed with FactoMineR using the R version 4.3.1. Data were standardised by scaling to have a mean of 0 and a standard deviation of 1 before plotting the PCA.

Unsupervised hierarchical clustering presented in chapter 7 was performed using the pheatmap package in R version 4.3.1.

Chapter 3. Development of standardised assays to investigate antibody effector functions

3.1. Introduction

Assessment of humoral immunity to enteric fever is a key part of understanding the mechanisms underpinning natural and vaccine induced protection. While extensive studies have been used to investigate quantitative and functional serological correlates of protection to *S*. Typhi after Vi vaccine, the application of these assays to assess *S*. Paratyphi A humoral immunity is still in its infancy.

Development of standardised, reproducible assays to measure antibody effector functions will more easily permit meaningful comparisons between vaccine responses in historical, current, and future clinical trial sample sets.

This chapter describes the steps taken to develop novel protocols to assess antibody bactericidal activity (SBA), ADMP, and ADNP using established cell lines.

Three independent SBA assays were set up:

1. *S*. Typhi specific assay for assessing responses to Vi vaccination in a challenge model.
2. *S*. Paratyphi A serum bactericidal activity (SBA) assay for assessing responses and oral vaccine *S*. Paratyphi A vaccine and response to Paratyphi exposure in a CHIM
3. *S*. Paratyphi A SBA for assessing responses to an *S*. Typhi-Paratyphi A bivalent conjugate vaccine in collaboration with SIIPL.

The chapter also describes the development steps used to set up an *S*. Paratyphi A specific antibody dependent monocyte phagocytosis (ADMP) and antibody dependent neutrophil phagocytosis

(ADNP) assays. The same assays were applied to all data sets collected in the clinical trials outline in **Table 2-1**.

3.2. Methods

The final assays methods for each for the protocols developed for this thesis are mentioned in **Chapter 2**, sections **2.5** (SBAs), **2.6** (ADMP), and **2.7** (ADNP). The optimisation steps taken to develop these protocols are explained within this chapter, deviations from the final methods for the sake of optimisation are detailed in each of the sections below and in the figure legends.

3.3. Results

3.3.1. Methods development of *Salmonella* Typhi luminescence based (L-)SBA

To measure the serum dependent bactericidal activity of our samples we adopted a luminescence based SBA protocol originally developed by Necchi *et al*[160]. I developed this assay with the *S*. Typhi Quailes strain, a wild type strain, and CVD909, which is modified so the Vi capsule polysaccharide is constitutively expressed. The aim was to examine changes in SBA after Vi vaccinations administered as part of the vaccines against *Salmonella* Typhi (VAST) controlled infection study, therefore using a bacteria with constitutive Vi expression (CVD909) would be advantageous in assessing the contribution of the Vi specific antibodies.

3.3.1.1. Selection of optimal complement conditions

SBA is a measure of antibody dependent complement mediated bacterial killing and to ensure the read out is primarily a measure of the antibody activity it relies on a uniform exogenous complement source. To optimise the complement conditions; bacteria were grown to log phase and incubated with different batches of rabbit complement, used at 5-50%, for 3 hours at 37°C, 220 rpm, and the surviving bacteria were quantified via luminescence. The ideal complement condition is one were you can see a small reduction in the bacterial growth, but not an overwhelming amount, i.e. biologically the complement is able to lyse the bacteria, but it is not added in such quantities that

would mask the antibody dependant enhancement of this reaction. In the presence of increasing complement amounts there is decreased growth of bacteria, these data show that the optimal conditions would be 10% for the Quailes strain and 5% for the CVD909 strain (**Figure 3-1**).

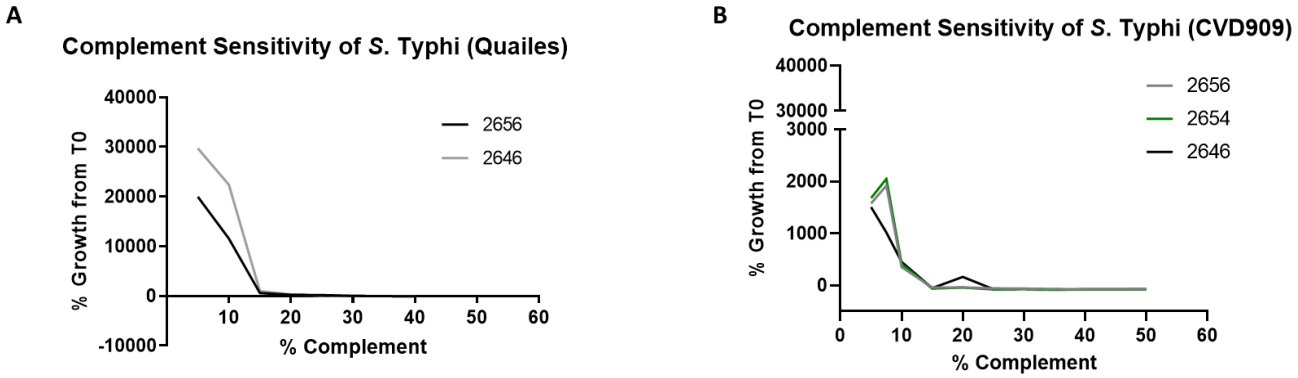


Figure 3-1 Optimisation of complement conditions for Salmonella Typhi specific L-SBA assay

Graphs show bacterial growth relative to starting bacteria quantity, measured via luminescence. Experiments were performed in the absence of serum, with complement at varying amounts. Note different scales for different strains (A) Quailes strain, (B) CVD909 strain. Different lines represent different batches of complement.

The L-SBA titre is calculated as the serum dilution needed to achieve 50% killing of the bacteria compared with a complement only control. To measure this, sera were serially diluted to generate bacterial survival/killing curves. Suitability of complement conditions were tested using the standard serum sample NIBSC 16/138, available from NIBSC, which is made from pool of sera collected from Vi vaccinated individuals. Serially diluted 16/138 was incubated with the bacteria and increasing concentrations of rabbit complement (5-25%). The survival curves are expressed as the luminescence in the test sera well relative to that in the complement only well. There is little difference in the curves generated with the Quailes strain when adding complement at 10-15% (**Figure 3-2** A), however, addition of 15% complement seems to induce less killing relative to the complement only control when the serum is more concentrated. This indicates that addition of higher concentrations of complement overwhelms the antibody dependent aspect (**Figure 3-2** A). When the same positive serum pool was added to the assay with CVD909 as the target strain,

marked differences in the killing profile at different complement concentrations were observed (**Figure 3-2** B). Addition of complement at 5% is the only condition were antibody dependent differences can be measured. These data indicate that 10% complement is adequate for the Quailes L-SBA and that 5% should be used for the CVD909 assay.

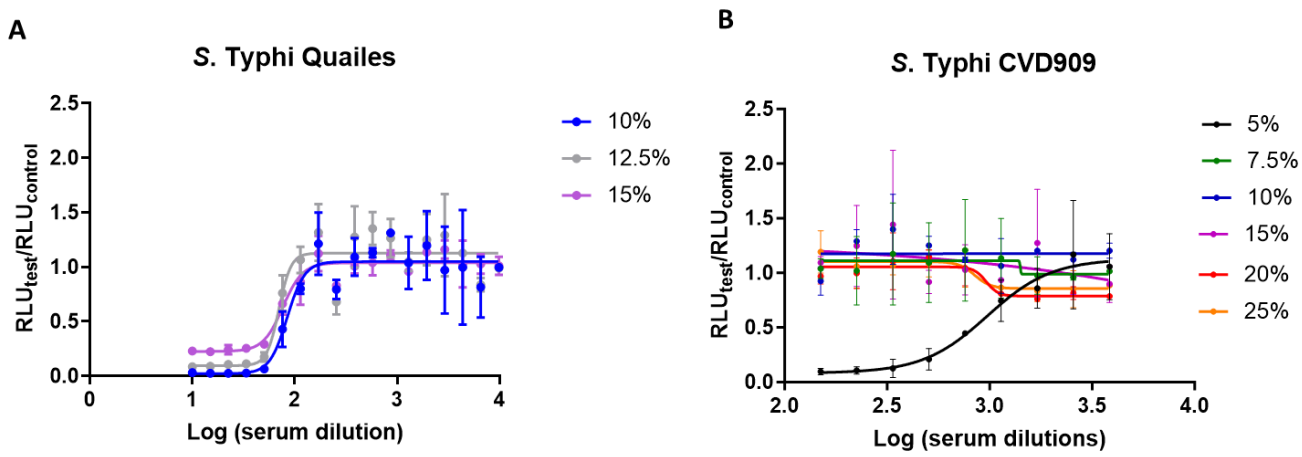


Figure 3-2 Bactericidal activity of NIBSC 16/138 serum standard with multiple complement amounts.

The L-SBA assay performed with either *S*. Typhi Quailes (A) or CVD909 (B) strain under different complement conditions. Graphs show the proportion of bacteria present in each condition (RLU_{test})compared with a no serum complement only control ($RLU_{control}$) as measured by luminescence (RLU) after a 3 hour incubation. Lower values on the y axis represent higher killing in the serum test condition compared with the control. Each point represents the average of 3 replicates and the error bars are indicative of the standard deviation.

Such stark differences in the curve profiles for CVD909 in the presence of increasing complement could be affected by other parameters of the assay such as incubation times. The serum incubation time was tested, using the positive serum pool 16/138 in the presence of various concentrations of complement. The assay developed by Necchi *et al* suggests a 3-hour reaction time, but we varied the reaction time to investigate the influence on the curve formation [161]. If the reaction is stopped before 3 hours then the serum dilution curve is not fully formed (**Figure 3-3Figure 3-4**). When the reaction continues for the full 3 hours the recognisable sigmoidal dilution curves are only present in the assay with the lowest complement, 5-7.5%.

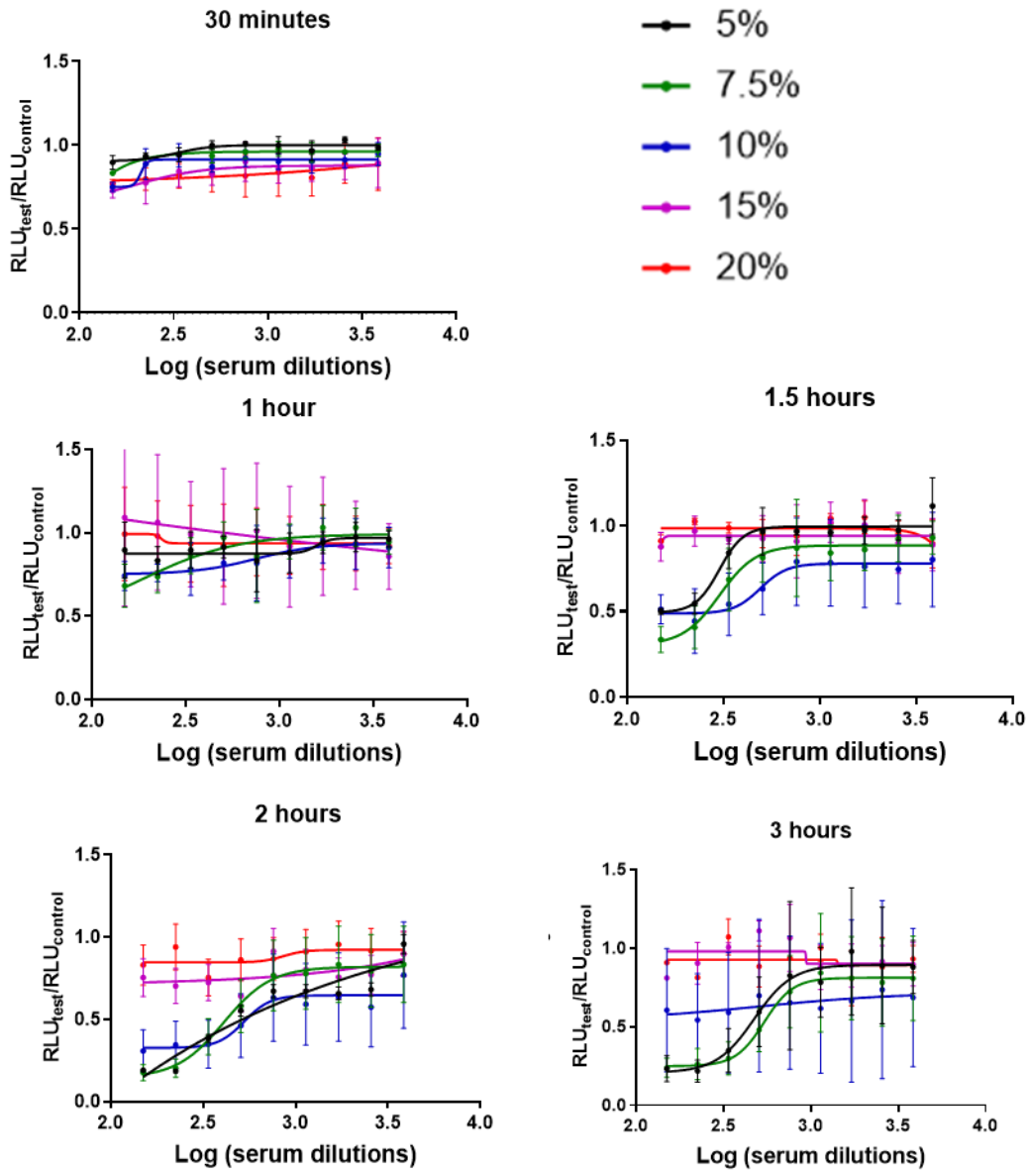


Figure 3-3 Comparison of bactericidal activity of NIBSC 16/138 at different time intervals

The L-SBA assay performed with either *S*. Typhi CVD909 strain under different complement conditions and with different time incubations (30 minutes – 3 hours). Graphs show relative ratio of surviving bacteria (*S*. Typhi, CVD909) in the presence of serially diluted positive control compared with complement only control. Each data point represents the duplicate average, and the error bars are standard deviation.

The exogenous complement source used in L-SBA assays is not purified, it comes from rabbit sera, therefore it is important to determine the presence of any cross-reactive antibodies as they could also lessen the quantification range of the assay. The rabbit complement was heat inactivated to remove any complement activity before testing in the L-SBA alongside the active complement source. The surviving bacteria in the heat inactivated complement (HIC) condition should be comparable to that of the active complement (AC) condition if there are no cross-reactive antibodies. **Figure 3-4** shows the optimal complement condition when taking this into account is 10% for Quailes and 5% for CVD909, which corroborates the data shown in **Figure 3-1** -**Figure 3-3**. Vi IgG levels were quantified using the VaccZyme ELISA also showed undetectable levels of antibodies in the complement source (data not shown).

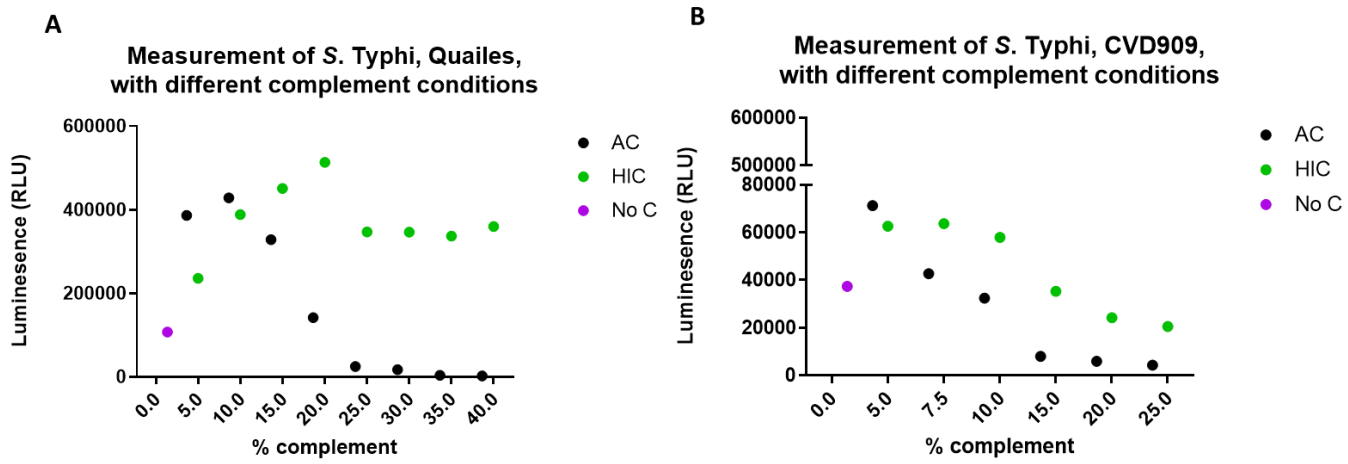


Figure 3-4 Surviving bacteria in active and heat-inactivated complement source without serum

Abundance of *S*. Typhi Quailes (A) or CVD909 (B) measured by luminescence after incubating for three hours with either active complement (AC) source or heat inactivated complement (HIC) source added at varying amounts, or with no complement (No C).

To determine if the assay could measure the bactericidal activity in our test samples rather than just a positive pool we tested the samples collected at two time points from 3 individuals from the VAST study, V1 is immediately prior to receiving Vi vaccine, and V2 is 4-6 weeks after receiving Vi conjugate vaccine (Vi-TT). The dilution curves generated against the Quailes strain showed a good level of fit to the 4-parameter sigmoidal regression curve as demonstrated by high R^2 values and the IC50 values could be calculated (**Figure 3-5**A, **Table 3-1**). For CVD909 the dilution curves were not sigmoidal and showed low R^2 values, the level of corroboration between the triplicates was also low as indicated by large error bars (standard deviation) at each data point (**Figure 3-5**B, **Table 3-1**). The purpose of developing this *S*. Typhi L-SBA assay is to measure bactericidal activity in samples collected in a Vi vaccine study. The advantage of assessing the bactericidal activity against CVD909 is that constitutive expression of the Vi capsule would better elucidate the contribution of Vi specific antibodies generated in response to vaccine. However, these data suggest that CVD909 is not a suitable choice for this assay, use of the Quailes strain is more stable.

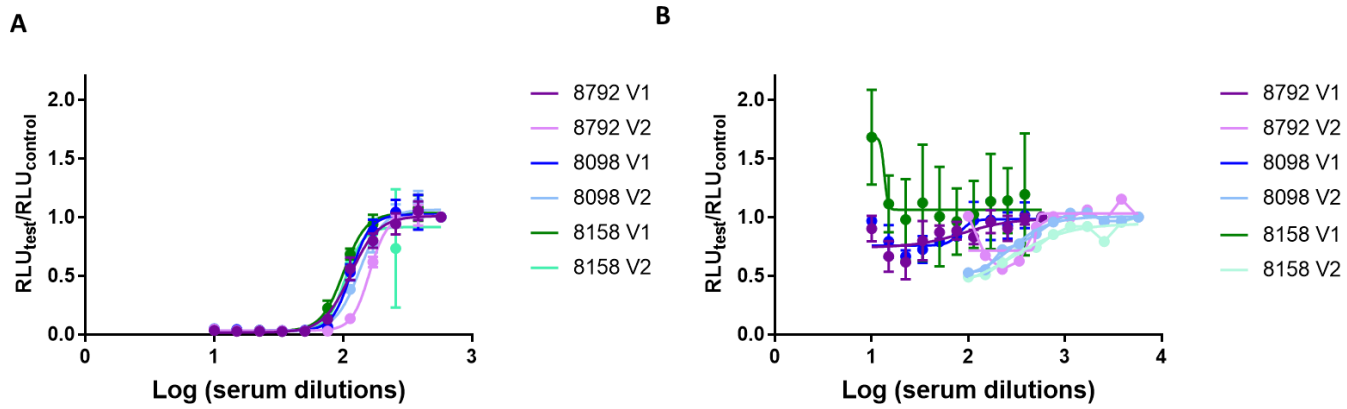


Figure 3-5 Comparison of bactericidal activity against S. Typhi Quailes and CVD909 strain, example experiment

Bactericidal activity curves of test samples from VAST run with either *S*. Typhi Quailes (A) or CVD909 (B). V1 = visit 1, pre-vaccination, V2 = visit 2, 28 days post Vi-Tetanus toxoid conjugate vaccination. Individual data points represent average of three replicates, and error bars are the standard deviation.

	S. Typhi (Quailes)		*S.* Typhi (CVD909)	
Sample and visit number	**Titre**	**R^2**	**Titre**	**R^2**
8792 V1	114.6	0.9851	87.60	0.3735
8792 V2	160.5	0.9954	500	0.3587
8098 V1	115.6	0.9828	~13.52	0.1987
8098 V2	134.6	0.9864	284.1	0.7077
8158 V1	101.2	0.9900	81.75	0.5100
8158 V2	104.0	0.8832	278.9	0.5986
NIBSC (16/138) Serum Standard	110.7	0.9586	859.8	0.6672

Table 3-1 Comparison of bactericidal activity against CVD909 and Quailes strains

Luminesence based serum bactericidal activity titres and corresponding R^2 values for identical samples run with either *S*. Typhi Quailes or CVD909.

3.3.1.2. Expression of Vi capsular polysaccharide by different *Salmonella* Typhi Strains

In wild type strains, Vi expression is highly regulated and is hypothesised to change in response to the osmolarity of the surroundings, with capsule synthesis being expressed preferentially at low and medium osmolarities [162]. To determine if the Quailes strain was expressing Vi in the assay conditions we used a Vi specific monoclonal antibody, developed as part of the VAST study, to assess the expression of the Vi capsule in multiple strains of *Salmonella* Typhi (**Figure 3-6**, **Table 3-2**). Vi expression was represented using a composite score calculated by multiplying the number of positive bacteria by the geometric mean fluorescence intensity. The composite score accounts for heterogeneity of a Vi expression within each bacterial population. CVD909 grown in DHB (0.1% 2,3-Dihydroxybenzoic acid) supplemented broth had the highest levels of Vi expression, as expected. CVD908, the parent strain of CVD909, had intermediate levels of Vi expression, similar to that of Quailes grown in regular salt LB. Changes in the salt conditions for Quailes resulted in lower expression confirming that regular LB broth with 85mM salt are the best growth conditions for Vi expression for the Quailes strain. Ty21a, an oral vaccine strain which contains a genetic deletion of the P_{viA} promotor of the Vi capsule, was tested alongside the other strains as a negative control.

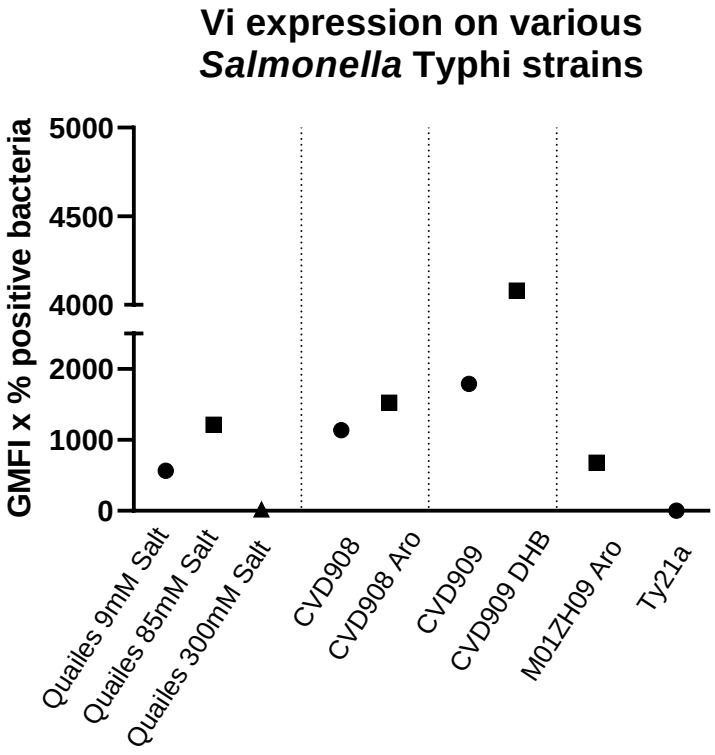


Figure 3-6 Vi capsular polysaccharide expression on various strains of S. Typhi

Expression of the Vi capsular polysaccharide, assessed on various *Salmonella* Typhi strains and differing growth conditions, was assessed by flow cytometry. Bacteria were grown in liquid Luria Bertani broth to log phase as in the L-SBA, to an OD_{600}=0.2. Expression was detected using a Vi specific monoclonal and secondary anti-human Fc antibody conjugated to alexa fluor (488nm). For comparison, a composite score of geometric mean fluorescence intensity multiplied by % positive bacteria was used.

S. Typhi Strain	Growth conditions
Quailes	Low salt 9mM Salt LB broth
Quailes	Regular (85mM Salt) LB broth
Quailes	300mM Salt LB broth
CVD908	Regular LB broth
CVD908	Regular LB broth supplemented with aromatic amino acids (aro)
CVD909	Regular LB broth
CVD909 DHB	Regular LB LB broth supplement with 0.1% 2,3-Dihydroxybenzoic acid (DHB)
M01ZH09	Regular LB broth supplemented with aromatic amino acids (aro)
Ty21a	Regular LB broth

Table 3-2 Details of S. Typhi strains and growth conditions used for the Vi expression assay

3.3.1.3. Performance of a NIBSC 16/138 international standard as a QC sample

To ensure the assay remains consistent across plates run on the same day in different batches or run on different days, a control sample, NIBSC 16/138, was included on each plate (**Figure 3-7**). The sample titre was calculated identically to the test samples. After running all the samples the upper and lower acceptable limit of the QC were calculated as the average titre +/- 1 standard deviation. Only runs where the dilution curve had an R^2 of >0.7, and had sufficiently diluted out the bactericidal activity were used. Samples run on plates where the QC samples fell out of the calculated acceptable range were re-run, samples were only included in the analysis if the QC sample fell within the designated range.

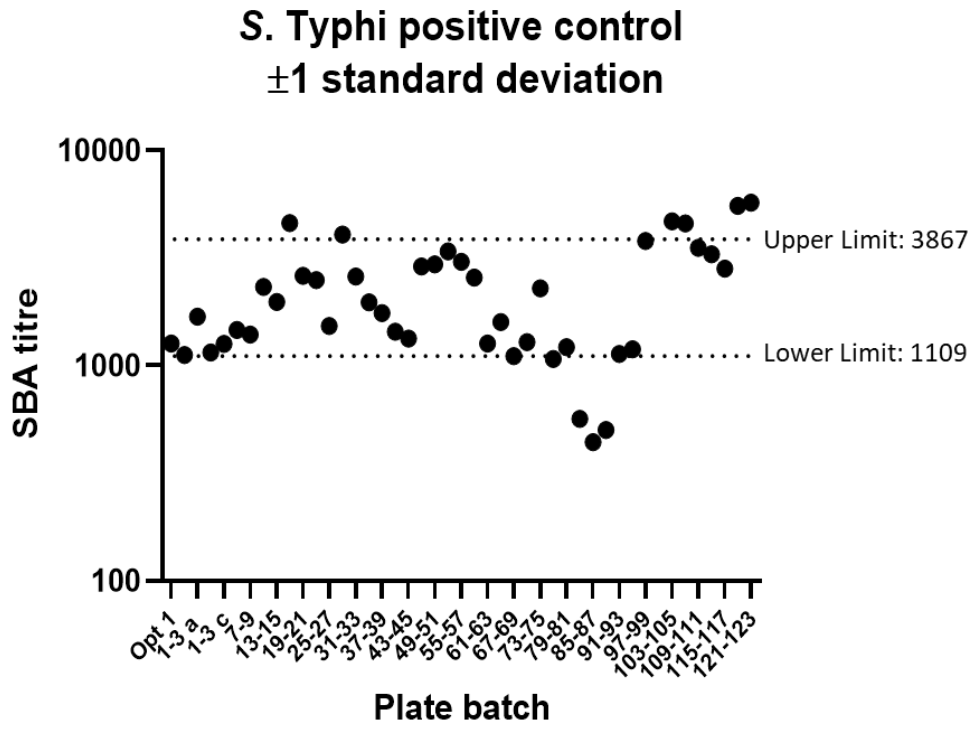


Figure 3-7 Summary of repeat titres of NIBSC 16/138

Bactericidal activity titre of NIBSC 16/138 against *S*. Typhi Quailes strain across multiple experiments. Dotted lines represent upper and lower limit of acceptable range, calculated as ±1 standard deviation of the average.

3.3.1.4. Vi specific antibody depletion optimisation

To assess the contribution of antigen specificities to the bactericidal activity of the test sera, I conducted some antibody depletion experiments. To deplete Vi specific antibodies, plates were coated with either BSA only, or Vi at various concentrations (**Figure 3-8**). After blocking the plates with 1x PBS + 0.5% BSA, undiluted samples were added and incubated for 30 mins at 37°C, this incubation was repeated for 10 iterations, the antibody titres were analysed via ELISA. The initial optimisation step was carried out using the positive control sample, NIBSC 16/138, and after 9 iterations there was an average 94% decrease in Vi specific IgG. The reduction was slightly less in the wells coated with just 2µg/mL, 88%, while the reductions observed for the other coating concentrations were comparable. Coating the plates with BSA only showed a reduction of Vi IgG by 20%. Bead and free antigen optimisation methods were also assessed, but they did not result in

reduced Vi antibody levels (data not shown). The same plate-based methods were used to deplete LPS antibodies to investigate if LPS antibodies were also contributing to vaccine related changes in SBA. LPS antibodies are known to contribute to bactericidal activity after challenge with *S*. Typhi, and interestingly in the VAST study we observed an increase in LPS specific antibody secreting cells 7 days post Vi vaccine [163]. Quantification of LPS specific IgG in serum after 9 incubations on plates coated with 40µg/mL of LPS antigen corresponded with a minimal 36% reduction in antibody, in comparison incubation with BSA coated plates showed a 23% reduction. Vi depleted samples were then used in the L-SBA assay to assess antigen specificity of bactericidal function in Vi vaccinated individuals (see Chapter 4, **Figure 4-3**). Due to the high volumes of serum needed for this depletion and the small reduction in LPS antibodies observed, the L-SBA assay was not carried out on LPS antibody depleted serum samples.

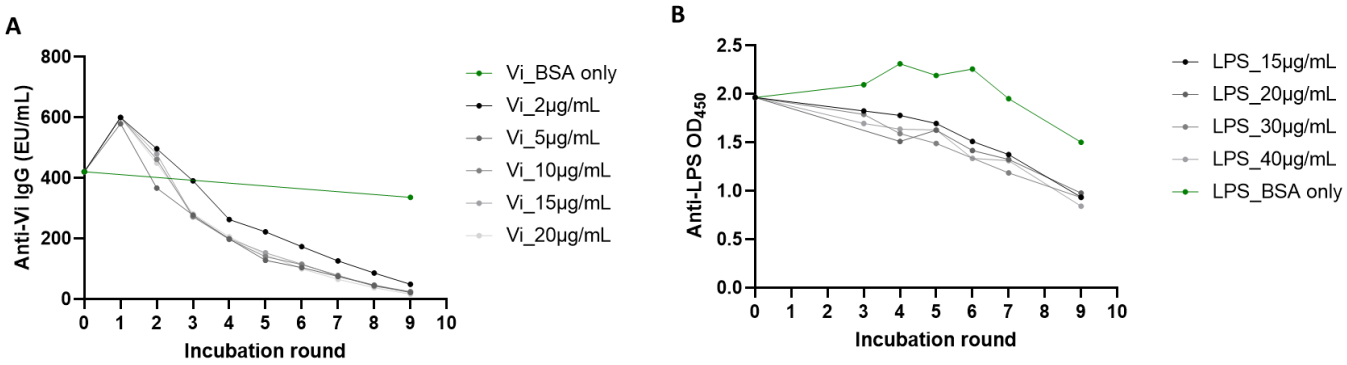


Figure 3-8 Optimisation of antigen specific antibody depletion in serum samples

Quantification of Vi (A) or LPS (B) specific IgG in NIBSC 16/138 after iterative incubations with plate bound Vi or LPS antigen at various coating concentrations, or BSA coated plates.

3.4. Method development of *Salmonella* Paratyphi A luminescence based SBA

For the assessment of the functional serological response to *Salmonella* Paratyphi A infection or vaccination a robust and reproducible assay for measuring the serum bactericidal activity is needed. Necchi *et al* describe the development of the L-SBA for high throughput measurement of bactericidal activity of *S*. Paratyphi A which was also validated for measuring *S*. Typhi activity as detailed in section **3.3.1**[161].

3.4.1. Optimisation of suitable complement conditions

Initial steps to validate the *S*. Paratyphi A specific assay were to find an appropriate complement condition. Using the same criteria as for the *S*. Typhi assay, the optimal complement conditions are those that show some level of killing/growth restriction in the absence of serum, and for the growth of the bacteria to be similar in the active complement and heat inactivated complement conditions (to confirm the absence of overwhelming cross-reactive antibodies). Growth of the bacteria was similar for the AC and HIC when adding 10-20% complement (**Figure 3-9**A), these conditions also demonstrated optimal complement mediated growth inhibition when measured in the absence of serum (**Figure 3-9**B).

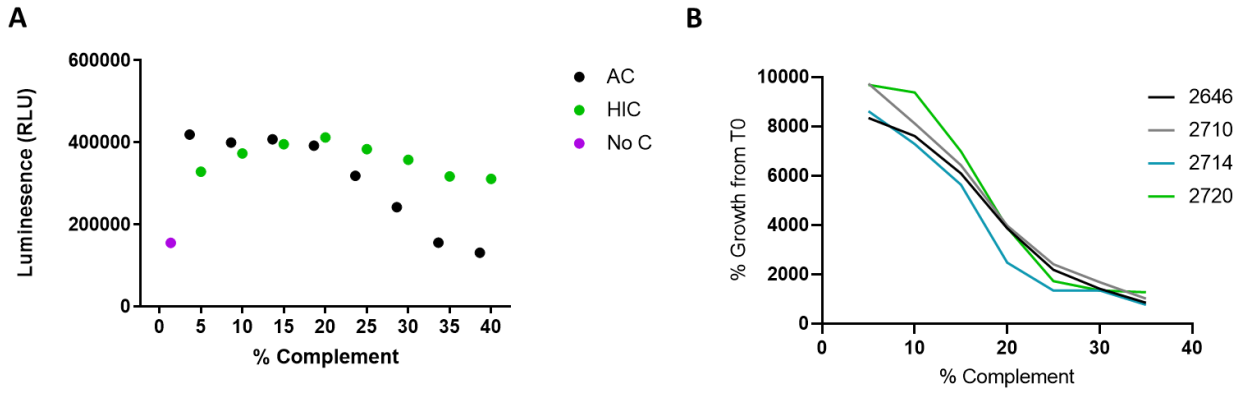


Figure 3-9 Optimisation of complement conditions for Salmonella Paratyphi A specific L-SBA assay

(A) Amount of bacteria as measured by luminescence after a 3 hour incubation with varying amounts of active (AC) or heat inactivated complement (HIC), or no complement (No C). (B) Bacterial growth relative to starting bacteria quantity, measured via luminescence, different lines represent different batch numbers of complement. Experiments were performed in the absence of serum.

3.4.2. Use of an in-house pool as an assay QC

As with the *S*. Typhi assay a positive sample was run as a QC for each plate. The QC sample was made of a combination of samples collected 90 days post *S*. Paratyphi A challenge from individuals enrolled in the P1/PATCH CHIM studies. Two independent pools were tested in the assay with addition of complement between 10-20%. Pool 2 consistently had a higher titre and both pools had highest titres when adding complement at 20%, suggesting that at 10-15% complement is not in excess and is restricting the serum dependant killing (**Figure 3-10** A). Pool 2 was included as the QC sample on each of the plates when assessing the test samples. The average titre measured on the 102 plates was used to define the acceptance criteria of the QC samples which was calculated as the average +/- 1 standard deviation, L-SBA titre of 91-949. Samples run on plates where the QC fell out of this range, did not dilute out or did not have an R^2 values of > 0.7 were repeated.

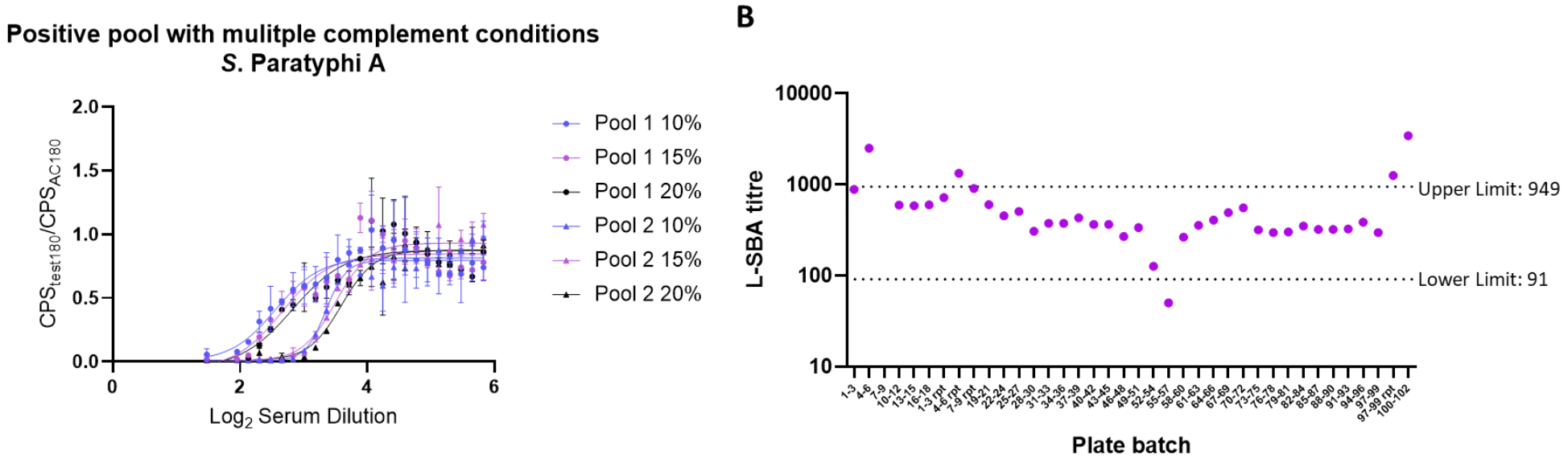


Figure 3-10 Selection and performance of an anti - S. Paratyphi A serum pool for use as a control in the L-SBA.

(A) Bacterial killing curves generated from performing the L-SBA assay with two independent pools of serum made from samples collected in the human challenge models (PATCH study, OVG2014/01) in the presence of varying amounts of complement. Relative survival of bacteria shown as the luminescence in the test sample normalised to the luminescence in the active complement only control well. Each data point represents three replicates, and error bars represent the standard deviation (B) L-SBA titres of Pool 2 run on each assay plate, dotted lines represent the lower and upper acceptable range, calculated at the average ± 1 standard deviation.

3.5. Development of a conventional, colony counting SBA in collaboration with SIIPL

To assess the immune response to a novel *Salmonella* Typhi and Paratyphi A bivalent conjugate vaccine developed and tested by SIIPL, we collaborated with them to develop a conventional, colony counting SBA against *S*. Paratyphi A to assess vaccine induced functional antibody changes.

3.5.1. Optimisation of SBA assay conditions

A stock of *S*. Paratyphi A, ATCC9150 strain was made as per **Table 2-2**. For the assay, sufficient bacteria must be added to each well to accurately determine antibody mediated differences in bacterial survival. Approximately 80-100 CFU are required per well/10µL. More than 100 colonies would make it difficult to count after the bacteria are spotted on to agar plates. Less than 80 would make it difficult to detect changes in the colony numbers due to the antibody effect. To determine the dilution needed to achieve this, bacteria were serially diluted from 1/6000 – 1/9000, spotted on LB agar plates and enumerated the following day using a semi-automated colony counter. A bacteria dilution of 1/6000 was sufficient to add at least 80 colony forming units per well (**Table 3-3**). To ensure correct complement conditions, active and heat inactivated complement was added at differing amounts (2-7µL) and incubated for 1 hour with the bacteria, but without serum. At lower complement amounts, the % difference between the active complement (AC) and the heat inactivated complement (HIC) is lower (**Figure 3-11**B), however the overall number of colonies is lower than desired, even at the most concentrated bacteria conditions, suggesting that we needed to use more concentrated bacteria for the assay (**Figure 3-11**A). These data also suggested that addition of 5µL of complement in a total 40µL (12.5%) reaction mixture was suitable for the assay. A 1/5000 dilution of the bacteria was later tested and showed > 80 colonies in all complement conditions and so this dilution was used in the final assay protocol (data not shown).

Dilution	R1	R2	Average
1/6000	90	74	82
1/7000	64	88	76
1/8000	67	68	68
1/9000	61	63	62
HBSS only	0	0	0

Table 3-3 Results of titration experiment showing the CFU of diluted bacteria, completed in duplicate

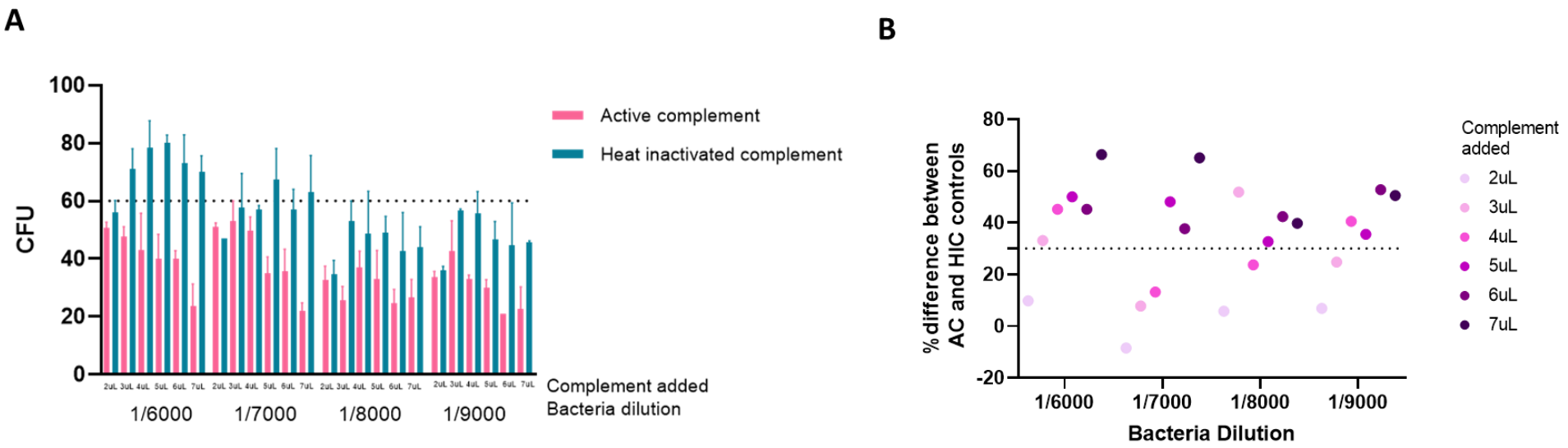


Figure 3-11 Quantification of colonies in various complement conditions and bacterial dilutions

(A) Surviving bacteria in active and heat inactivated complement conditions with varying amounts of complement and different bacterial dilutions. Dotted line at 60 CFU; desirable minimum number of colonies in complement control wells. Mean and SD of duplicates are plotted. (B) Percentage difference between active complement and heat inactivated complement. Dotted line at 30%; maximum accepted % difference. Percentage difference = ((HIC-AC)/HIC)*100

SBA titres in this protocol are calculated as the serum dilution required to achieve 50% killing compared with the HIC. The assay was tested using preclinical samples and the original protocol shared by SIIPL, whose titres had already been assigned in the using the SIIPL in house SBA. Using this protocol we saw an average % difference between the AC and HIC of 47% in initial experiments. The maximum desired % difference for this assay is 10%. To see if the difference between could be reduced from 47%, the assay was performed with buffers containing blocking agents, on different plates, and with an increased reaction volume. A combination of increasing the overall reaction mixture volume from 40µL to 80µL, switching to U-bottom plates and addition of blocking agents to the reaction buffer decreases AC/HIC % difference. In the experiments summarised in **Table 3-4**, % differences are acceptable in the original SIIPL conditions with addition of a shaking step, however these conditions had a high standard deviation. Doubling the volume of all the components proportionally to a total volume of 80µL increased the CFU in the control wells, which is preferable because it means the counts are less sensitive to small fluctuations in CFU (which can be introduced at the pipetting or counting stages). Final conditions were decided as, a total reaction volume of 80µL, performed in U-bottom plates and using HBSS+ buffer with 0.5% FBS, incubated at 37°C without shaking.

Plate type	Buffer HBSS+	Total reaction volume (µL)	Plate shaking during reaction	Average AC/HIC % difference	SD	n
Flat	NA	40	Yes	8.2	37.3	10
Flat	0.5% BSA	40	Yes	32.7	8.0	4
Flat	1.0% BSA	40	Yes	50.0	4.5	2
U	NA	40	Yes	58.6	7.2	4
U	0.5% BSA	40	Yes	32.4	6.4	4
U	NA	80	Yes	43.5	14.3	10
U	0.5% BSA	80	Yes	37.4	13.6	10
U	0.5% FBS	80	Yes	36.4	19.9	8
U	1.0% FBS	80	Yes	39.8	23.4	8
U	0.5% FBS	80	No	6.8	16.5	8

Table 3-4 Summary of the % difference between the active complement (AC) and heat inactivated complement (HIC) condition

Percentage difference in surviving bacteria in the SBA assay performed with active (AC) and heat inactivated (HIC) complement and no serum, calculated using: ((HIC-AC)/HIC)*100. Results with various buffers, volumes, and plate types. Experiment was performed with a 1/5000 bacteria dilution. HBSS+ = Hanks balanced salt solution. SD = Standard deviation. n = number of replicates.

3.5.2. Qualification of SIIPL SBA assay

For the qualification of the assay, as required by SIIPL, 10 serum samples collected at baseline (D0), and 90 days after exposure (D90) from P1/PATCH CHIM studies were tested alongside a D90 pool and the NIBSC 16/138 standard sample. Samples were analysed by two independent operators, on three different days (**Table 3-5**). If the titres from the sample replicates were within 2-fold dilutions of each other they were accepted. In 10/12 of the samples tested, each operator was within 2-fold dilutions on the different days tested demonstrating the assays inter day precision. The average titre obtained across 3 replicates for each operator is within 2-fold dilutions for 11 of the 12 samples tested. Therefore, the assay demonstrates inter-operator precision (**Table 3-5**).

	Operator 1					Operator 2					Operators within 2 dilutions
	R1	R2	R3	Average	Within 2 dilutions of the average	R1	R2	R3	Average	Within 2 dilutions of the average	
0027 D0	3072	5760	11520	6784	Yes	8192	2880	5760	5611	Yes	Yes
0027 D90	11520	11520	11520	11520	Yes	5760	46080	5760	19200	No	Yes
0042 D0	1024	2880	5760	3221	No	4096	7680	4080	5285	Yes	Yes
0042 D90	11520	7860	11520	10300	Yes	2880	7680	7680	6080	Yes	Yes
0053 D0	1280	768	768	939	Yes	512	384	128	341	No	No
0053 D90	2048	1024	288	1120	No	640	512	384	512	Yes	Yes
0036 D0	3840	5760	1920	3840	Yes	1920	2400	2880	2400	Yes	Yes
0036 D90	7680	7680	3840	6400	Yes	3840	3840	17280	8320	Yes	Yes
0077 D0	2048	768	760	1192	Yes	768	1280	1536	1195	Yes	Yes
0077 D90	1024	1536	1024	1195	Yes	2560	768	1280	1536	Yes	Yes
NIBSC 16/138	2560	2560	1024	2048	Yes	2044	2560	3072	2559	Yes	Yes
SPtA QC	9600	7680	5760	7680	Yes	5760	5760	15360	8960	Yes	Yes
					Pass: 10/12					Pass: 10/12	Pass: 11/12

Table 3-5 Summary data for SBA qualification samples against S. Paratyphi A

SBA titres measured in 12 qualification serum samples, run in multiple replicates by two different operators. The average titre obtained across 3 replicates for each operator is with 2-fold dilutions for 11 of the 12 samples tested. SPtA QC = pool of D90 samples from *Salmonella* exposed individuals (from Oxford challenge study).

3.6. Methods development for an antibody dependent monocyte phagocytosis assay

The role of antibodies in phagocytosis and clearing *S*. Paratyphi A infection is not well defined. To study this effector function in response to paratyphoid infection and vaccination we developed a reproducible, standardized flow cytometry-based monocyte phagocytosis assay, using GFP expressing *S*. Paratyphi A bacteria and the THP-1 monocyte cell line.

The THP-1 cell line is a widely characterised leukemia monocyte cell line which has been extensively utilised to study a variety of monocyte functions including phagocytosis[164]. The aim of the assay is to measure antibody mediated uptake of GFP expressing bacteria that have been pre-opsonised with antibodies in our participant test sera. Standardization of assay components is essential for comparison of results between studies. To minimise variability, we aimed to develop an assay using a frozen THP-1 cell bank, and a stock of fixed bacteria.

3.6.1. Selection of suitable reaction time and MOI

A preliminary matrix experiment which varied the number of bacteria per cell (multiplicity of infection, MOI) and the incubation time showed that an incubation of 30 - 60 minutes was enough to see considerable phagocytic uptake when using a MOI of 10 or 100 (**Figure 3-12** A). Using the SPtA positive pool we saw serum dependent uptake of bacteria and importantly, there were minimal observable differences in the phagocytosis when using fresh culture or thawed, pre-banked THP-1 cells following an overnight rest (**Figure 3-12** B & C).

Technical variation could also come from day-to-day changes in the cell surface components of the bacteria when cultured. Along with the fact that *S*. Paratyphi A is a hazard group 3 organism in the UK and all experiments with live bacteria are performed in a biosafety level 3 laboratory, making a stock of fixed, non-culturable, frozen bacteria would be advantageous in the standardisation of the ADMP assay. While serum dependant phagocytosis of fixed bacteria (**Figure 3-12** B) was 10-fold lower compared with live bacteria (**Figure 3-12** C), there was still measurable serum dependent uptake indicating that this is a viable option for a standardized assay.

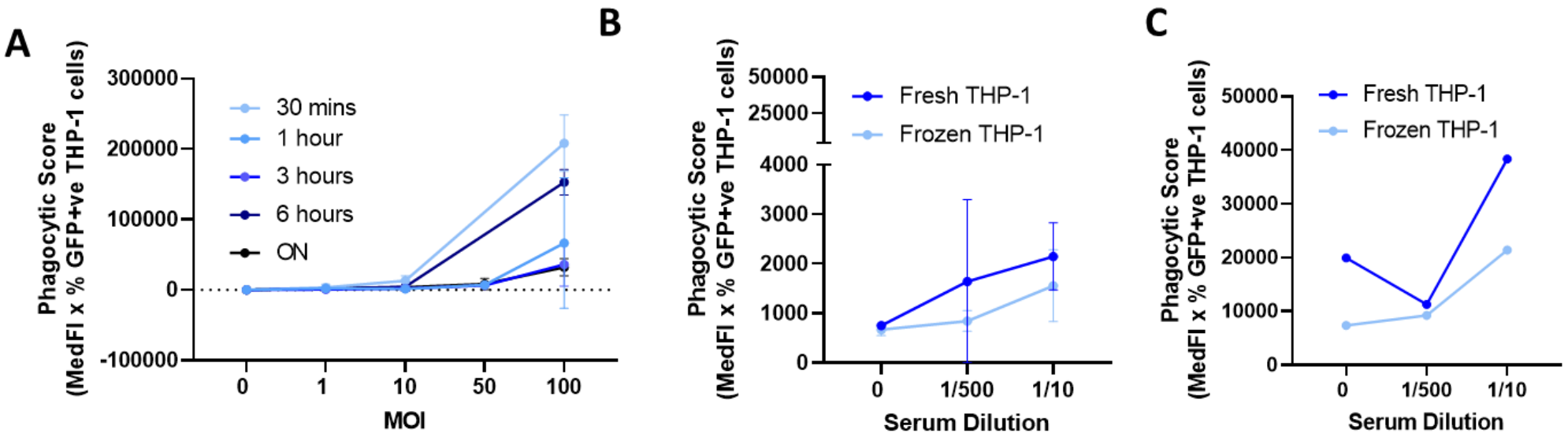


Figure 3-12 Initial optimisation of ADMP conditions

(A) Representation of two matrix experiments measuring phagocytic uptake of bacteria by THP-1 monocyte cells under various incubation times and with different multiplicity of infection conditions. Phagocytosis measured using composite score, termed phagocytic score (B+C) Initial measurement of serum enhanced phagocytosis using THP-1 cells in fresh culture or a frozen stock of THP-1 cells that have been thawed immediately prior to use, using either fixed bacteria (B) or live bacteria (C). Error bars are standard deviation of replicates.

3.6.2. Optimisation of bacterial fixing conditions

Figure 3-13 A shows differences in fluorescence of THP-1 cells that have engulfed bacteria pre-treated with different fixatives compared with live, non-fixed bacteria. Frozen BD cell fix treated stocks of bacteria showed highest median fluorescence after incubating with THP-1 cells for 1-hour, but the live bacteria had higher levels of phagocytosis as calculated using the composite phagocytic score (**Figure 3-13** B). Measurement of ingested bacteria as opposed to adherent bacteria was confirmed by trypan blue quenching of fluorescence of adherent bacteria. Within the BD cell fix group there was a minimal difference in phagocytic score with a 1-hour reaction time after quenching compared with not quenched (-27%) suggesting that the majority of bacteria are internalised rather than adhered to the cell surface (**Figure 3-13** C & D, **Table 3-6**). This pattern was conserved when looking at the serum dependant uptake (**Figure 3-13** E), at the 1 hour timepoint BD cell fix treated bacteria show less reduction in phagocytosis after quenching compared with the live bacteria or other prefixed conditions.

The overnight condition was performed as a comparator time point, it was hypothesised that the bacteria were bound to the cell surface in the shorter incubations but could take longer to be phagocytosed. Similar reduction in phagocytosis after quenching in the overnight condition suggests this is not the case. When incubated overnight the serum dependant uptake was reduced, confirming that shorter incubations with the bacteria are more suitable for measuring antibody mediated phagocytosis.

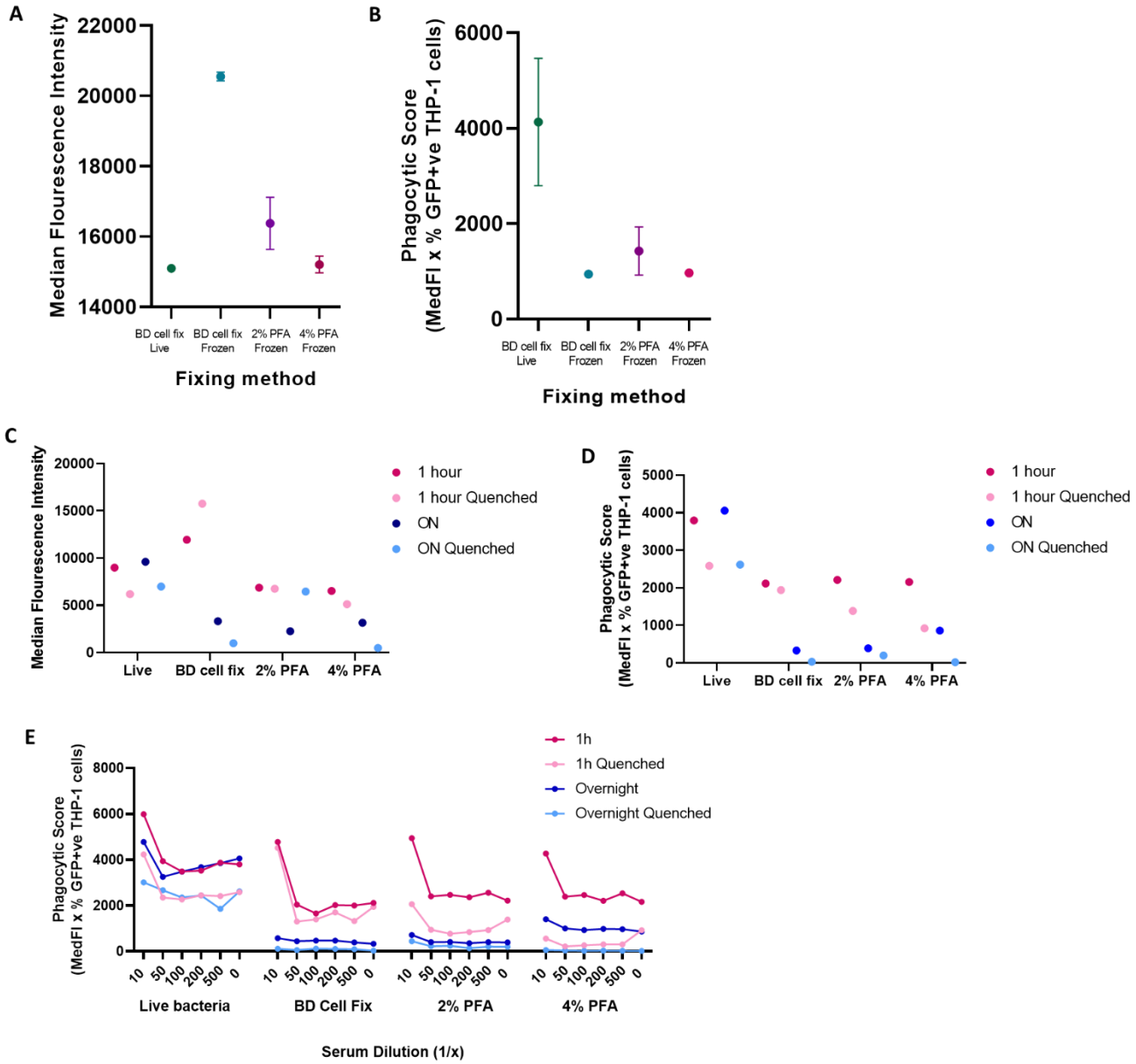


Figure 3-13 Optimisation of fixative conditions

(A) Fluorescence intensity of THP-1 cells after 1 hour incubation of live bacteria or bacteria fixed with BD Cell Fix, 2% paraformaldehyde, or 4% paraformaldehyde (PFA). (B) Measurement of phagocytic uptake using the composite phagocytic score of fixed bacteria without serum. Error bars show standard deviation. (C-E) Flow cytometric analysis of median fluorescence intensity or phagocytic score of GFP positive THP-1 cells after either a 1 hour or overnight incubation with fixed bacteria, with or without quenching via addition of trypan blue, in the absence (C-D) of serum or after a 2-hour pre-opsonisation step with a positive pool of serum (E). Error bars are standard deviation of replicates.

	Live	BD cell fix	2% PFA	4% PFA
Average % Difference from live (1 hour)	NA	-80%	-50%	-56%
Average % Difference between quenched – unquenched (1 hour)	-52%	-27%	-155%	-669%

Table 3-6 Comparison of different fixative methods and quenching

The percentage difference from the live bacteria condition was calculated in the absence of serum: (Phagocytic score of condition/ score of live)*100. The percentage difference between the quenched and unquenched was calculated similarly: (score of quenched / score of unquenched) * 100. Calculations shown are for the one hour incubation only.

Further dilutions of test samples, showed the serum dependant uptake of live bacteria to be similar to bacteria pre-fixed with BD cell fix (**Figure 3-14**). Samples with low levels of uptake were low in both conditions, and samples with high uptake were high in both conditions, indicating that the assay is suitable for measuring a range of antibody functions and supporting the use of a fixed and frozen stock of bacteria.

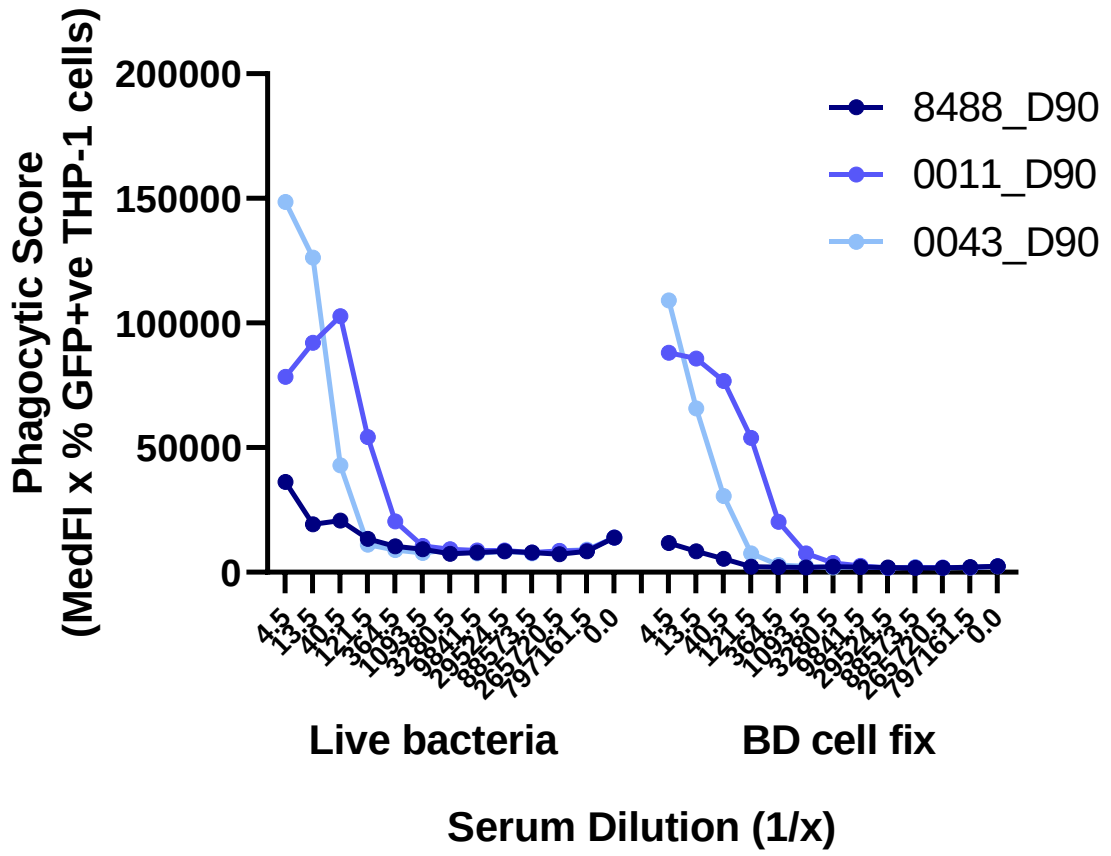


Figure 3-14 Comparison of serum dependant phagocytosis of fixed with live bacteria

The ADMP assay was performed with three different samples using either live bacteria or fixed and frozen bacteria.

3.6.3. Effect of complement addition on phagocytosis

Addition of complement has been shown to enhance antibody mediated phagocytosis of other pathogens. I tested human, guinea pig, standard rabbit and baby rabbit complement to in this assay. Addition of human, guinea pig, or baby rabbit complement to the assay when using a positive pool enhanced the prozone/Hook effect, where functional output appears reduced when the concentration of serum is higher due to over presence of antibodies blocking down-stream functional effects (**Figure 3-15** A). Addition of standard rabbit complement (5-25%) showed a dose dependant uptake, similar to the no complement condition. When testing this with 2 samples, one positive and one negative as defined by *S*. Paratyphi A specific SBA titre, addition of 10-15% standard rabbit complement enhanced uptake and resulted in better resolution between the positive and negative sample, compared with the no complement conditions. Addition of human complement did not result in the same enhancement and the prozone effect was still prominent (**Figure 3-15** B).

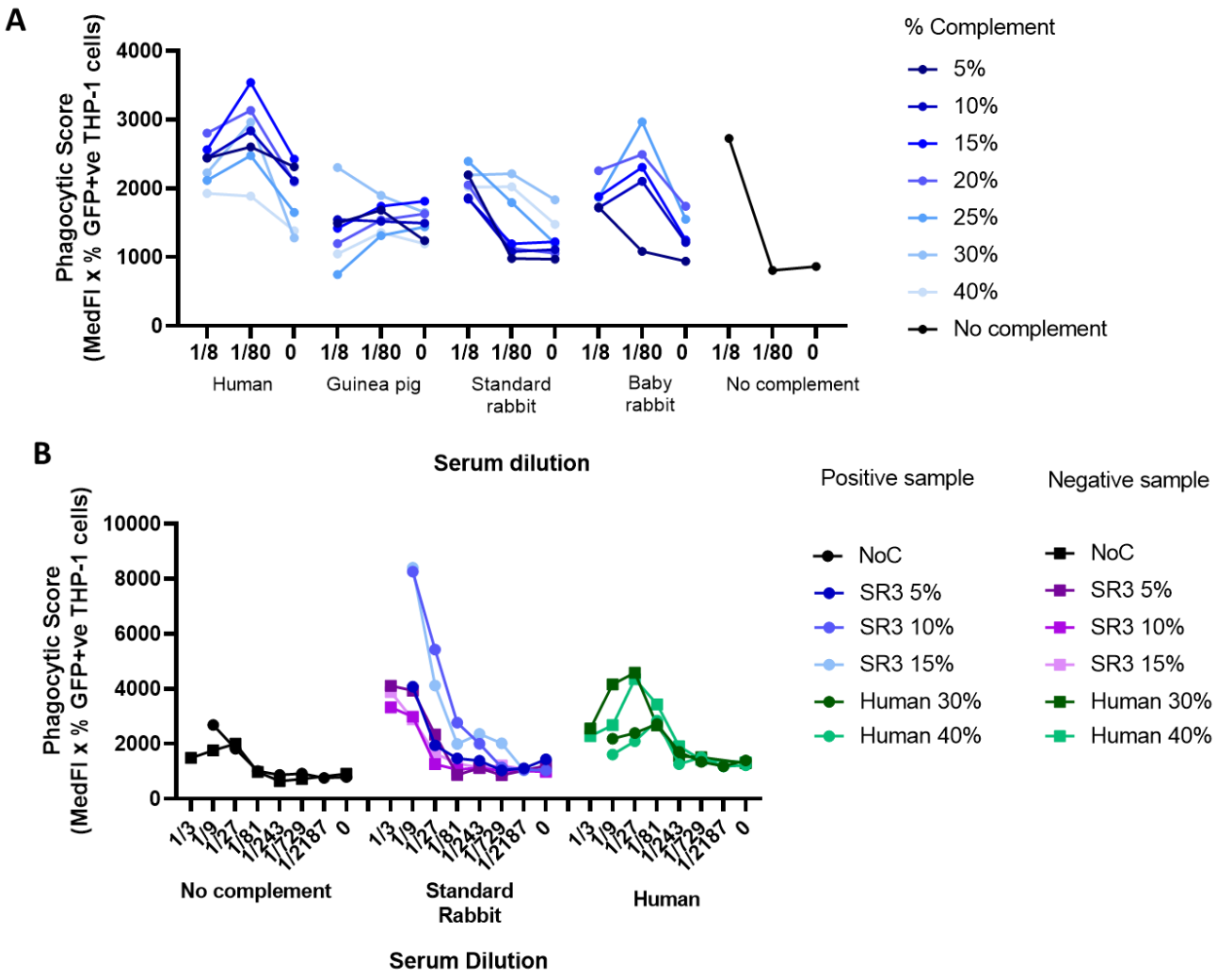


Figure 3-15 Optimisation of complement conditions

(A) The ADMP assay performed with a positive pool at three dilutions in the presence of complement from different sources and at varying final amounts, or with no complement. (B) ADMP assay performed with two samples a baseline (negative sample) and a post exposure (positive sample). Samples were tested in the presence of standard rabbit complement (SR3) at 5-15%, or human complement 30-40%.

The goal was to produce a standardised assay to measure ADMP across multiple projects and populations. We aimed to limit the technical variation by developing a protocol that used a frozen stock of bacteria and THP-1 cells. Frozen THP-1 cells showed similar levels of phagocytosis when compared with cells in conventional culture (**Figure 3-12**C & D). The post-thawing rest times for the frozen stock of THP-1 cells were tested in the absence of serum, longer pre-assay rest times resulted in fewer live cells (**Figure 3-16** A), when rested overnight the % live cells was 56-60% compared with the freshly thawed cells which had 87-90% viability. Addition of 10% complement reduced the cell viability further, although conversely, longer rest times and addition of complement increased the levels of bacterial phagocytosis even though there were fewer viable THP-1 cells (**Figure 3-16** B). Measurement of apoptosis by annexinV staining revealed more cells in the in freshly thawed condition were actively undergoing apoptosis compared with the rested cells, possibly hindering the phagocytic capacity of this cell population. These observations were consistent across different reaction incubation times. From this I concluded that an overnight rest would be optimal for the assay.

Use of freshly thawed THP-1 cells in the assay showed limited serum dependant bacteria uptake in the presence of a positive pool of serum compared with overnight rested cells, this pattern was true independent of the complement status (**Figure 3-16** D, **Figure 3-17**). Furthermore, addition of complement masked serum dependant uptake of bacteria in overnight rested cells when incubated for 30 minutes. Most likely due to there being fewer live cells after the overnight rest and also complement addition also seemed to reduce the number of viable cells. Therefore, complement was omitted for the analysis of test samples (**Figure 3-16** E).

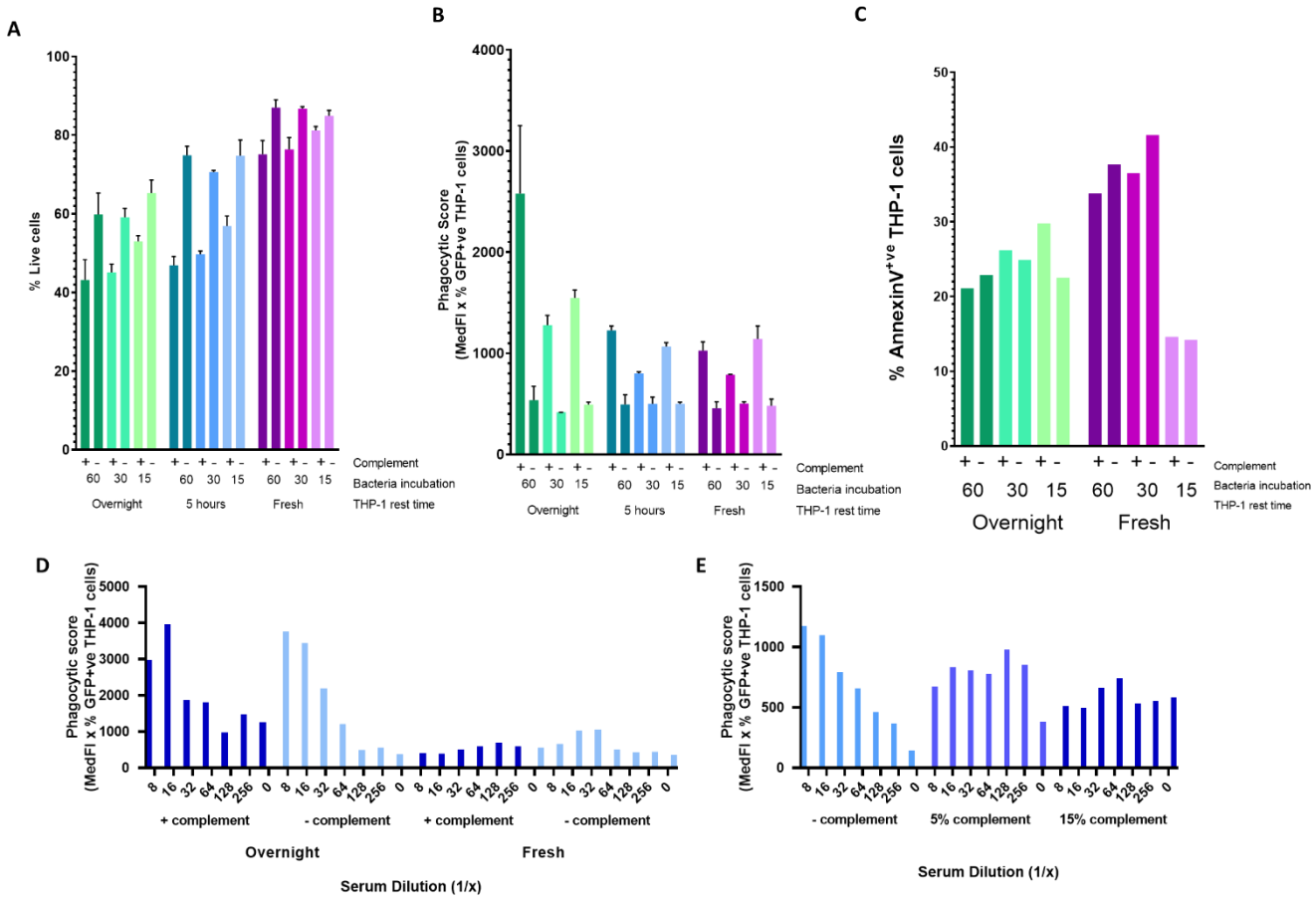


Figure 3-16 Optimisation of time incubations in the presence of complement

The ADMP assay was performed using a stock of frozen THP-1 cells. THP-1 cells were thawed and rested overnight, for 5 hours, or used in the assay immediately (<30mins rest). These cells were incubated with bacteria, with or without complement, for 15, 30, or 60 minutes. (A) Shows % live cells, (B) shows the phagocytic score, and (C) shows the % THP-1 cells that are AnnexinV positive. Error bars represent standard deviation.
(D) The ADMP assay performed with a 30 minute incubation time, with or without complement (15%), using overnight rested or freshly thawed cells, in the presence of a positive pool. (E) The serum dependant uptake at multiple complement amounts with overnight rested cells.

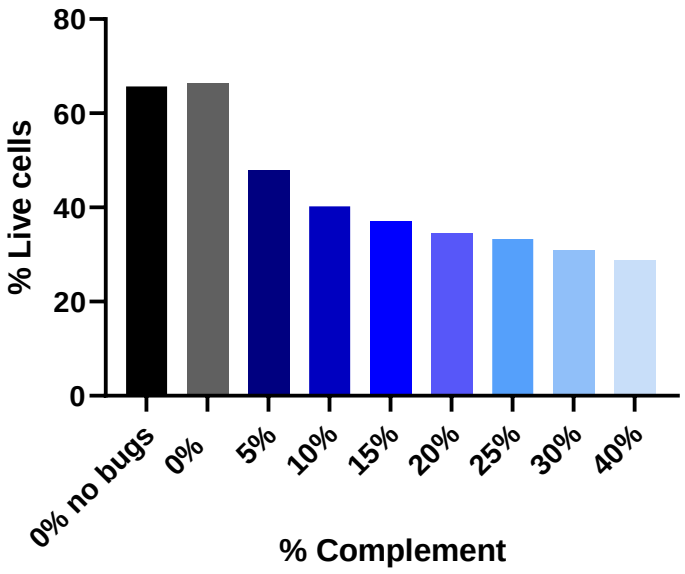


Figure 3-17 Percentage live cells with changing complement amounts

Percentage live cells of overnight rested THP-1 cells after 30 min incubation with standard rabbit complement at various amounts

3.6.4. Final ADMP assay conditions and reproducibility assessment

During initial experiments analysing participant samples, cell viability varied after the overnight rest stage and the number of live cells dropped to <40% this was deemed not suitable and THP-1 cells in conventional culture were used for the subsequent assays and reproducibility testing (described in section **2.3.1**).

To calculate the titre of our test samples a positive pool was used as a standard. An initial dilution of ¼ of the standard was assigned an arbitrary 1000 phagocytic units, the standard was diluted ½ for 7 further dilutions, the phagocytic score from the serial dilution were plotted using a 4-parameter sigmoidal curve and used to interpolate the ADMP titre of test samples (**Figure 3-18** A-C). A panel of 10 samples were run on three independent occasions (**Figure 3-18** D, **Table 3-7**). Inter-day analysis showed a high levels of reproducibility with CV% from 14.23-30.66%. This was true for both samples with low phagocytic capacity or those with high phagocytic capacity that needed further pre-diluting (**Table 3-7**). This assay was deemed suitable for accurately measuring antibody dependent monocyte

phagocytosis. Two control samples were run on each plate, the upper and lower limit of each QC sample were calculated as the average ± 1 standard deviation (**Figure 3-18** F).

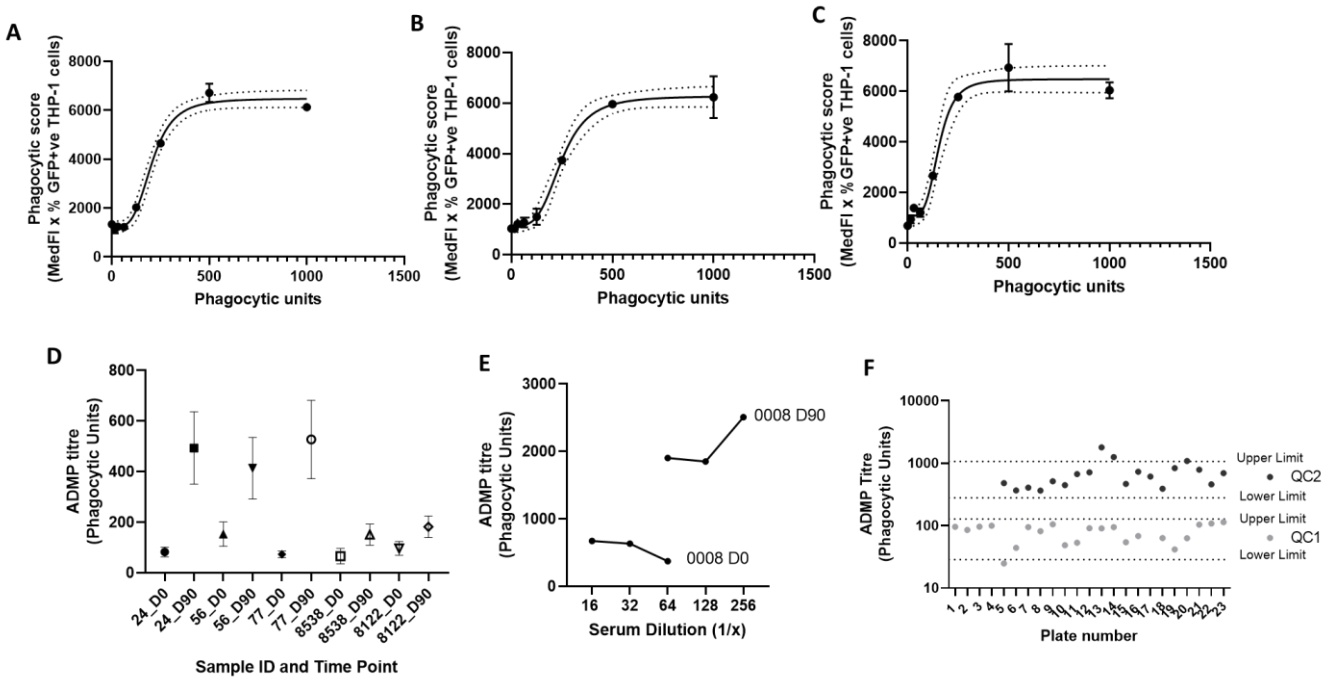


Figure 3-18 Reproducibility of the ADMP and standard curve interpolation

(A-C) Example ADMP standard curves: A positive pool, SPtA, was serially diluted and interpolated using a 4-parameter sigmoidal regression curve, a starting 1/8 dilution of the pool was assigned an arbitrary titre of 1000 phagocytic units. Curves represent data collected over the period of 3 months. Error bars represent the standard deviation between the replicates.
(D) ADMP titres of a panel of ten samples, run on three different days, plotted with error bars representing the standard deviation.
(E) Example of a linearity test of two samples, ADMP titre plotted accounting for dilution.
(F) ADMP titres of the two quality control samples run on each plate (QC1 & QC2), dotted lines denoting the lower and upper acceptable limit, calculated as the average ± 1 standard deviation.

Sample	Time Point	Ave	%CV
24	D0	78.23	22.46
24	D90	688.20	30.66
56	D0	153.66	24.90
56	D90	418.63	23.28
77	D0	73.40	14.23
77	D90	538.77	26.98
8538	D0	66.59	25.14
8538	D90	162.69	27.42
8122	D0	97.12	23.03
8122	D90	178.77	20.54

Table 3-7 ADMP titres of the reproducibility sample panel

ADMP titre, average and % CV of ten samples used to assess the reproducibility of the ADMP assay and standard curve interpolation.
% CV calculated as (standard deviation/ average) x 100.

3.7. Methods development for an antibody dependent neutrophil phagocytosis assay

I aimed to develop a standardized assay to measure phagocytosis of pre-opsonised *S*. Paratyphi A by neutrophils. Neutrophils play an important role in bacterial clearance and antibody dependant neutrophil phagocytosis correlates with protection from typhoid after Vi-vaccination [141]. Donor neutrophils are sensitive and easily activated meaning they can be difficult to work with. To create a standardised antibody dependant neutrophil phagocytosis assay I used fixed GFP expressing bacteria as described in section **2.2**, and a cell line. The HL60 cell line is a promyleoblast cell line, differentiation of the cells to a neutrophil like cell is achieved through incubation with a dimethylformamide (DMF) for 5 days, see section **2.3.2** for methods and gating strategy.

3.7.1. Selection of suitable reaction time and assay standard

I adopted the MOI of 50 bacteria per cell as per the ADMP assay. Initial testing showed the serum dependent bacterial uptake could be measured after just 30 minutes using differentiated HL60s (dHL60s) either in fresh culture, or using rested thawed, pre-banked dHL60s. There was minimal uptake by non-differentiated cells when using the same composite score used for the ADMP assay, GFP MedFI x % GFP positive dHL60 cells = phagocytic score (**Figure 3-19** A). However, pre-banked frozen dHL60 cells were not used for the assay in case we saw the same thawing induced cell death that was observed in in the THP-1 cell line. Testing of further incubation times ranging from, 15 mins to overnight, using baseline and D90 samples showed good resolution between positive and negative samples, it also showed little variation in phagocytosis for incubations times between 15 – 60 minutes (**Figure 3-19** B). Minimal levels of phagocytosis were measured after overnight incubation. From this data a reaction time of 30 minutes was decided for the final assay.

For culturing the cells, ATCC recommend a culture media of IMDM with 20% FBS initially and subsequently 10% once the cell line is established. Testing of 10-20% FBS during the differentiation and incubation steps showed that this made little change to the outcome of the assay, serum

dependent uptake using an *S*. Paratyphi A positive pool could be measured in both conditions (**Figure 3-19** C).

Three D90 samples and an *S*. Paratyphi A positive pool (SPtA) were serially diluted to find a suitable standard for the assay which could be used as a standard curve to interpolate the test samples (**Figure 3-19** D). The curve generated by sample 8949 spanned a larger range compared with the other samples tested, which would be beneficial for more accurately measuring a wider range of titres in the test samples. At dilutions higher than 1/32, 8949 D90 demonstrated the hook effect as the level of phagocytosis decreased with increasing serum. Sample 8949 D90 was used as the ADNP standard, a 1/32 dilution was assigned an arbitrary titre of 1000 phagocytic units and the phagocytic score from subsequent ½ serial dilutions were plotted using a 4-parameter sigmoidal curve and used to interpolate the ADMP titre of test samples.

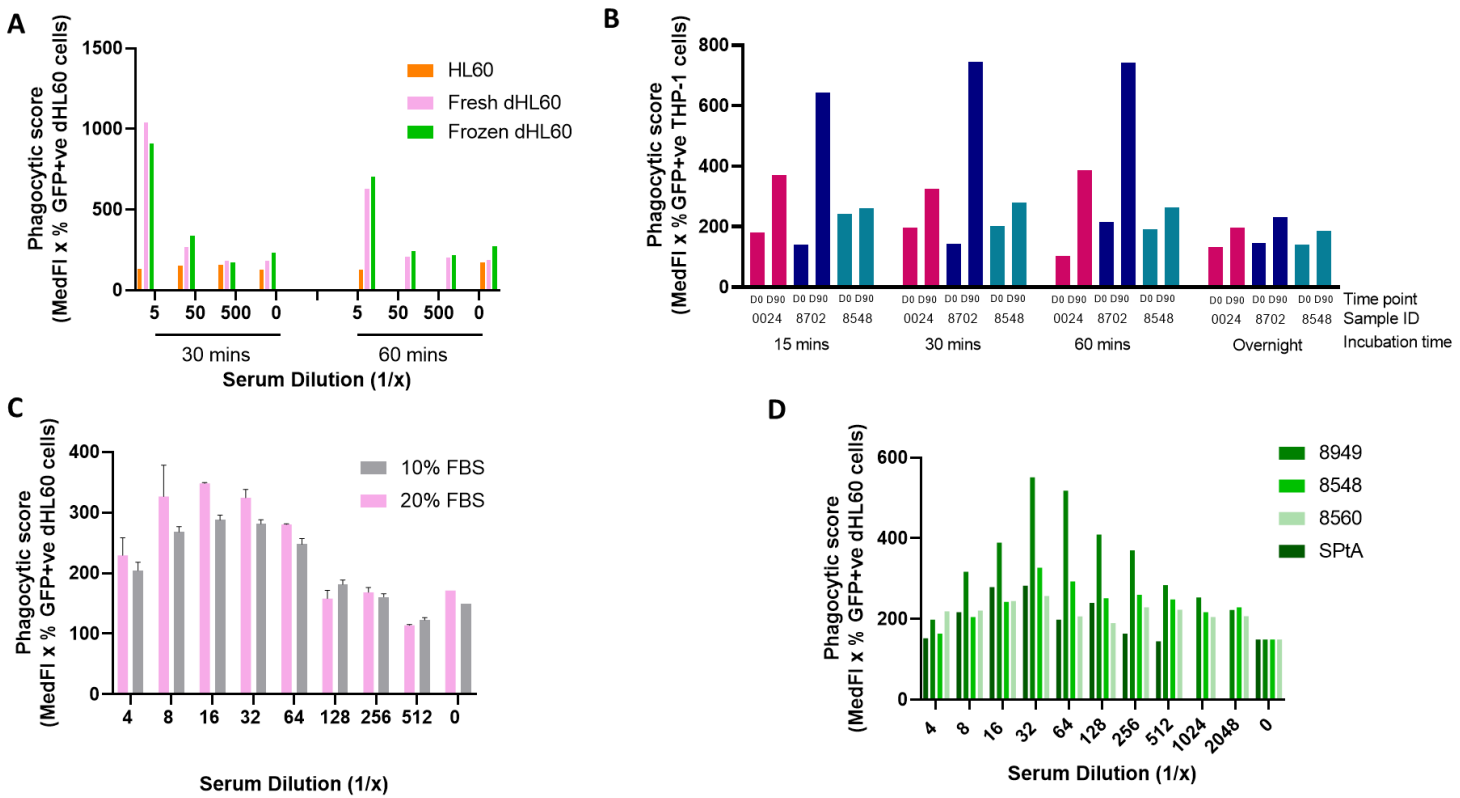


Figure 3-19 Optimisation of a neutrophil phagocytosis assay

Phagocytic score analysis from various optimisation experiments. (A) *S*. Paratyphi A pre-opsonised with positive pool sera incubated for 30 or 60 minutes with either HL60, fresh differentiated or frozen HL60 cells tested against. (B) Baseline (D0) and 90 days post challenge (D90) samples were tested from three participants at 1/8 dilution, incubating for either 15, 30, 60 minutes or overnight. (C) A positive pool was tested using HL60 cells differentiated in the presence of either 10% or 20% FBS. Error bars represent standard deviation of replicates. (D) Extended serial dilution of three D90 samples or positive pool

3.7.2. Final ADNP assay conditions and reproducibility assessment

Example standard curves shown in **Figure 3-20** A-C demonstrate good reproducibility between different days, plates were only passed if the R^2 of the standard curve was > 0.90. A panel of 10 samples were used to determine the reproducibility of the assay. Titres were calculated from the 10 samples run on 3 separate occasions, the CV% was under 40% for each sample and under 30% for the majority of samples indicating an acceptable reproducibility cut off for a cell-based assay, **Table 3-8**. Two QC samples were run on each plate, the samples were only included if the titre of the QC samples was within range, as determined by the average titre from all plates with a valid standard curve ± 1 standard deviation (**Figure 3-20** E).

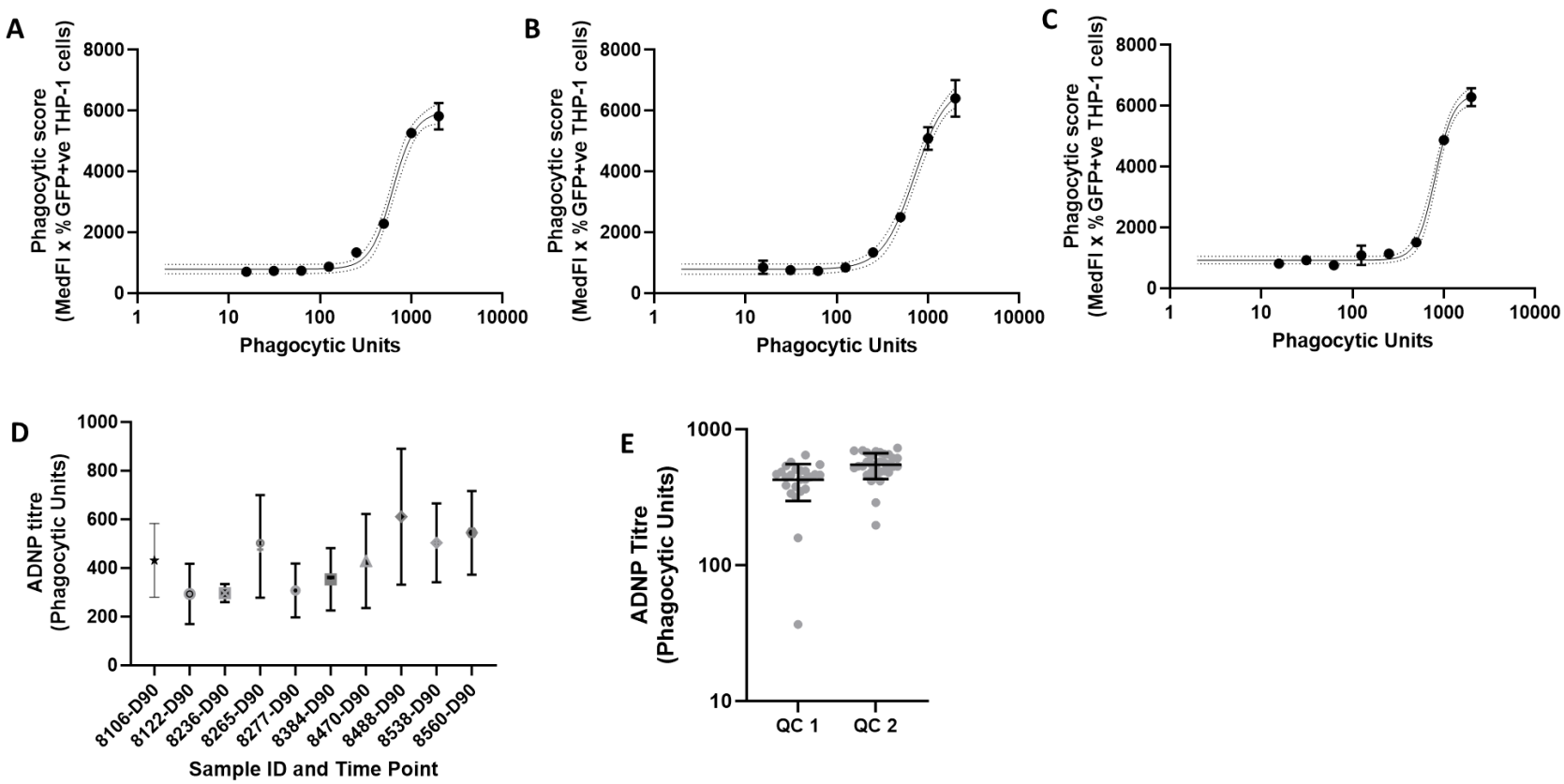


Figure 3-20 Reproducibility of the ADNP assay and standard curve interpolation

(A-C) Example ADNP standard curves collected over a period of 3 months: A positive pool was serially diluted and interpolated using a 4 parameter sigmoidal curve, a starting 1/16 dilution of the standard was assigned an arbitrary titre of 2000 phagocytic units. Error bars represent the standard deviation between the replicates.
(D) ADNP titres of a panel of 10 samples, run on three different days, plotted with error bars representing the standard deviation.
(E) ADNP titres of the two quality control samples run on each plate, error bars represent the average, and 1 standard deviation used as the upper and lower acceptable limit

Sample	Time Point	Ave	%CV
8106	D90	431.6	28.7
8122	D90	293.5	34.5
8236	D90	297.1	10.2
8265	D90	489.3	35.1
8277	D90	307.9	29.5
8384	D90	353.8	29.6
8470	D90	428.7	36.8
8488	D90	611.2	37.3
8538	D90	503.8	26.2
8560	D90	545.1	25.7

Table 3-8 ADNP titres of the reproducibility sample panel

Average ADNP titre and % CV of 10 samples used to assess the reproducibility of the ADNP assay and standard curve interpolation.
% CV calculated as (standard deviation/ average) x 100

3.8. Conclusion

This chapter describes the successful optimisation and relevant QC criteria of multiple standardized assays to measure SBA, ADMP, and ADNP against *S*. Typhi and Paratyphi A. These assays show good inter-day reproducibility and can distinguish between samples with positive and negative titres for each function. In the following chapters these assays will be applied to the samples collected from multiple clinical trials outlined in section **2.1** to assess the changes in antibody effector functions in response to *Salmonella* vaccination or infection.

Chapter 4. Changes in Serum bactericidal activity after *S*. Paratyphi A exposure or vaccination

4.1. Introduction

Serum bactericidal activity (SBA) assays are an *in vitro* measure of classical complement pathway activation. They quantify the ability of antibodies to bind to bacteria, recruit complement effector proteins, activating the complement cascade resulting in bacterial lysis. The complement cascade is a triggered-enzyme cascade, and is an effective link between the innate and adaptive immune system. In the classical pathway, recruitment and binding of C1q to the Fc portion of antibodies bound to bacterial cell surface triggers recruitment and cleavage of downstream effector proteins, namely C3-C9, in a sequential manner. Collectively the cleaved sections of the effector proteins, C5b-C9, bind to the bacteria surface forming a pore named the membrane attack complex (MAC). This ruptures the bacteria outer membrane causing lysis and death [165].

Bactericidal antibodies are important for neutralising and promoting clearance of bacteria, and have a key role in preventing dissemination and establishment of systemic infection. The SBA assay is a valuable tool for measuring functional antibody responses to natural infection and in response to vaccination for Gram-negative bacteria. SBA is an accepted correlate of protection for the encapsulated Gram-negative bacterium, *N. meningitidis*, it is widely interpreted as a surrogate of vaccine efficacy and is used to license new meningococcal vaccines [166,167].

SBA is thought to be one potential protective mechanism in *S*. Typhi infection. In Kathmandu, Nepal, increases in *S*. Typhi SBA with age have been associated with decreasing typhoid fever incidence [135]. In an *S*. Typhi CHIM study, SBA induced after a single dose of oral vaccine candidate M01ZH09 correlated with less severe disease, although did not protect human volunteers from developing infection after oral challenge with bacteria [163]. While expression of the Vi capsule is thought to help

the bacteria evade the immune system and make it less sensitive to complement mediated killing, anti-Vi antibodies have been shown to support complement mediated bacterial killing *in vitro* [162]. Research into Vi specific serological correlates of protection revealed a role for IgA quantity and fold change, and neutrophil phagocytosis in protection from acute typhoid fever in a controlled human infection study, as well as a role for higher anti-Vi-IgG1 aviditiy in mitigating disease severity [141,168]. These studies highlighted the multifaceted nature of immune responses underpinning protection from disease. How bactericidal antibodies fit into this dynamic system is not known.

Our comprehension of *S*. Paratyphi A infection is largely extrapolated from our understanding of *S*. Typhi infection, but *S*. Paratyphi pathogenesis is not well understood. The lack of Vi capsule on *S*. Paratyphi serovars A and B, which are responsible for the majority of paratyphoid fever cases, and other variations in surface antigens mean that antibody derived protection against paratyphoid fever might be mediated by distinct mechanisms compared with typhoid. The specific role of bactericidal antibodies in the context of paratyphoid has not been extensively characterised.

Pre-clinical studies of paratyphoid vaccine candidates have demonstrated a potential role of bactericidal antibodies in vaccine mediated protection. A vaccine comprising of a recombinant *S*. Typhi invasin, an outer membrane protein expressed by *S*. Typhi and *S*. Paratyphi, was demonstrated to be protective against 10x the lethal dose of *S*. Paratyphi A challenge in an adapted murine model [169]. A MAPS (Multiple Antigen Presenting System) vaccine that uses rhivavadin-biotin linking technology to generate polysaccharide-protein complexes, including Vi and *S*. Paratyphi O specific polysaccharide, showed significant increases in SBA post-vaccination in rabbits, although no formal analysis has been performed in a preclinical challenge setting [170].

The aims of this chapter are:

1. Does Vi vaccination induce changes in bactericidal antibodies? Is there a relationship between Vi vaccine induced SBA titres and diagnostic outcome in an *S*. Typhi CHIM?

2. Does bactericidal activity against *S*. Paratyphi A change in response to *S*. Paratyphi A challenge and homologous rechallenge? Does baseline SBA activity at either challenge correlate with protection from paratyphoid infection?
3. Does live attenuated oral *S*. Paratyphi A vaccine (CVD1902) induce changes in bactericidal antibodies? Is there a correlation between CVD1902 vaccine induced SBA titres and diagnostic outcome in an *S*. Paratyphi CHIM?
4. Does novel S. Typhi – Paratyphi A bivalent conjugate vaccine induce changes in SBA?

4.2. Methods

Quantification of bactericidal antibodies was performed on all data sets outlined in **Table 2-1**.

Methods for measuring SBA titres against *S*. Typhi in VAST study samples are outlined in section **2.5.1**. Methods for measuring SBA titres against *S*. Paratyphi A in P1, PATCH and VASP CHIM study samples using a luminescence based SBA are outlined in **2.5.2**. Methods for measuring SBA titres against *S*. Paratyphi A in the SIIPL bivalent vaccine phase I study using a conventional colony counting SBA protocol are outlined in sections **2.5.3 - 2.5.4**.

L-SBA titres are correlated with other immune markers, the methods for measuring each immune markers are noted in chapter **2.8** (binding antibodies Quantified by ELISA).

4.3. Results

4.3.1. L-SBA responses to *S*. Typhi after Vi vaccine in a CHIM

Serum bactericidal activity was measured against *S*. Typhi, at baseline (prior to Vi vaccination) and 28 days later, immediately prior to challenge, in participants enrolled in the VAST study. A total of 72 vaccinated volunteers who completed the 14-day challenge period were analysed. There was a significant increase in SBA titre in both Vi-PS and Vi-TT vaccine groups (**Figure 4-1 L-SBA titre at baseline (Pre–vac) and 28 days later, at the point of S. Typhi challenge**A). To investigate whether SBA was associated with protection from disease in the challenge model, titres at the point of

challenge (D28) were compared between individuals who were later diagnosed and those who did not meet the diagnosis threshold. There were no significant differences in SBA titre related to diagnosis outcome in either vaccine arm (Vi-PS: $p = 0.88$, Vi-TT: $p = 0.29$, **Figure 4-1** B). There was no significant difference in fold change from pre-vac to day 28 between any groups, although there was a trend towards significance comparing fold change in Vi-PS nTD group (n = 22) with Vi-PS TD group (n = 13), p = 0.09. Data not shown).

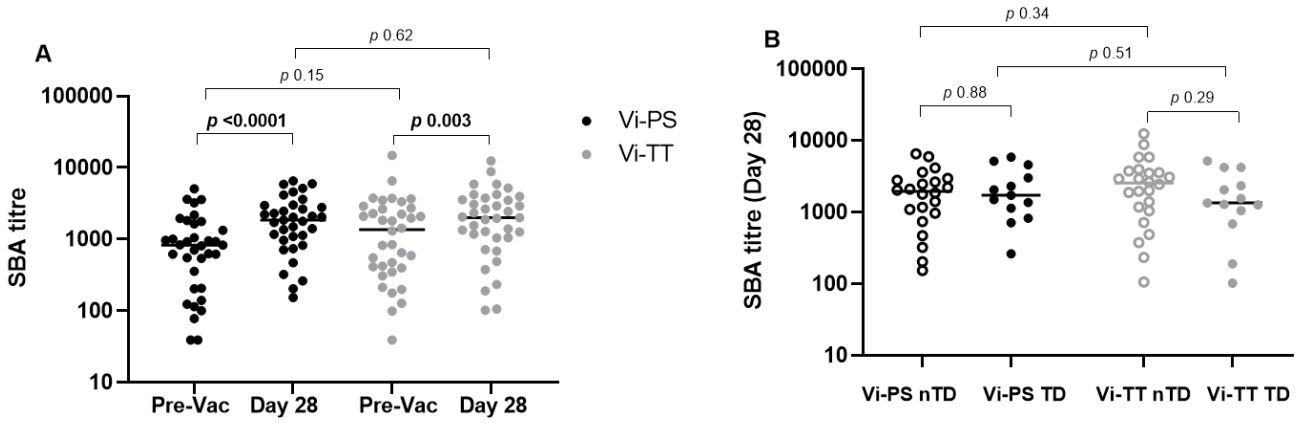
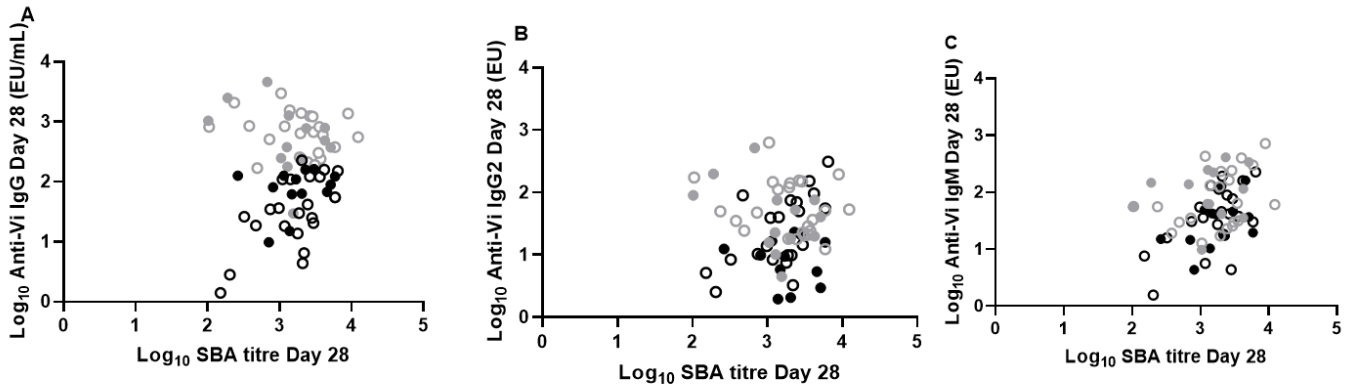


Figure 4-1 L-SBA titre at baseline (Pre−vac) and 28 days later, at the point of S. Typhi challenge

(A) Comparisons between time points, split by vaccine (Vi−PS n=35, Vi−TT n=37). (B) Comparisons of day 28 titres, split by vaccine and challenge outcome (TD; typhoid diagnosed, closed circles. nTD; not typhoid diagnosed/remained well, open circles). Limit of detection is an L-SBA titre of 39. Between time point comparisons analysed using Wilcoxon matched−pairs signed rank test, between group comparisons analysed by Mann−Whitney. Bars represent median SBA titre.

SBA titres at the point of challenge were compared with alternative variables and assessed for correlation. SBA titres at D28 in Vi-PS vaccinated individuals correlated with Vi specific IgG, IgG2 and IgM (**Figure 4-2** A-C) conversely SBA titres in the Vi-TT vaccinated group did not significantly correlate with any measured variables (**Figure 4-2** A-C, **Table 4-1**). There were no significant correlations with any of the clinical outcomes or parameters of disease severity.



Spearman rho (*p* value)	
Vi–PS (all, *n*=35)	**0.356 (0.036)**
Vi–PS (nTD, *n*=22)	**0.521 (0.013)**
Vi–PS (TD, *n*=13)	0.256 (0.407)
Vi–TT (all, *n*=37)	−0.306 (0.066)
Vi–TT (nTD, *n*=24)	−0.329 (0.116)
Vi–TT (TD, *n*=13)	−0.330 (0.269)

Spearman rho (*p* value)	
Vi–PS (all, *n*=35)	**0.386 (0.022)**
Vi–PS (nTD, *n*=22)	**0.603 (0.003)**
Vi–PS (TD, *n*=13)	0.000 (>0.999)
Vi–TT (all, *n*=37)	−0.213 (0.205)
Vi–TT (nTD, *n*=24)	−0.210 (0.325)
Vi–TT (TD, *n*=13)	−0.322 (0.282)

Spearman rho (*p* value)	
Vi–PS (all, *n*=35)	**0.478 (0.004)**
Vi–PS (nTD, *n*=22)	**0.462 (0.035)**
Vi–PS (TD, *n*=13)	0.473 (0.106)
Vi–TT (all, *n*=37)	0.296 (0.084)
Vi–TT (nTD, *n*=24)	0.397 (0.067)
Vi–TT (TD, *n*=13)	0.223 (0.461)

Figure 4-2 Correlation between anti−Vi IgG, IgG2, and IgM levels and SBA titre at the time of challenge

Correlations between L-SBA titre at the point of challenge with anti−Vi IgG (A), anti−Vi−IgG2 (B), and anti−Vi−IgM (C). TD; typhoid diagnosed, closed circles. nTD; not typhoid diagnosed/remained well, open circles. Correlation assessed using the non-parametric spearman rank correlation coefficient assessment

	Spearman rho (*p* value)					
	Vi–PS (all, *n*=35)	Vi–PS (nTD, *n*=22)	Vi–PS (TD, *n*=13)	Vi–TT (all, *n*=37)	Vi–TT (nTD, *n*=24)	Vi–TT (TD, *n*=13)
Anti−Vi IgG1	0.226 (0.192)	0.261 (0.241)	0.102 (0.740)	−0.152 (0.371)	−0.040 (0.845)	−0.382 (0.197)
Anti−Vi IgG3	0.230 (0.183)	0.245 (0.272)	0.280 (0.354)	−0.070 (0.682)	0.044 (0.837)	−0.429 (0.144)
Anti−Vi IgA	0.079 (0.657)	0.286 (0.205)	−0.223 (0.461)	−0.092 (0.593)	−0.232 (0.287)	−0.052 (0.867)
Antibody Dependant Complement Deposition	0.178 (0.307)	0.350 (0.110)	−0.107 (0.763)	0.051 (0.763)	−0.005 (0.982)	0.070 (0.818)
Time to Typhoid Fever Diagnosis (Days)	0.064 (0.716)	NA	0.252 (0.404)	−0.218 (0.196)	NA	−0.209 (0.490)
Duration of Fever (Days)	0.089 (0.617)	0.333 (0.140)	−0.772 (0.365)	−0.038 (0.825)	−0.243 (0.253)	0.333 (0.262)
Duration of Bacteraemia (Days)	−0.062 (0.731)	NA	−0.182 (0.589)	−0.178 (0.298)	NA	0.470 (0.125)
Symptom Severity	0.133 (0.4454)	0.132 (0.558)	0.011 (0.978)	−0.266 (0.112)	−0.312 (0.138)	−0.124 (0.684)
Bacterial Burden (CFU/mL)	0.127 (0.733)	NA	0.127 (0.733)	0.112 (0.760)	NA	0.112 (0.759)

Table 4-1 Table of correlation outcomes of log10 SBA titre 28 days after vaccination compared with various parameters

Anti−Vi antibody levels and antibody dependent complement deposition data all 28 days post vaccination, at the point of challenge.

To assess whether Vi vaccine induced SBA is mediated by Vi specific antibodies, serum samples were depleted of Vi specific antibodies and then assessed for bactericidal activity. A subset of samples from 6 participants, who were vaccinated but not challenged were chosen based on large sample volume availability. Vi IgG titre at pre-vaccination went from 45.8 EU/mL to 17.5 EU/mL following depletion, while for D28 the GMT went from 669.1 EU/mL to 106.4 EU/mL following depletion. Analysis of SBA in these samples showed a corresponding significant reduction in activity after Vi antibody depletion at D28 only, with titres comparable to pre-vaccination levels (**Figure 4-3** B; p = 0.56). SBA GMT pre-vaccination versus D28; 1190-2002 originally, 809.2-1232 in depleted samples).

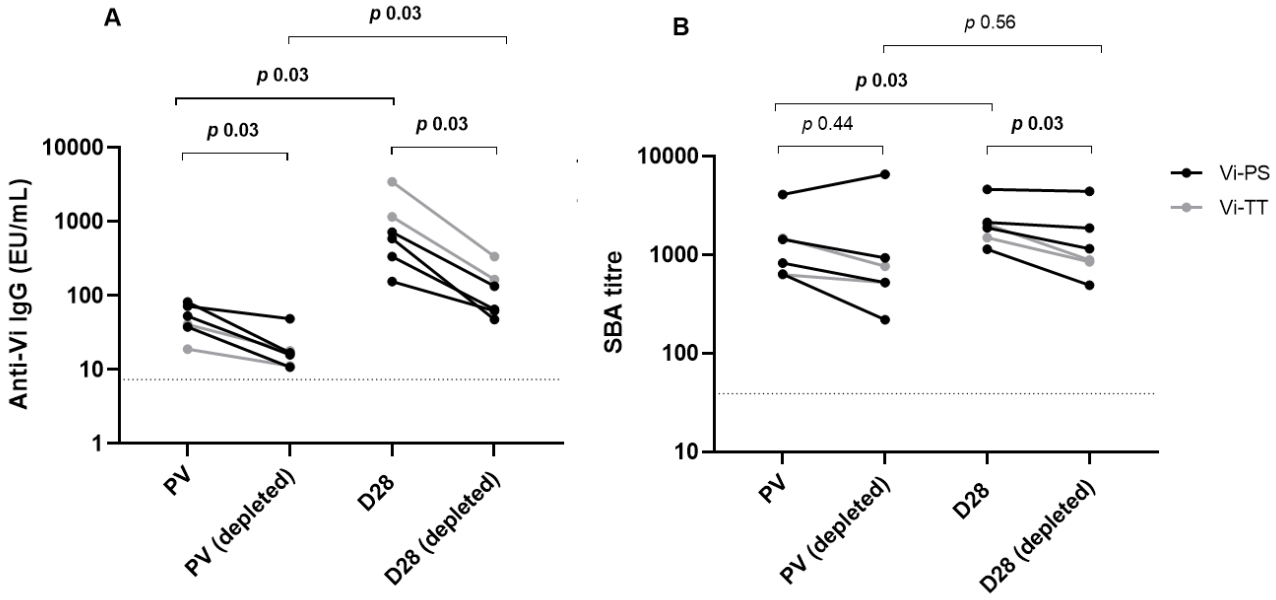


Figure 4-3 Anti−Vi IgG and SBA titres before and after Vi antibody depletion

(A) Anti−Vi IgG titre determined by ELISA, pre and post Vi antibody depletion, in a subset of Vi−PS or Vi−TT vaccinated individuals. Dotted line marks the limit of detection,7.4 EU/mL. (B) Corresponding SBA titres from pre and post depleted serum. Dotted line marks the limit of detection, 39. (n=6).

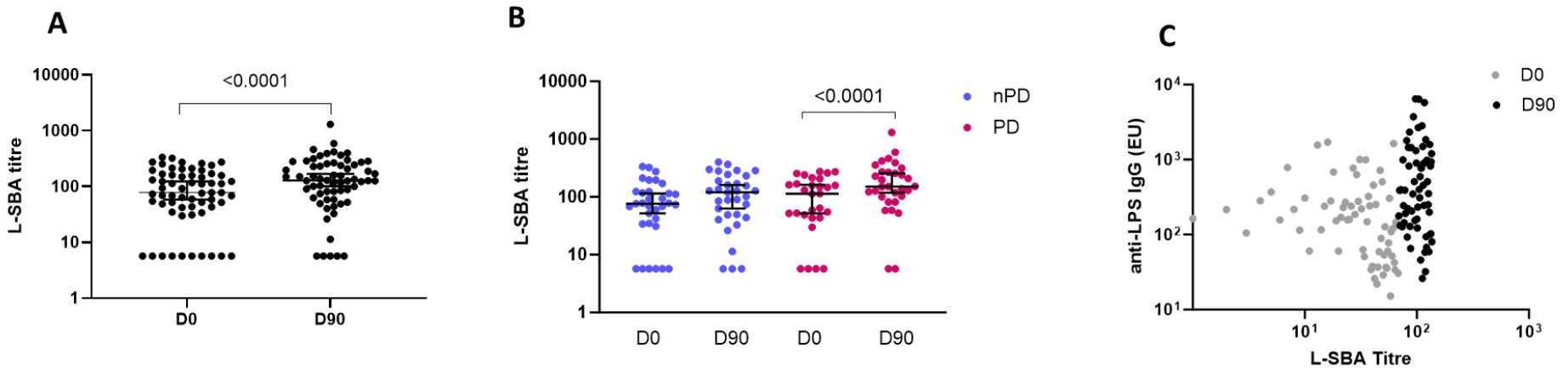
4.3.2. L-SBA responses to *S*. Paratyphi after exposure in a CHIM

Bactericidal antibodies against *S*. Paratyphi A were evaluated in healthy volunteers at baseline (D0) and 90 days post *S*. Paratyphi A challenge (D90) in 62 individuals enrolled in two CHIM studies. The P1 study which was a dose finding trial investigating the challenge dose needed to achieve an attack rate of 60-70%, and the PATCH study which was a trial examining the immunological responses to *S*. Typhi and *S*. Paratyphi A challenge and the effect of homologous or heterologous rechallenge.

Ninety days after challenge SBA titres were significantly increased. When analysed by diagnosis outcome this elevation was only significant in the group who developed paratyphoid fever ($p < 0.0001$, **Figure 4-4** A & B). However, this was not statistically significant when analysed using a regression model (Odds ratio 1.002, p 0.0745). 24/62 challenged individuals showed no change in SBA or decreased SBA activity post challenge.

L-SBA titres at the point of challenge (D0) did not correlate with protection from infection after S. Paratyphi A exposure (Odds ratio 1.002, p 0.4391).

Correlation of L-SBA titres with anti LPS-IgG showed that L-SBA at D90 correlate with anti LPS-IgG (**Figure 4-4** C).



Time point	Spearman's r (p value)
D0	-0.0354 (0.7834)
D90	**0.2851 (0.0224)**
All	**0.2167 (0.0144)**

Figure 4-4 L-SBA titres against S. Paratyphi A

L-SBA titres against *S*. Paratyphi A NVGH308 strain at baseline (D0) and 90 days (D90) after oral challenge with *S*. Paratyphi NVGH308 strain. (A) All participants grouped together. (B) split by diagnosis outcome (C) correlation analysis between L-SBA titre and anti-LPS IgG. Correlation assessed using non-parametric spearman's rank correlation coefficient, tabulated underneath the graph. (PD; paratyphoid diagnosed, nPD; not paratyphoid diagnosed). Between time point comparisons analysed using Wilcoxon matched−pairs signed rank test, between group comparisons analysed by Mann−Whitney. Bars represent median and 95% confidence interval. Comparisons between all timepoints and groups were performed, but only those that reached statistical significance are presented.

Forty five out of the 62 participants analysed received the high dose (1-5 x 10^3 CFU) and 17 received the low dose (500-1000 CFU). There was a significant increase in SBA titre at D90 compared with D0 irrespective of challenge dose (**Figure 4-5** A). 3/17 participants who received the low dose didnt show any changes in L-SBA between D0 and D90, whereas 21/46 participants in the high dose group did not have an L-SBA response. In the high dose group, challenge induced increases in L-SBA titre were only significant in the diagnosed group (PD; p = 0.0009). In contrast, there was a statistically significant increase in titre by D90 irrespective of diagnosis outcome in the low dose group (nPD; p = 0.0117, PD; p = 0.0078). This could be due to the fact that the time to diagnosis was delayed in the low dose group.

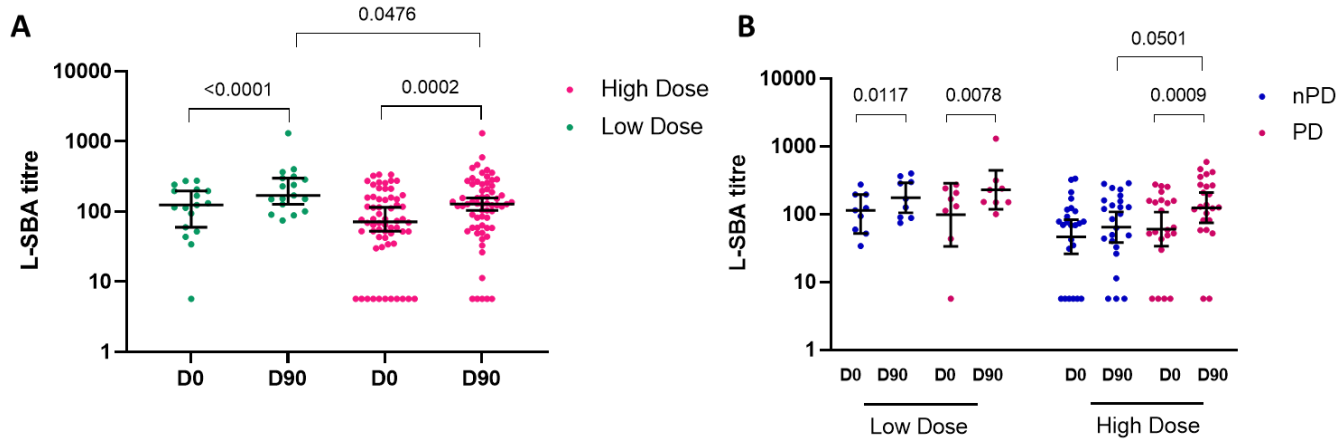


Figure 4-5 Comparison of L-SBA titres in individuals challenged with S. Paratyphi at two different doses

L-SBA titres at baseline (D0) and 90 days after challenge (D90) with *S*. Paratyphi A NVGH308 strain split by dose (A) or dose and diagnosis outcome (B). High dose; 1-5 x 10^3 CFU, low dose; 500-1000 CFU. PD; paratyphoid diagnosed, nPD; not paratyphoid diagnosed. Between time point comparisons analysed using Wilcoxon matched−pairs signed rank test, between group comparisons analysed by Mann−Whitney. For Panel A comparisons were performed between all groups and time points, only those that reached statistical significance are presented on the graph. For Panel B, comparisons were performed between all time points and diagnosis groups within each dose arm, only those that reached statistical significance are presented. No comparisons between dose groups were performed for panel B.

4.3.2.1. Effect of prior exposure to *S.* Paratyphi A

In a challenge model setting, previous exposure to *S.* Paratyphi A was associated with a 57% reduced risk of developing paratyphoid fever upon second exposure[134]. To investigate the relationship between bactericidal antibodies and protection after homologous rechallenge, titres at D0 and D90 were compared between 45 naïve and 12 rechallenged volunteers. A significant increase in SBA was measured between baseline (D0) and D90 in the group which were naïve at the point of challenge only ($p < 0.0001$), in contrast no significant changes in SBA titre were detected 90 days after rechallenge (**Figure 4-6** A). SBA titres had decreased significantly at the point of rechallenge compared with D90 of the first challenge ($p = 0.0303$, **Figure 4-6** B, median interval between challenges was 17 months), indicating waning of bactericidal antibodies. Although this might be at least partially attributable low numbers of rechallenged participants (n=12).

To determine if SBA changes over time were different based on outcome after initial exposure, SBA titres in rechallenge participants who were diagnosed (PD) were compared with those who were not diagnosed (nPD). In participants who were developed paratyphoid fever after their first challenge SBA titres were significantly higher after 17 months (D0 of rechallenge) compared with those who were not did not develop clinical disease ($p = 0.0303$, **Figure 4-6** B). The median SBA titre at the point of rechallenge was lower in those who went on to develop disease however it did not reach statistical significance (median nPD 68.7, PD 17.8, p 0.4713, **Figure 4-6** D). Furthermore, baseline titres measures at the point of rechallenge did not correlate with protection after a second exposure as assessed by logistic regression (Odds ratio 0.9929, p 0.6028)

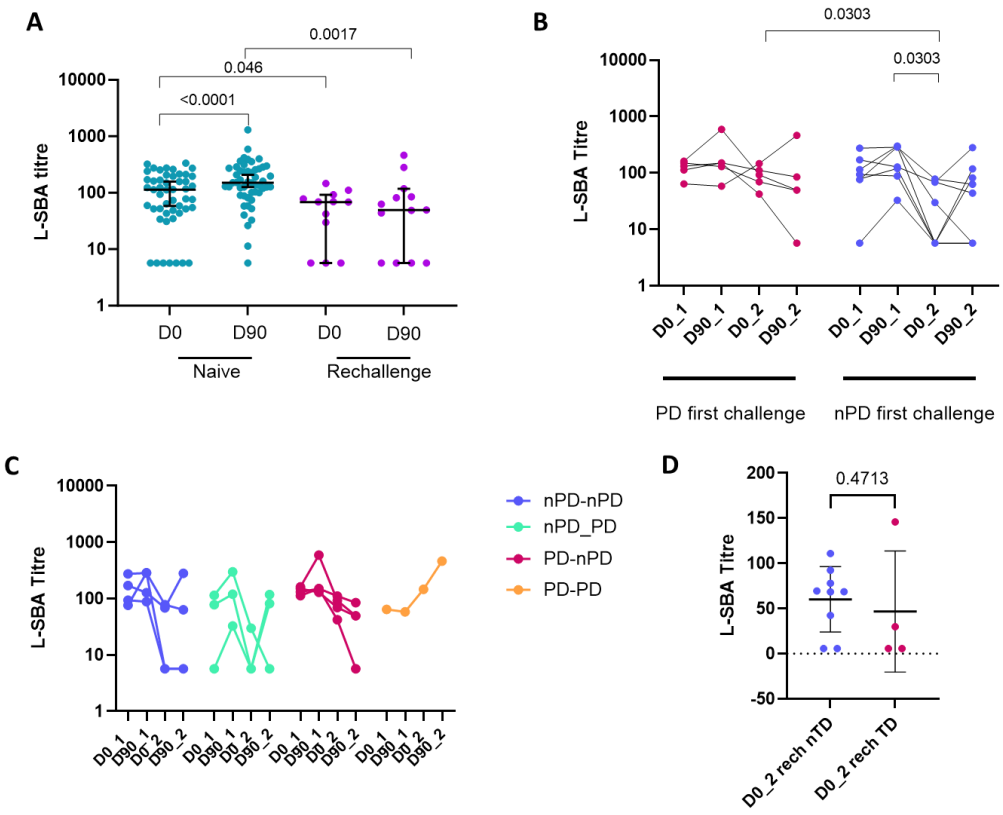


Figure 4-6 Comparison of L-SBA titres after homologous rechallenge with S. Paratyphi

L-SBA titres at baseline (D0) and 90 days after challenge (D90) with *S*. Paratyphi A NVGH308 strain. (A) Results plotted by challenge iteration. (B) Timeline of L-SBA titres in the rechallenge group only, plotted based on initial challenge outcome. (C) L-SBA titres in the rechallenge group only separated based on the diagnosis status of both challenges. (D) L-SBA titre at the point of rechallenge, split based on outcome of the second challenge.
PD; paratyphoid diagnosed, nPD; not paratyphoid diagnosed. Time point from the first challenge are denoted as D0_1 and D90_1, time points from the second challenge are denoted as D0_2 and D90_2.
Between time point comparisons analysed using Wilcoxon matched−pairs signed rank test, between group comparisons analysed by Mann−Whitney. Bars represent median and 95% confidence interval. On panel A all groups were compared, p-values are presented only from comparisons that reached

statistical significance. No formal comparisons were performed on panel B or C, due to the low numbers of participants in each group these data are for descriptive purposes only.

4.3.2.2. Changes in L-SBA in participants in an oral *S*. Paratyphi A vaccine and challenge study

Bactericidal antibodies against *S*. Paratyphi A were measured in 27 healthy volunteers enrolled in a live attenuated oral paratyphoid vaccine, CVD1902, CHIM study (VASP). Samples were taken prevacination (D-42), and then 42 days later immediately prior to challenge (D0), from participants who received either two doses of CVD1902 or placebo administered two weeks apart. At the point of writing this thesis the VASP study is actively recruiting therefore I am blinded to the vaccine status of the participants and data are analysed in a blinded fashion. Forty-two days after primary vaccination/placebo there was a significant increase in SBA titre (p 0.0025, **Figure 4-7** A). To evaluate the potential protective effect of vaccine induced bactericidal antibodies, SBA titres in diagnosed individuals were compared with titres in individuals that were not diagnosed during the 14 day challenge follow up period. There was a significant increase in titre in the diagnosed group between D-42 and D0 (p 0.0117). Although L-SBA at the point of challenge (D0) was not significantly related to the development of paratyphoid fever (Odds ratio 0.9988, p 0.4680)

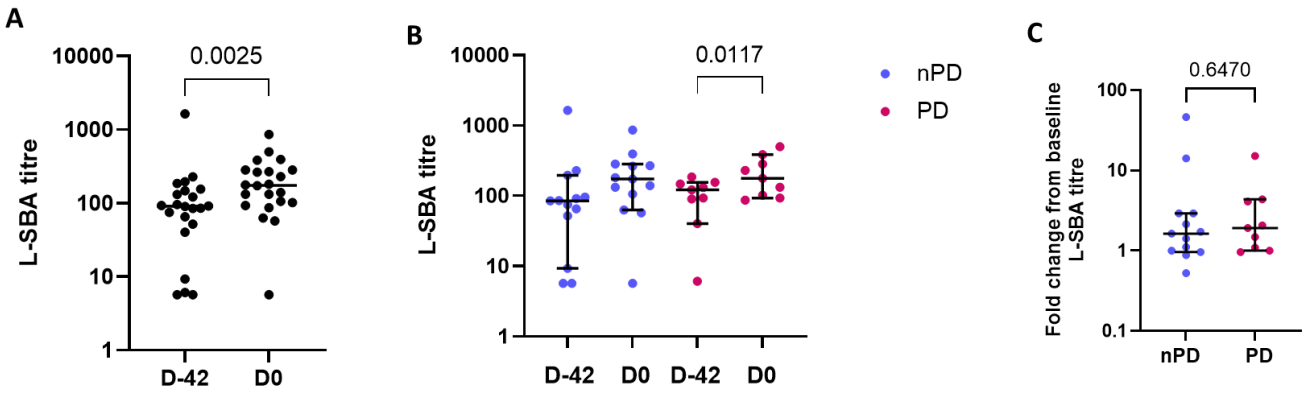


Figure 4-7 L-SBA titres in participants in an oral S. Paratyphi A vaccine trial

L-SBA titres in individuals at baseline (D-42) and 42 days after vaccine or placebo immediately prior to challenge with *S*. Paratyphi A NVGH308 strain (D0) for all participants combined (A), or divided by diagnosis outcome after challenge (B). (C) Fold change in L-SBA titre from D-42 to D0 split by diagnosis outcome. PD; paratyphoid diagnosed, nPD; not paratyphoid diagnosed. Between time point comparisons analysed using Wilcoxon matched−pairs signed rank test, between group comparisons analysed by Mann−Whitney. Bars represent median and 95% confidence interval. For Panel B, comparisons were performed between all groups and time points, only those that reached statistical significance are presented.

Fold change in SBA titre was calculated as D90/D0 in P1/PATCH, and as D0/D-42 in VASP participants. No significant differences in fold change were observed when comparing subgroups (**Figure 4-8**).

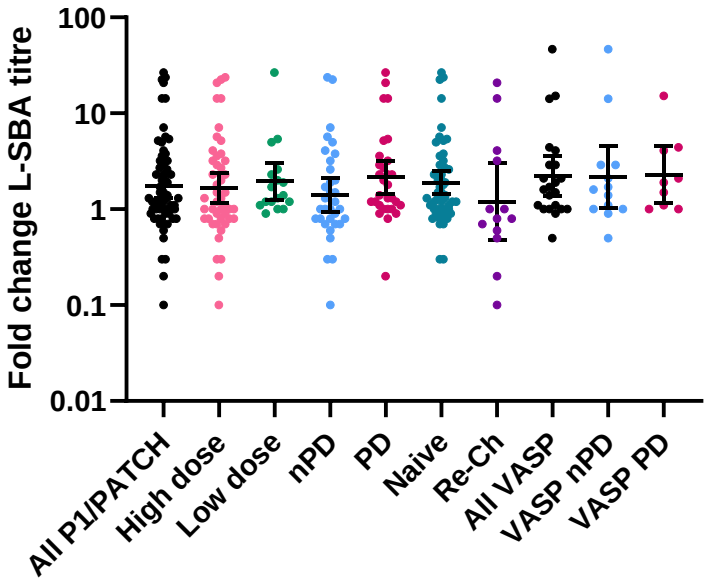


Figure 4-8 Comparison of L-SBA titre fold change

Fold change in L-SBA titres at baseline (D0) and 90 days after challenge (D90) with *S*. Paratyphi A NVGH308 strain split by dose, diagnosis outcome, or challenge iteration. High dose; 1-5 x 10^3 CFU, low dose; 500-1000 CFU. PD; paratyphoid diagnosed, nPD; not paratyphoid diagnosed. Or fold change in L-SBA titre at baseline and 42 days after vaccine with CVD1902 or placebo (VASP) split by diagnosis outcome.

4.3.3.SBA responses to *S*. Paratyphi after bivalent *S*. Typhi - Paratyphi A conjugate vaccine

Bactericidal antibodies against *S*. Paratyphi A were evaluated in healthy volunteers enrolled in a phase I clinical trial evaluating the safety and immunogenicity of the novel typhoid-paratyphoid bivalent conjugate vaccine (Sii-PTCV). This vaccine contains S. Typhi Vi CPS conjugated to tetanus toxoid and S. Paratyphi A O specific polysaccharide conjugated to diphtheria toxoid. Samples were analysed at baseline (D0) and 29, and 181 days post vaccine in 60 individuals. Inoculation with Sii-PTCV induced a significant increase in SBA titre ($p < 0.0001$) measured 29 days post vaccine, titres showed signs of waning by D181 (p 0.0007) but remained significantly elevated above baseline levels ($p < 0.0001$, **Figure 4-9** A). There were no significant changes in the SBA titres against *S*. Paratyphi A measured in the Typbar-TCV® group. These findings are corroborated when comparing fold change between time points and groups. Fold change in SBA titre from D0 to D29 and D181 were significantly higher in Sii-PTCV recipients compared with Typbar-TCV recipients ($p < 0.0001$ at both time points, **Figure 4-9** B). SBA titres significantly correlated with anti-LPS IgG (**Figure 4-9** C).

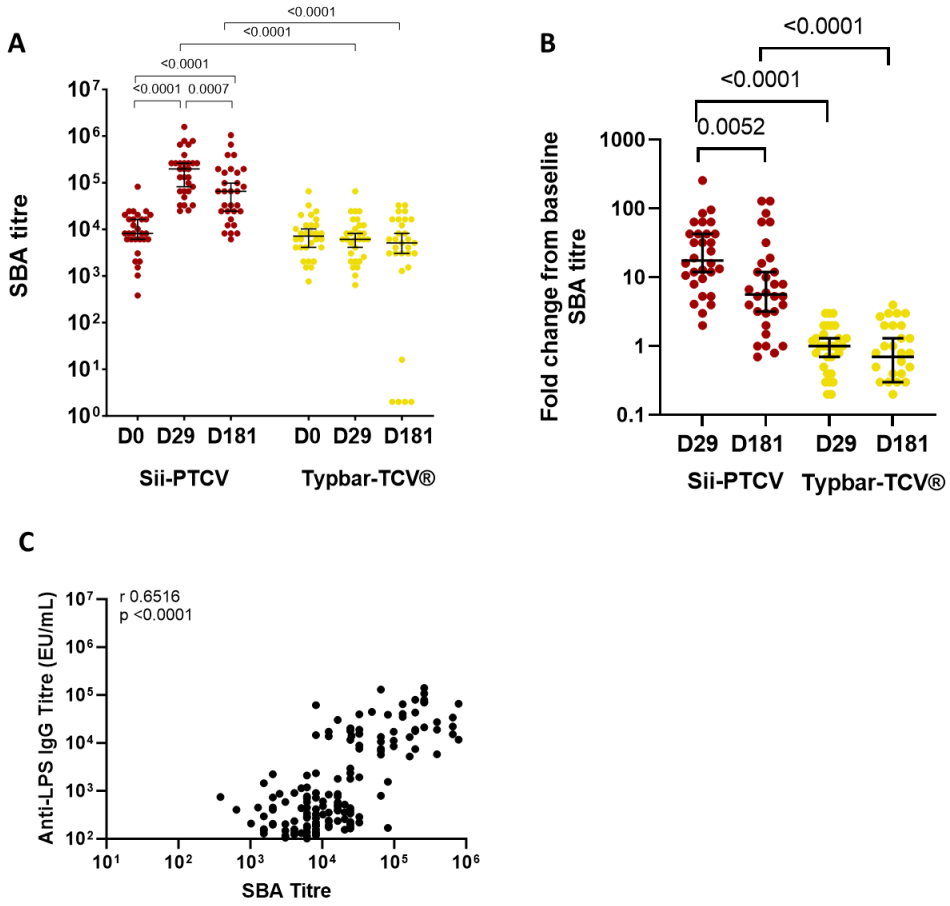


Figure 4-9 Bactericidal activity in SIIPL phase I study participants

SBA titres against *S*. Paratyphi A measured at baseline (D0), and then 29 (D29) or 181 (D181) days after vaccination with either SIIPL Typhoid – Paratyphoid A bivalent conjugate vaccine (Sii-PTCV), or typhoid Vi conjugate vaccine (Typbar-TCV®) (A).(B)Fold change SBA titres measured 29 or 181 days from baseline in participants who received a single dose of Sii-PTCV or Typbar-TCV®. (C) Correlations between SBA titre and anti-LPS IgG all time points combined. Between time point comparisons analysed using Wilcoxon matched−pairs signed rank test between group comparisons analysed by Mann−Whitney U test. Correlations assessed using a spearman's rank correlation coefficient assessment. Bars represent median and 95% confidence interval. For Panels A and B, comparisons were performed between all groups and time points, only those that reached statistical significance are presented.

4.3.4.Comparison of SBA titres with difference methods and strains

SBA titres in the Sii-PTCV study were measured against *S*. Paratyphi A ATCC9150 strain, the same strain used for the LPS purification during the vaccine manufacturing. In order to provide comprehensive protection against paratyphoid it's important that functional responses are cross reactive against other strains. We evaluated bactericidal titres in serum samples collected in the Sii-PTCV study against the NVGH308 strain. Selected samples which had a range of titres against ATCC9150, and when tested against NVGH308 there was a strong, positive correlation (spearman r 0.9493, p <0.0001) indicating that bactericidal antibodies are cross-reactive (**Figure 4-10** A).

Bactericidal antibodies in samples collected in the CHIM studies were measured using the luminescence based assay, whereas samples collected in the SIIPLTD bivalent vaccine study were evaluated using the conventional colony counting methods. This difference in protocol means that no statistical comparisons can be made between these datasets. To allow descriptive comparisons, a Spearman rank correlation coefficient assessment was performed to determine the degree of correlation between the two methods. Samples collected in the P1/PATCH study with a range of L-SBA titres were run on the colony counting SBA using ATCC9150. There was a significant, positive correlation between the two methods (r 0.7333, p 0.0311), indicating that samples with a high L-SBA titre would also have a high conventional SBA titre.

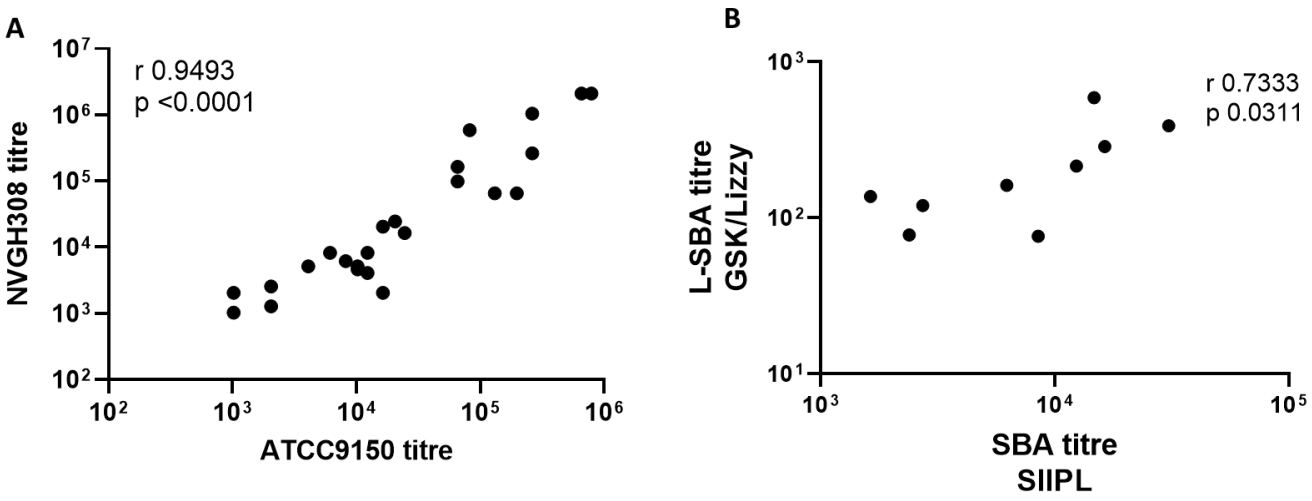


Figure 4-10 Correlation of bactericidal activity using different assays

(A)SBA titres in participants enrolled in the SIIPL Phase I study measured using the colony counting method with either NVGH308 challenge strain, or ATCC9150 vaccine strain. (B) Correlation of SBA titres of participants enrolled in the P1/PATCH studies using the luminescence SBA methods and the colony counting SBA methods. Correlation assessed using the non-parametric spearman rank correlation coefficient assessment.

4.4. Discussion

In this chapter I describe and compare changes in bactericidal antibodies in response to vaccination and experimental infection with typhoidal *Salmonella* serovars. These analyses represent the first time SBA has been measured post Vi vaccine in a human challenge model, the first time SBA responses have been characterised in a paratyphoid human challenge model, and the first time SBA responses have been quantified in human volunteers after receiving a typhoid-paratyphoid bivalent conjugate vaccine.

4.4.1.Bactericidal antibodies after Vi vaccination in an *S*. Typhi CHIM

SBA titres were measured in healthy volunteers enrolled in a CHIM study where participants received a single dose of either Vi-PS or Vi-TT 1 month before being challenged with *Salmonella* Typhi. Vaccination with either Vi-PS or Vi-TT caused significant induction of SBA antibodies after 28 days, consistent with findings for other *S*. Typhi vaccines: M01ZH09, CVD910, and intramuscular Vi-purified capsular polysaccharide in animals and humans which showed significant increases in SBA

post vaccination [138,171,172]. However, there were no significant differences in SBA titre at the point of challenge between individuals who went on to develop acute typhoid fever compared with those who did not, nor was there a significant correlation between SBA and disease severity. This highlights the heterogeneity of bactericidal antibodies to different vaccine types.

SBA titre at the point of *S*. Typhi challenge was assessed for correlation with other antibody measures. After Vi-PS vaccination SBA titre significantly correlated with anti-Vi IgG, IgG2 and IgM. This is in line with the known importance of IgM as an activator of complement due to its pentameric structure and ability to bind multiple epitopes and IgG2 being a dominant isotype induced following polysaccharide vaccination [165,173]. No significant correlations were found between SBA titre and markers of disease severity after either vaccine, providing no evidence for a role of Vi vaccine-induced SBA in mitigating acute typhoid fever within our challenge model. One possible explanation for this is the mechanism *S*. Typhi utilizes to evade antibody dependent killing by being facultative intracellular pathogen. The exact lifecycle is not fully understood but bacteria are thought to hide intracellularly within the *Salmonella* containing vacuole (SCV) and disseminate to multiple sites using the reticuloendothelial system, causing systemic infection [174]. Expression of the Vi capsule is tightly regulated by genes encoded on SPI-7, it is mainly thought to be expressed in response to low osmolarity, for example in the small intestine where expression of the capsule aids translocation from the intestinal epithelium[175]. The low level of complement in the intestinal epithelium and predominant presence of IgA, a non-complement fixing isotype, mean that when Vi is expressed there is little bactericidal activity capacity. Even though SBA-capable Vi antibodies are abundant in circulation after vaccination, their target antigen might not be visible, and so they cannot bind and therefore have a limited role *in vivo* in protecting against systemic infection.

Another reason for the lack of correlation between SBA titres and protection is that the measured SBA titres could be an underestimate of the true titre. It is thought that as little as 2% of the Vi capsule remains attached to the bacterial surface *in vitro* [175]. Sloughing of Vi antigen could bind

antibodies in our test samples, abrogating the measurable SBA titres, this would explain why we see only a small increase in SBA titres after vaccine but a much larger increase in Vi specific binding antibodies.

A previous study which demonstrated a link between higher SBA following M01ZH09 and reduced disease severity showed that vaccine-induced SBA in this setting was largely mediated by LPS antibodies [163]. Vibriocidal antibodies, predominately targeting O-polysaccharide, have been shown correlate with protection after cholera vaccination and infection, and bactericidal antibodies primarily specific to O-polysaccharide are associated with reduced disease severity after vaccination in a *Shigella* CHIM [176–179]. Depletion of Vi specific antibodies lead to a corresponding significant reduction in SBA. In depleted samples titres at D28 were comparable to pre-vaccination levels confirming that the majority of vaccine elicited bactericidal activity is mediated by Vi specific antibodies. Recent data suggest a common virulence mechanism of long chain LPS and Vi for evading the immune system, and while *in vitro* Vi antibodies have bactericidal properties, perhaps *in vivo* LPS specific antibodies have greater potency for SBA dependent disease severity mitigation [92,93]. This highlights the antigen specific nature of antibody effector functions, and the variable mechanisms of protection of different vaccines, which is particularly important when investigating correlates of protection after single antigen subunit vaccines.

The high SBA function seen *in vitro* without a corresponding role mitigating disease could also be due to differences in antigen density. To activate the complement cascade, antibodies need to bind the surface at a high enough density to allow C1q activation [165]. In vivo, antibodies could be binding the surface at a density that activates limited amounts of C1q, whereas in vitro the assay conditions could be skewed giving lower SBA titres which do not then correlate with protection.

4.4.2. Bactericidal antibodies against *S.* Paratyphi A

SBA titres against *S*. Paratyphi A were quantified in 64 volunteers enrolled in two CHIM studies, and in 60 participants of a Phase I *S*. Typhi-Paratyphi Bivalent vaccine study.

Comparisons between day of challenge and 90 days later showed a significant increase in SBA activity (p <0.0001). This observation was dependant on challenge outcome, comparisons between D0 and D90 were only statistically significant in the group who met the diagnostic threshold (PD; p <0.0001, nPD; p 0.1023). Consistent with previous observations of serological immunity in both *Salmonella* Typhi and Paratyphi A CHIM studies which show that changes in antibodies and antibody secreting cells are limited to those who developed acute paratyphoid fever [133,134]. Post challenge increases in SBA were consistent irrespective of challenge dose, higher dose: 1-5x10^3, or lower dose: 500-1000 CFU (**Figure 4-5**), however when receiving the lower dose, the SBA increase was significant irrespective challenge outcome. This observation could be attributable to low numbers of low dose recipients (n=17, PD; 8, nPD; 9). However, we also saw that the time to diagnosis in the low dose group was longer compared with those in the high dose group (median time to diagnosis; high dose; 6.4, low dose; 8.3), suggesting that functional responses might be partially driven by low levels of exposure over a longer period of time. This has important implications when comparing with real life settings where natural, acquired immunity is thought to arise from multiple, subclinical exposures of various doses.

Data from a challenge – rechallenge study showed a reduced risk of getting acute paratyphoid fever upon re-exposure. Titres on the day of the first *S*. Paratyphi A challenge were marginally higher compared with the day of second challenge (p 0.046), suggesting that after a median interval of 17-months, SBA antibodies wane to at least pre-existing levels. However, again this observation was dependent on challenge outcome as those who developed paratyphoid fever after initial exposure showed sustained levels of SBA during the 17-month interval compared with those who did not meet diagnosis criteria (D90 1st challenge – D0 2nd challenge; PD; p 0.3152, nPD; p 0.0303), indicating

that systemic infection drives longer term immunity. SBA titres on the day of second challenge were generally lower in those who went on to develop acute paratyphoid disease upon rechallenge, although this did not meet statistical significance (median titre nPD; 68.7, PD; 17.8, p 0.4713). The number of participants who underwent rechallenge were low (n = 12). Although this trend indicates a potential role of SBA in protection from reinfection, the study was not powered to assess serological correlates of protection. In endemic settings, enteric fever incidence is highest in school age and decreases with age, it is thought that immunity is acquired through repeated exposure to *Salmonella* in the environment. These findings support this theory and demonstrate that a single infection is enough to drive significant changes in functional humoral immunity to *S*. Paratyphi A, which in turn further supports the potential role of an oral vaccine against paratyphoid fever. Interestingly, no significant increases were present 90 days after second exposure.

SBA titres were evaluated in 27 volunteers in the VASP trial where participants received either two doses of an oral live attenuated *S*. Paratyphi A vaccine CVD1902, or sodium bicarbonate placebo. The primary endpoint of the study was not complete at the time of writing this thesis and data were analysed in a blinded fashion. SBA titres were significantly elevated 42 days after the initial vaccine/placebo (p 0.0025). Assuming these differences are driven by immunological change sin those who have received the vaccine, and not the placebo. This observation is consistent with findings from other oral vaccine studies, such as typhoid vaccine, M01ZH09, which was associated with a significant rise in bactericidal activity against *S*. Typhi (p 0.0001) or cholera vaccine, CVD103HgR , which is associated with a 5.9 geometric fold rise in SBA titre after one month [180]. Paradoxically, when divided by challenge diagnosis outcome increases in SBA titre post vaccine were only significant in the group who went on to develop acute paratyphoid fever (D-42 – D0, nPD; p 0.0771, PD; 0.0117). This observation is confounded by the inclusion of placebo recipients, likely diluting the vaccine specific responses. Furthermore, only 22 samples were tested and included in the analysis, once this is divided into vaccine and placebo arms and further divided in to diagnosed and not diagnosed, the numbers become small and the statistical comparisons become less

meaningful. Titres at D0 were not significantly different between nPD and PD groups, indicating that an absolute higher abundance of bactericidal antibodies is not directly associated with an increased probability of getting diagnosed. Preclinical studies demonstrated a live attenuated oral vaccine CVD1902 had a protective efficacy of 90% using a murine adapted paratyphoid model, however complement mediated kill titres at the point of challenge were no different in protected mice compared with those who succumbed to infection [181]. In conjunction, these observations suggest a redundant role for SBA after oral paratyphoid vaccines. One mechanism of action of oral vaccines is activation of T helper cells by antigen presenting cells (APC) residing in the Peyer's patches, causing downstream activation of B cells, promoting class switching to IgA, [182,183]. Secretory IgA works by blocking pathogens or toxins from binding to the epithelium. Increases in potentially protective secretory IgA would not be reflected in SBA titres as it is not a complement fixing isotype [184]. The corresponding increase in SBA titres in non-protected individuals could be indicative of a subpopulation of vaccine responders, that have not undergone class switching and have a predominately IgM response.

Interestingly, while SBA increases after challenge were only significant in those who developed systemic infection, there was a general increase in SBA titre after oral vaccine in VASP despite there being no evidence of systemic dissemination of the vaccine strain. This could be due to the vaccine dose being much higher, not less than 2×10^9 CFU, compared with the maximum challenge dose of 5×10^3 CFU. Higher titres of the vaccine strain could be more potent in driving systemic immune responses without the need for dissemination.

A single dose of typhoid paratyphoid bivalent conjugate vaccine induced significant increases in SBA titres as measured at 1 month and 6 months post vaccine. It's well documented that conjugate vaccines elicit strong functional antibody responses including SBA which is associated with disease mitigation from various pathogens such as *N. meningitidis* and *H. influenzae* [185]. Although induction

of SBA indicates this novel vaccine is capable of driving functional immune responses, which is often a requirement for vaccine licensure, it is unknown if vaccine induced SBA can protect against paratyphoid fever. Correlation of SBA and protection from disease will be assessed in an upcoming CHIM study investigating the efficacy and immunogenicity of the bivalent vaccine, which will be particularly exciting as LPS specific antibody mediated SBA has been shown to reduce symptom severity in a typhoid fever model [163]. Unsurprisingly there were no changes in SBA against *S*. Paratyphi after Vi-TT vaccine in the SIIPL phase I study. Significant correlations of SBA titres in SIIPL samples against NVGH308 strain as well as ATCC9150 strain demonstrates cross reactive functional responses, supporting the use of the bivalent vaccine for protection against paratyphoid fever in endemic settings in which the vaccine would need to be efficacious against many circulating wild type strains.

4.5. Conclusion

In this chapter, I describe the variable nature of bactericidal antibodies in the context of enteric fever infection and vaccination. Vi-PS or Vi-TT vaccine are strong inducers of SBA, however there is no evidence to support a role for Vi specific bactericidal antibodies in protection in a human challenge model. Exposure to *S*. Paratyphi A alone is not enough to drive an increase in bactericidal antibodies but requires systemic infection. In a homologous *S*. Paratyphi A rechallenge model, there is a general trend for lower SBA titres in participants who develop acute paratyphoid fever upon reexposure although the low number of participants in this study limits meaningful interpretation from these results. Interestingly, these findings are not mimicked in a study looking at SBA responses after oral vaccine CVD1902, which shows a complex relationship between SBA and protection.

Vaccination with an *S*. Typhi-Paratyphi bivalent conjugate vaccine is a strong driver of SBA responses, although it is not feasible to assess the protective nature of *S*. Paratyphi LPS specific bactericidal antibodies in the phase I study. The varied SBA responses described here demonstrate the unique

nature of immune response to different *S*. Paratyphi stimuli, and suggest this area warrants further assessment to aid our understanding of the role of SBA in paratyphoid fever.

Chapter 5. Changes in antibody dependant monocyte phagocytosis after *S*. Paratyphi A exposure or vaccination

5.1. Introduction

Antibody dependent monocyte phagocytosis (ADMP) is a coordinated process linking the adaptive and innate immune system and plays an important role in the clearance of pathogens. Antigen specific antibodies opsonise pathogens by binding to their target antigen, the Fc domain of the antibody binds to the relevant Fc receptor on the phagocyte activating intracellular signalling pathways, resulting in actin cytoskeleton rearrangement engulfing the bacteria. Within the cell, bacteria reside in membrane bound vesicles called the phagosome, or the *Salmonella* containing vacuole (SCV) in the case of *Salmonella enterica*. Bacteria within the phagosome are destroyed by acidification and hydrolytic enzymes contained within lysosomes which fuse with the phagosome[186].

Historically, most studies into humoral correlates of protection have focused on singular antibody functions such as SBA and neutralisation; but recent advances in the data driven, systems serology approach have highlighted the importance of cooperative antibody effector functions in infection control [187]. While phagocytosis is broadly recognised as an effective method of pathogen clearance, it's importance in immunity for specific pathogens has been somewhat understudied. Recently, the role of monocyte and macrophage phagocytosis has been highlighted in viral infections, such as HIV, where studies linked monocyte phagocytosis to reduced disease severity and vaccine mediated protection [188–190]. A role for ADMP has also been implicated in protection from infection with parasite *P. falciparum*, and bacteria *S. aureus* [191,192].

Intracellular bacteria, like *S*. Paratyphi have evolved effective virulence mechanisms to avoid being detected and destroyed within the intracellular niche, however it has also been shown that bacteria specific antibodies can overcome this resulting in effective clearance [193,194]. Monocytes and macrophages provide a unique intracellular niche for *Salmonella* at various points in the infection life cycle. *Salmonella* reside in monocyte SCVs and disseminate from the gut to other sites causing systemic infection. *Salmonella* can also reside long term within macrophages in an altered metabolic state, a phenomena known and "*Salmonella* persisters", which is thought possibly contribute to reinfection and the chronic carriage state, although the contribution of *Salmonella* persisters in enteric fever is unknown [195].

Studies of a live attenuated oral *S*. Typhi vaccine M01ZH09, show that in the presence of post vaccination sera rates of *S*. Typhi uptake by macrophages was up to 2.3 fold higher compared with pre vaccination sera [138]. Simultaneously, survival of bacteria was 50% less in the presence of post immunisation sera compared with pre-immunisation sera [138]. The reported efficacy of Ty21a against *S*. Paratyphi A infection ranges from 0 – 75%, and role of phagocytosis in the cross protection against paratyphoid provided by oral typhoid vaccines has not been defined [196,197]. Interestingly, sera collected after 4 doses of oral *S*. Typhi vaccine Ty21a or a single dose of oral vaccine CVD909 showed cross-reactive responses to *S*. Paratyphi A and B. Both vaccines elicited an increase in phagocytic activity against *S*. Paratyphi, compared with sera collected prior to immunisation [143]. Increases in monocyte phagocytosis have also been observed after parental Vi vaccine, although this did not correlate with protection in an experimental *S*. Typhi human challenge study [141]. It is often assumed that there is considerable overlap between immune mechanisms underpinning protection from enteric fever serovars but increasing evidence suggests unique virulence factors and distinct host pathogens interactions for typhoidal *Salmonella* serovars.

Therefore, further studies in to the role of monocytes in context *S*. Paratyphi A infection and immunity would provide important insight into this potential mechanism of protection.

The aims of this chapter are:

1. Does monocyte phagocytosis activity against *S*. Paratyphi A change in response to *S*. Paratyphi A challenge and homologous rechallenge? Does baseline ADMP activity at either challenge correlate with protection from paratyphoid infection?
2. Does live attenuated oral *S*. Paratyphi A vaccine (CVD1902) induce changes in ADMP? Is there a correlation between CVD1902 vaccine induced ADMP titres and diagnostic outcome in an *S*. Paratyphi CHIM?
3. Does novel S. Typhi – Paratyphi A bivalent conjugate vaccine induce changes in ADMP?

5.2. Methods

The samples included in this analysis are detailed in section **2.1**, **Table 2-1**. Samples were included from the P1 and PATCH oral challenge studies, the VASP oral vaccine and challenge study, and the phase 1 S. Typhi – Paratyphi bivalent study.

The methods for the ADMP assay are described in detail in section **2.6**.

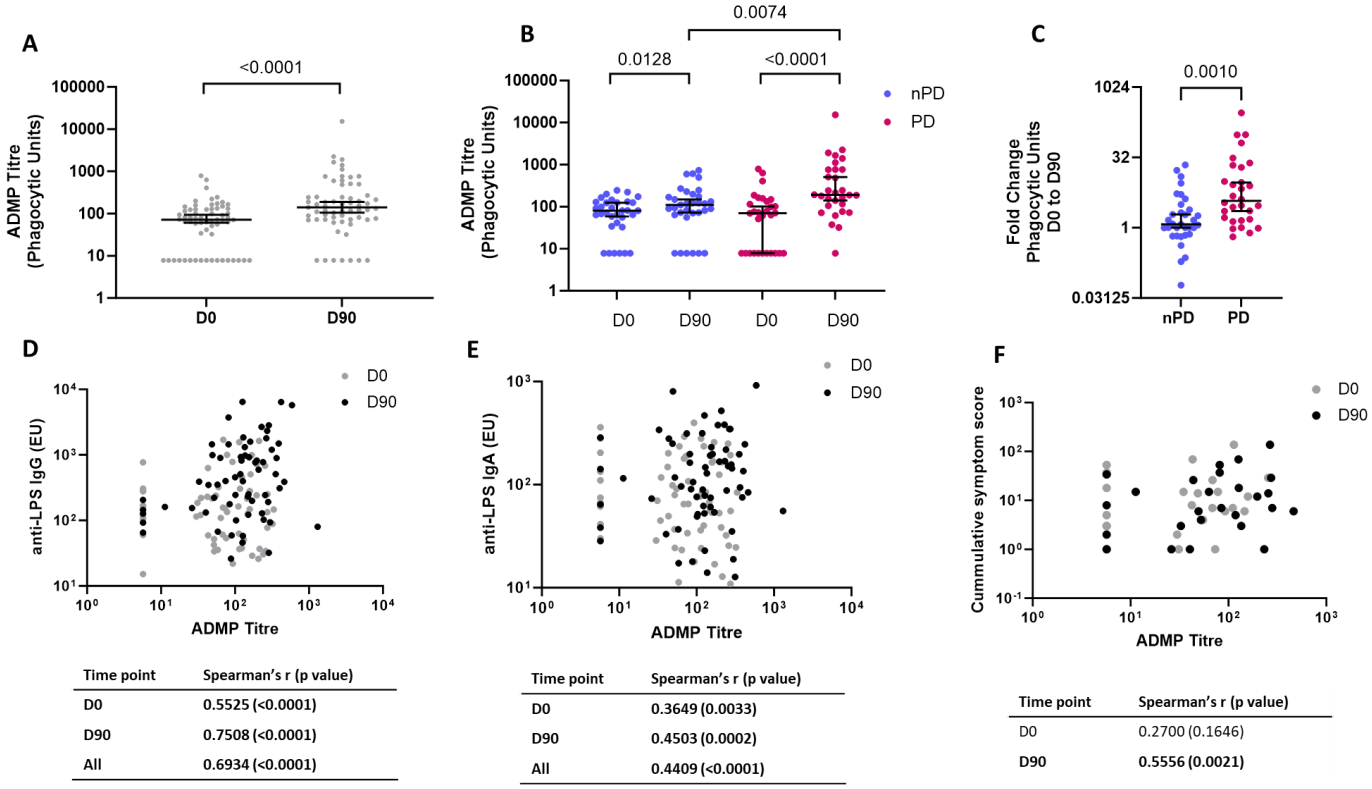
ADMP titres are correlated with other data obtained from other assays, the methods for measuring each immune marker are noted in chapter **2.8** (binding antibodies quantified by ELISA).

5.3. Results

5.3.1.Changes in ADMP in participants in an *S*. Paratyphi A challenge model

Phagocytic uptake of S. Paratyphi A was evaluated in healthy volunteers at baseline (D0) and 90 days post challenge (D90) in 62 individuals enrolled in two CHIM studies. Ninety days after challenge,

phagocytosis titres were significantly elevated above baseline (

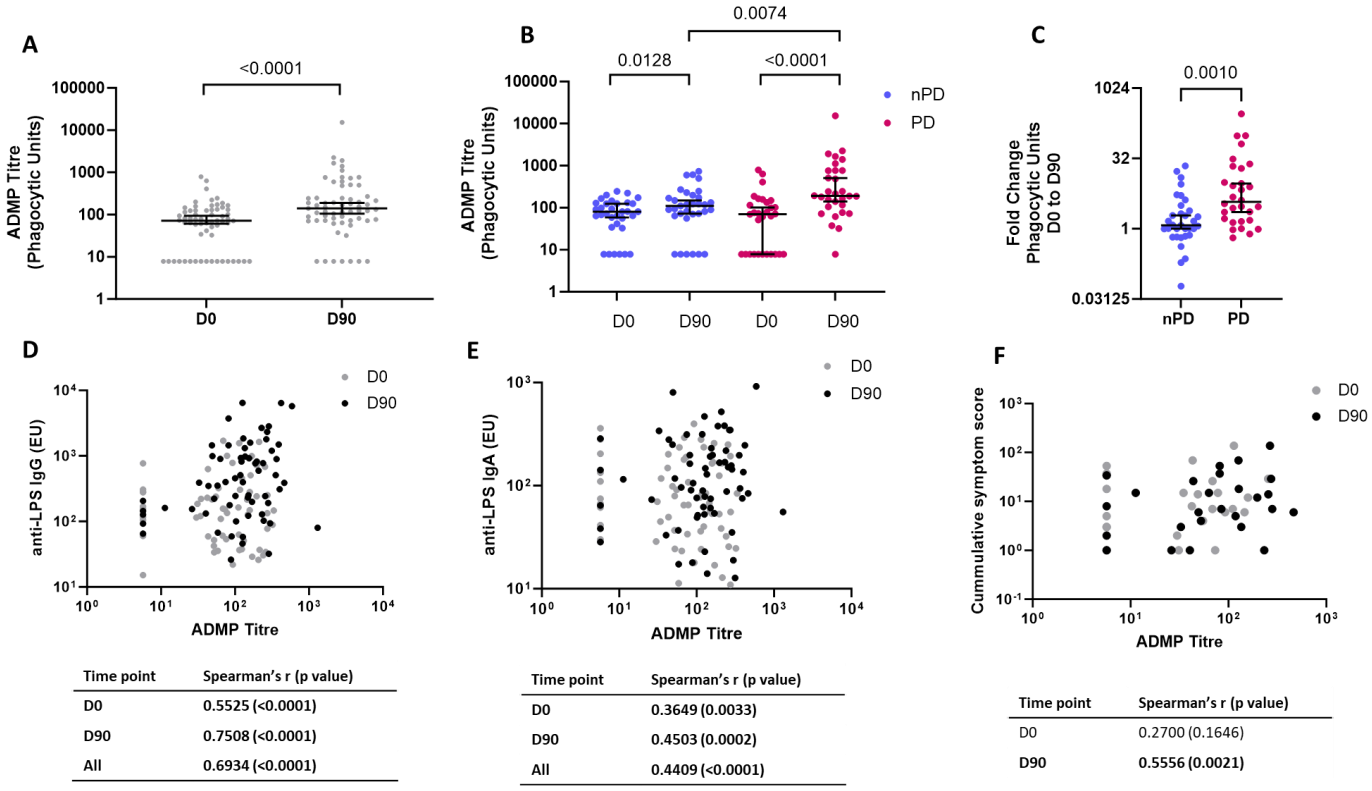


Time point	Spearman's r (p value)
D0	0.5525 (<0.0001)
D90	0.7508 (<0.0001)
All	0.6934 (<0.0001)

Time point	Spearman's r (p value)
D0	0.3649 (0.0033)
D90	0.4503 (0.0002)
All	0.4409 (<0.0001)

Time point	Spearman's r (p value)
D0	0.2700 (0.1646)
D90	0.5556 (0.0021)

Figure 5-1 A, p <0.0001). Differences in ADMP titre 90 days after challenge were significant irrespective of challenge outcome (**Figure 5-1** B, nPD; 0.0128, PD; <0.0001). This is also reflected when analysing FC data, those who developed disease after challenge had a significantly higher fold rise in titre after 90 days (**Figure 5-1** C, p 0.0010). ADMP titres significantly correlated with LPS IgG and IgA titres (**Figure 5-1** D & E), there was a general pattern of positive correlation between D90 ADMP titres cumulative symptom severity score, suggesting more severe disease stimulates stronger ADMP responses. Although this was only available from PATCH participants and did not meet statistical significance (**Figure 5-1** F). There was no difference in ADMP at baseline in those who developed disease compared with those who did not (Odds ratio: 0.9992, p 0.8038).



Time point	Spearman's r (p value)
D0	0.5525 (<0.0001)
D90	0.7508 (<0.0001)
All	0.6934 (<0.0001)

Time point	Spearman's r (p value)
D0	0.3649 (0.0033)
D90	0.4503 (0.0002)
All	0.4409 (<0.0001)

Time point	Spearman's r (p value)
D0	0.2700 (0.1646)
D90	0.5556 (0.0021)

Figure 5-1 ADMP titres against S. Paratyphi A in a human challenge model

ADMP titres at baseline (D0) and 90 days (D90) after oral challenge with *S*. Paratyphi NVGH308 strain. (A) All participants grouped together. (B) split by diagnosis outcome. (C) Fold change in ADMP titre from D0 to D90 split by diagnosis outcome after challenge (PD; paratyphoid diagnosed, nPD; not paratyphoid diagnosed). (D) Correlation of ADMP titres and LPS IgG titres (D), or LPS IgA titres (E),or cumulative symptom score (F) Correlation assessed using non-parametric spearman's rank correlation coefficient, tabulated underneath each graph. Between time point comparisons analysed using Wilcoxon matched−pairs signed rank test, between group comparisons analysed by Mann−Whitney U test. Bars represent median and 95% confidence interval. For panel B all groups and time points were compared, only those that reached statistical significance were presented on the graph.

5.3.1.1. Changes in ADMP in an *S*. Paratyphi A challenge dose finding study

The effect of exposure *S*. Paratyphi A dose on ADMP titres was assessed by comparing titres between recipients of the low dose (500-1000 CFU) and the high dose (1-5 x10^3 CFU) (**Figure 5-2**). ADMP titres were significantly elevated 90 days after challenge in both dose groups (low dose; p 0.0155, high dose; p<0.0001, **Figure 5-2** A).

When divided by challenge outcome, increases in ADMP titre following low dose, only reached significance in those who were diagnosed (PD; p 0.0156, nPD; p 0.8438) while, high dose group increases in ADMP were significant irrespective of the development of paratyphoid fever (PD; p 0.0103, nPD; p 0.8438). However, the 95% confidence intervals of the these findings significantly overlap (low dose PD; D0 7.8 – 633.3, D90 7.8 – 1903.0), high dose nPD; D0 56.0 – 131.7, D90 72.8 – 199.0).

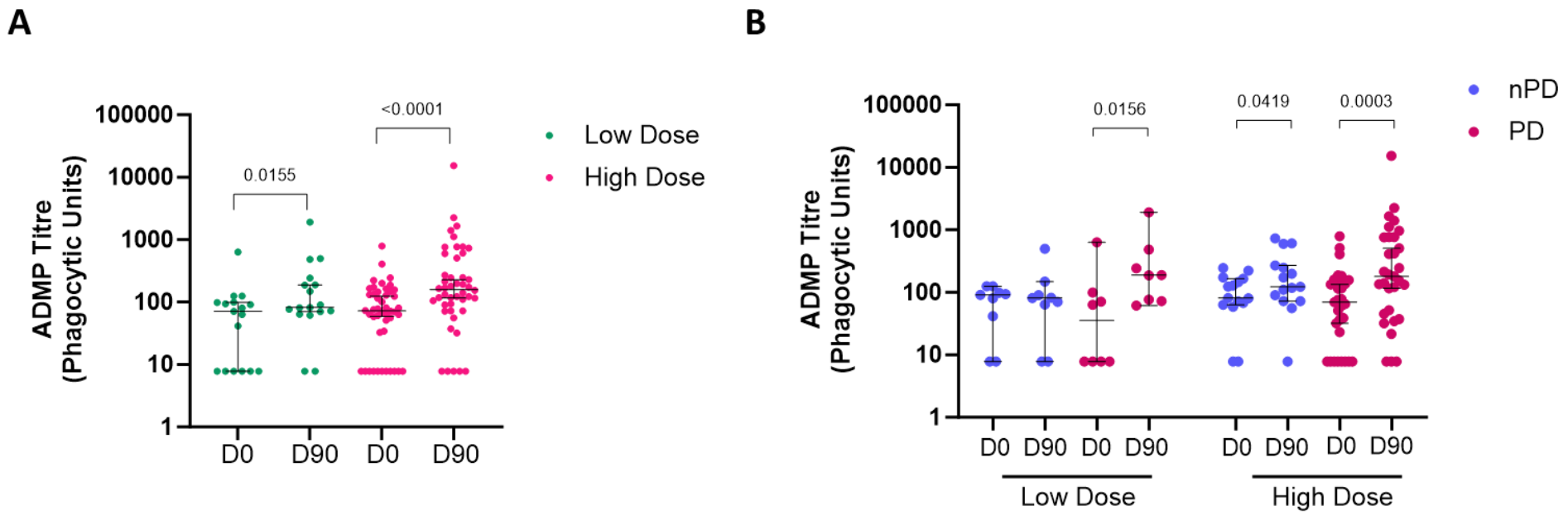


Figure 5-2 Comparison of ADMP titres in individuals challenged with S. Paratyphi at two different doses

ADMP titres measured in healthy individuals at baseline (D0) and 90 days after oral challenge (D90) with *S.* Paratyphi A NVGH308 strain split by dose (A) or dose and diagnosis outcome (B). High dose; 1-5 x 10^3 CFU, low dose; 500-1000 CFU. PD; paratyphoid diagnosed, nPD; not paratyphoid diagnosed. Between timepoint comparisons analysed using Wilcoxon matched−pairs signed rank test, between group comparisons analysed by Mann−Whitney U test. Comparisons were performed between all groups and time points, only those that reached statistical significance are presented.

5.3.1.2. Effect of prior exposure to *S.* Paratyphi A and homologous rechallenge

To investigate the effect of prior exposure on ADMP titres, titres at D0 and D90 were compared between 45 naïve and 12 rechallenged volunteers. A significant increase in ADMP was measured between baseline and D90 in the group which were naïve at the point of challenge only ($p < 0.0001$), in contrast, no significant changes in ADMP titre were detected 90 days after rechallenge (**Figure 5-3** A). There was a significant decrease in ADMP titres at the point of rechallenge compared with D90 of the naïve challenge (D90 naïve vs D0 rechallenge $p = 0.0054$, median interval between challenges was 17 months), indicating waning of phagocytosis promoting antibodies.

There were no differences in titres upon rechallenge relating to the outcome from the initial challenge (**Figure 5-3** B & C). To determine if ADMP antibodies induced by prior exposure were protective upon rechallenge, titres at the point of rechallenge were compared, while there were generally higher ADMP titres in those who developed acute paratyphoid fever after rechallenge, the difference did not reach statistical significance (p 0.1007, **Figure 5-3** D). There was no significant association between ADMP titre at D0 and development of paratyphoid fever after re-exposure when assessed by odds ratio(Odds ratio 1.015, p 0.1713).

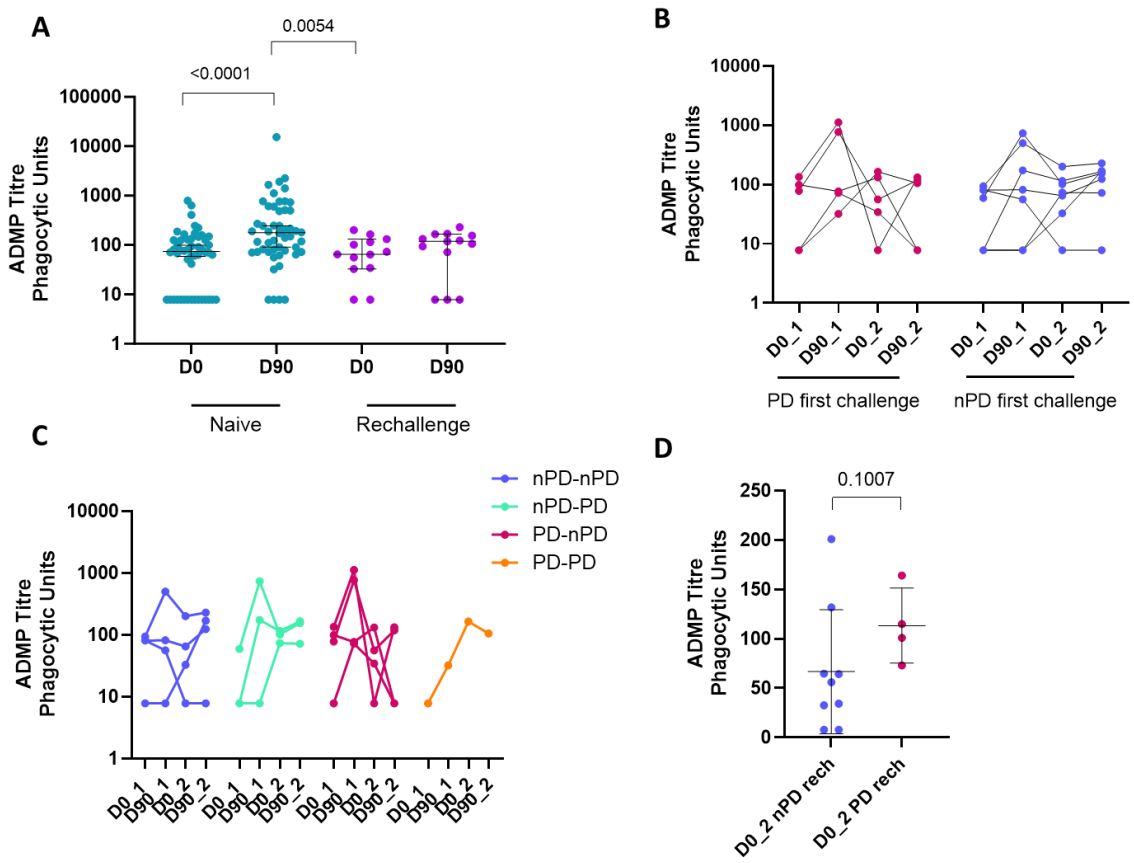


Figure 5-3 Comparison of ADMP titres after homologous rechallenge with S. Paratyphi

ADMP titres at baseline (D0) and 90 days after oral challenge (D90) with S. Paratyphi A NVGH308 strain. (A) Results plotted by challenge iteration. (B) Timeline of ADMP titres in the rechallenge group only, plotted based on initial challenge outcome. (C) Time line of SBA titres plotted based on challenge outcome of both challenges. PD; paratyphoid diagnosed, nPD; not paratyphoid diagnosed. Time point from the first challenge are denoted as D0_1 and D90_1, time points from the second challenge are denoted as D0_2 and D90_2.

Between time point comparisons analysed using Wilcoxon matched−pairs signed rank test, between group comparisons analysed by Mann−Whitney U test. Bars represent median and 95% confidence interval. On panel A all groups were compared, p-values are presented only from comparisons that reached statistical significance. No formal comparisons were performed on panel B or C, due to the low numbers of participants in each group these data are for descriptive purposes only.

5.3.2.Changes in monocyte phagocytosis in participants in an oral *S*. Paratyphi A vaccine and challenge study

ADMP titres were measured at baseline (D-42) and 42 days later (D0) in 27 healthy volunteers enrolled in an oral vaccine study. Participants in this study received 2 doses of live attenuated oral *S*. Paratyphi A vaccine, CVD1902, or sodium bicarbonate placebo alternative, two weeks apart. As this study is ongoing at the point of writing so only a subset of individuals have been analysed, and includes vaccine and placebo participants. ADMP titres 42 days after oral vaccine/placebo were significantly higher compared with baseline, and titres were comparable to those induced by oral challenge with wild type *S*. Paratyphi (**Figure 5-4** A & B). This increase is only significant in the group who do not develop paratyphoid fever after they receive oral challenge (**Figure 5-4** C & D). Despite this, there was no significant association between protection from paratyphoid fever and baseline ADMP titres when calculating the odd's ratio(Odds ratio 0.9992, p 0.8038)

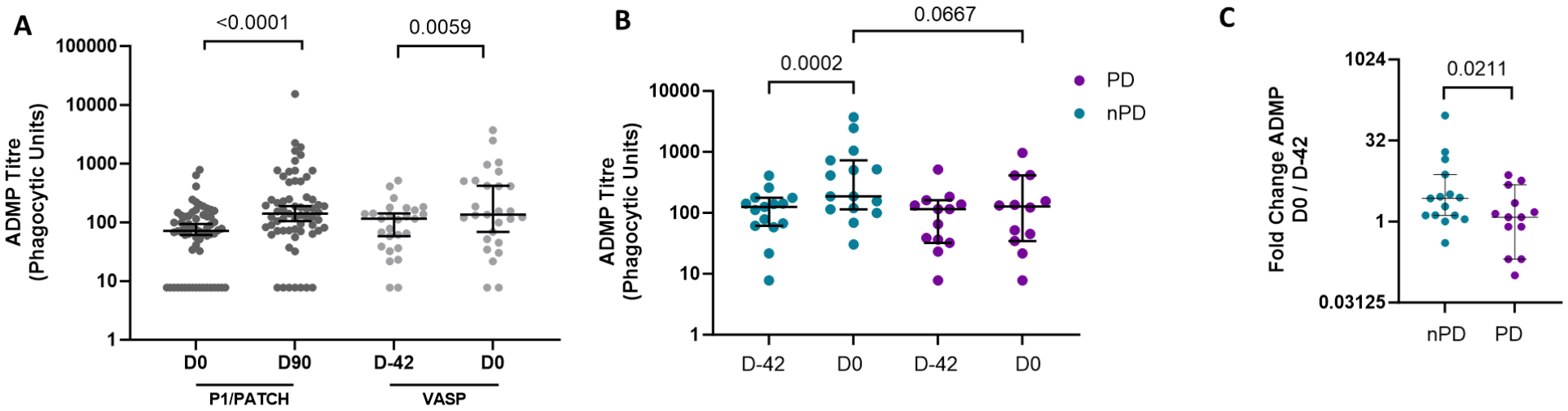


Figure 5-4 Comparison of ADMP titres after oral S. Paratyphi vaccine (VASP study)

ADMP titres at baseline (D-42) and 42 days after vaccination with either an oral S. Paratyphi A vaccine (CVD1902) or placebo (sodium bicarbonate) at the point of challenge with *S*. Paratyphi A NVGH308 strain (D0). (A) all results plotted together, ADMP titres in VASP participants compared with challenge participants from the P1/PATCH studies. (B) ADMP titres in VASP vaccinated/placebo participants separated based on challenge outcome. (C) Fold change in ADMP titre from D0 to D90 split by diagnosis outcome after challenge. PD; paratyphoid diagnosed, nPD; not paratyphoid diagnosed. Between time point comparisons analysed using Wilcoxon matched−pairs signed rank test, between group comparisons analysed by Mann−Whitney. Bars represent median and 95% confidence interval. For panels B and C comparisons were performed between all time points and groups, only those that reach statistical significance are presented on the graph.

5.3.3. Changes in monocyte phagocytosis in participants in a typhoid – paratyphoid bivalent conjugate vaccine study

ADMP titres against *S*. Paratyphi A NVGH308 were quantified at baseline and 29 days after receiving either Typbar TCV vaccine or Sii-PTCV, bivalent *S*. Typhi - Paratyphi A conjugate vaccine, in volunteers enrolled in a phase I clinical trial. ADMP titres were significantly elevated above baseline levels in individuals who received Sii-PTCV ($p < 0.0001$), while titres remained comparable with baseline levels 29 days post Typbar-TCV vaccine (p 0.1757, **Figure 5-5** A). There were significantly elevated titres 29 days post Sii-PTCV compared with 90 days post challenge in the P1/PATCH CHIM studies ($p < 0.0001$). Baseline ADMP titres were also significantly elevated in Indian residents enrolled in the SIIPL Phase I study compared with UK residents enrolled in the P1/PATCH CHIM studies ($p < 0.0001$). Fold change (FC) increase in ADMP titres 29 days after Sii-PTCV were significantly higher than after Typbar-TCV and 90 days after challenge (**Figure 5-5** B, $p < 0.0001$), FC post challenge were also significantly higher compared with post Typbar-TCV (p 0.0094). Spearman's rank correlation coefficient assessment showed a strong, positive correlation between ADMP titres and anti-LPS IgG IgG titres in the SIIPL study population using data collected from both D0 and D29 (**Figure 5-5** C).

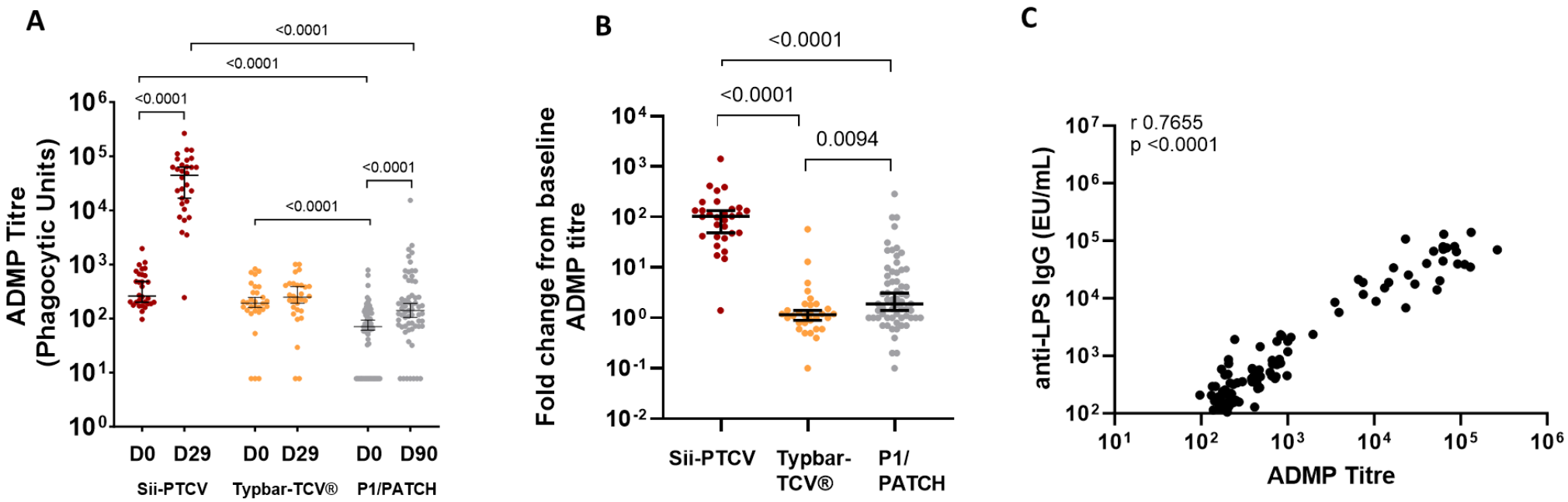


Figure 5-5 Comparison of ADMP titres in S. Paratyphi A challenged individuals or vaccine recipients

ADMP titres against *S*. Paratyphi A challenge strain, NVGH308 measured in individuals who received either Sii-PTCV, Typbar-TCV, or were challenged in the controlled human infection studies (P1/PATCH). (A) ADMP titres measured at baseline, D0, and 29 days after vaccine, or D0 and 90 days post challenge (P1/PATCH). (B) Fold change ADMP from baseline measured at D29 in vaccinees and D90 in challenge study participants. (C) Correlations between ADMP titre and anti-LPS IgG in Sii-PTCV recipients assessed using data collected at D0 and D29. Between time point comparisons analysed using Wilcoxon matched−pairs signed rank test between group comparisons analysed by Mann−Whitney U test. Correlations assessed using a spearman's rank correlation coefficient assessment. Bars represent median and 95% confidence interval. For panel A comparisons were performed between all time points and groups, only those that reached statistical significance are presented.

5.4. Discussion

In this chapter, I have described the application of a standardised ADMP assay to samples collected in 4 *S*. Paratyphi A clinical trials with the aim of investigating changes in monocyte phagocytosis after vaccination and experimental challenge. These analyses represent the first time ADMP responses have been characterised in a paratyphoid human challenge model, and in human volunteers after receiving an oral *S*. Paratyphi vaccine or typhoid-paratyphoid bivalent conjugate vaccine.

5.4.1. ADMP responses to experimental *S*. Paratyphi A challenge in a CHIM

ADMP was quantified pre and post *S*. Paratyphi A challenge in healthy adults enrolled in 2 CHIM studies. Experimental challenge with *S*. Paratyphi A lead to significant increases in ADMP titres, measured after 90 days ($p < 0.0001$). ADMP titres rose significantly irrespective of challenge diagnosis outcome, although the magnitude of the response was greater in individuals who developed paratyphoid fever after challenge (as defined by: bacteraemia and/or fever ≥38°C for ≥12 hours, nPD; p 0.0128, PD; $p < 0.0001$). This indicates that exposure to *S*. Paratyphi A in a challenge setting is enough to stimulate the changes in the phagocytic capacity of antibodies, in contrast with binding antibodies measured by Gibani *et al* and bactericidal antibodies (chapter 4), which only show significant changes after challenge in individuals who developed acute paratyphoid fever [134].

Differences in these observations could be attributable to multiple biological and methodological reasons. Historically, measurement of *S*. Paratyphi specific binding antibodies is primarily through IgG and IgA specific to the O antigen, or flagella (O2, Ha respectively), however the ADMP assay uses whole bacteria. Therefore, the ADMP might be a more sensitive technique because it can detect changes in functional antibodies targeted against multiple polysaccharide and protein antigens, which are not detected in single antigen specific tests. It could also be the case that the threshold for detection of ADMP antibodies is lower than bactericidal antibodies due to other biophysical properties such as antibody affinity and avidity. Observations from antibody quantification and SBA studies have lead us to believe that exposure alone is not a potent stimulus of the humoral immune

system, however the findings described here support the theory that a single exposure, or subclinical infection is enough to stimulate functional changes in the immune system.

Another theory is that induction of SBA and ADMP occur at different stages of infection. Perhaps ADMP is induced at the intestinal epithelium level, and therefore exposure alone is enough to stimulate ADMP. Whereas SBA could be induced during the blood stage phase of the infection and therefore systemic infection and is a prerequisite for stimulating SBA.

These discrepancies could also be explained by the limitations of clinical diagnosis within the model. While blood culture is a widely used diagnostic technique, even in experimental settings it is thought the sensitivity may only reach 80%, within the challenge model there are a small number of individuals that could have undetectable infection [198]. It is also recognised that some individuals would meet the diagnostic criteria if the challenge follow-up was not stopped at day 14 by the commencement of antibiotics. ADMP changes in the group who did not meet the diagnostic threshold appear to be primarily driven by a small number of individuals (n=4), who could potentially belong to either of these two groups with undetectable or early infection.

ADMP titres in in challenged individuals significantly correlated with LPS IgG titres, and weakly correlated with LPS specific IgA titres (IgG; p <0.0001, r 0.6822, IgA; p 0.0010, r 0.2903). Both IgG and IgA are capable of enhancing phagocytosis through binding FcγRI-III and FcαRI expressed on the surface of monocytes (expression on THP-1 cells shown in section **2.3.1**, **Figure 2-2**B). While the antigen specificity of serum IgA is known to reflect that of secretory IgA, produced by stimulation of intestinal B cells, the function of serum IgA is not fully understood and IgA mediated activity exists on a spectrum between anti- (IgA1) and pro-inflammatory (IgA2) depending on the monomer involved[184,199,200]. Which could explain why there is a significant but weak correlation between phagocytic activity and IgA titre.

Challenge with low or high dose *S.* Paratyphi A was associated with a significant increase in titre after 90 days (low dose; p 0.0155, high dose; p <0.0001). A more significant increase in recipients of the

higher dose indicates a positive relationship between exposure dose and induction of phagocytic antibodies. Further supporting this, changes in ADMP titre in the low dose group were dependent on diagnosis outcome. In the low dose group, ADMP titre at D90 compared with baseline titres were only significant in individuals who met the diagnosis threshold (PD; p 0.0156, nPD; p 0.8438). Whereas in the high dose group, ADMP titres were significant in individuals irrespective of diagnosis outcome (PD; p 0.0003, nPD; p 0.0419). Indicating that at low doses, without breakthrough infection there is little to no stimulation of phagocytic antibodies. How accurately this reflects the kinetics of functional immunity in natural infection is unknown due to difficulties in estimating exposure dose because of limitations in environmental sampling sensitivity. However, it is likely that natural exposure dose has a range of CFUs and these observations suggest a dose dependent stimulation of the immune response.

Homologous rechallenge was not associated with any significant changes in phagocytic activity. There were no associations between ADMP and paratyphoid fever development at the point of rechallenge. By the point of rechallenge ADMP titres had returned to baseline levels (**Figure 5-3** A-D). This indicates that while homologous rechallenge was associated with a reduced risk of developing acute paratyphoid fever, there were no measurable correlations in ADMP associated with this outcome. This somewhat contradicts the observation described above that a single exposure event can induce functional changes.

Prior challenge with *S*. Paratyphi A mimics the role of an oral paratyphoid vaccine. A desirable outcome of vaccination is long lasting protection, currently we don't know which antibody properties correlate with protection from paratyphoid, if any, but long lasting functional responses would be a promising indicator of lasting immunity. However, at challenge doses of up to 5 x 10^4 CFU ADMP at 17 months (rechallenge D0) are comparable with baseline titres prior to first challenge. The duration of responses induced by oral vaccine, which have much higher doses, is unknown.

5.4.2. ADMP responses to oral *S.* Paratyphi A vaccine, CVD1902, and challenge

ADMP SBA titres were measured in 27 volunteers enrolled in the VASP trial where participants received either two doses of an oral live attenuated *S.* Paratyphi A vaccine CVD1902, or sodium bicarbonate placebo. The primary endpoint of the study was not complete at the time of writing this thesis and data were analysed while still blinded. Significant increases in ADMP titre were measured 42 days post vaccine (p 0.0059), even with the inclusion of the placebo recipients. Changes in ADMP titres were only significant in those who did not subsequently develop acute disease post challenge (PD; p 0.5186, nPD; p 0.0004). Interestingly, titres at the point of challenge were generally higher in individuals that did not develop acute paratyphoid fever compared with those who did (GMT; nPD; 295.9 phagocytic units, PD; 97.95 Phagocytic units, p 0.0667). Although this did not reach statistical significance, possibly due to the inclusion of placebo recipients, it does indicate a potential role for ADMP in protection from infection. It will be exciting to see if the significance of this comparison changes with the addition of more study samples, and study vaccine status unblinding.

Titres measured 42 days after vaccine were comparable to those measured 90 days after challenge (D90 post challenge median; 135.6 phagocytic units, D0 post vaccine median; 141.2, p 0.8886) despite the 5 log difference in vaccine dose (2 x 10^{10} CFU) compared with challenge (1-5 x 10^{4} CFU). This is an important comparison since we know that phagocytic antibodies induced by oral challenge do not remain elevated beyond 17 months, and did not associate with protection upon *S.* Paratyphi A rechallenge. This observation is important since the desired outcome from vaccination would be long-term protection against paratyphoid fever. Furthermore this observation somewhat contradicts the dose dependant response theory, or at least suggests that there is perhaps an upper limit to how much ADMP can be stimulated through exposure to whole bacteria.

Antibody enhancement of monocyte phagocytsis could provide protection at the intestinal epithelium destroying bacteria before they can leave the gut compartment or it could be acting at a later stage in the infection lifecycle, killing bacteria in the blood, preventing establishment of

systemic infection. In a study investigating cross-reactive humoral responses to Ty21a, *S*. Paratyphi A specific antibody secreting cells measured 7 days after vaccination were predominantly IgA and IgM and expressed gut homing marker α4β7[201]. In another study, cytotoxic activity of donor derived peripheral blood monocytes towards *S*. Paratyphi A measured 15 days after Ty21a was strongly associated with IgA, but not IgG, indicating strong response at the intestinal level [202]. While it's believed that THP-1 function correlates with activity of human derived macrophages and monocytes, gut resident macrophages are unique compared with other tissue resident macrophages and are known to be hyporesponsive as a mechanism to build tolerance to the microbiome, and prevent chronic gut inflammation [164,200,203].

The inclusion of placebo participants (prior to unblinding) dampens the statistical power of this analysis and therefore the significance of these comparisons is likely an underestimate. While this interim analysis provides exciting insight into a potential mechanism of protection of oral paratyphoid vaccines, these results should be interpreted with caution until the data can be analysed in an unblinded way.

5.4.3. ADMP responses to a novel *S*. Typhi – Paratyphi bivalent conjugate vaccine

ADMP titres were quantified in 60 volunteers enrolled in a phase I study evaluating the safety and immunogenicity of an *S*. Typhi – Paratyphi A conjugate vaccine. A single dose of bivalent vaccine elicited a significant increase in monocyte phagocytosis (D0 – D29; $p < 0.0001$). When compared with samples collected from the challenge studies, the bivalent vaccine was a more potent ADMP stimulus (D29 post vaccine vs D90 post challenge; $p < 0.0001$, FC from baseline vaccine vs challenge; $p < 0.0001$). This indicates that vaccine induced LPS antibodies are functional against *S*. Paratyphi A bacteria, although since this was a phase I study no correlations with disease incidence or severity could be performed. Future studies of this vaccine will assess the relationship between ADMP and

protection from infection, given the magnitude of response elicited by Sii-PTCV, this will be an exciting analysis.

Baseline titres measured in the Indian population as part of the bivalent vaccine trial were significantly higher than those measured in UK residents ($p < 0.0001$). Both studies recruited healthy adults, this observation likely reflects boosting of functional antibodies by exposure in individuals living in an enteric fever endemic area, compared with the baseline functional activity in naïve individuals. This further supports the theory that multiple exposures can induce functional antibody responses which in turn, may play a role in the age related incidence decline [135].

Both populations show a range of titres at baseline. Variations in baseline activity could be attributable to the presence of antibodies from prior exposure, the presence of cross reactive antibodies, or non-specific monocyte activation via PAMPs [204].

5.5. Conclusion

The data presented in this chapter describe the first evaluation of monocyte phagocytosis in experimental paratyphoid infection and in response to two unique *S*. Paratyphi A vaccinations. The observations reported here show that oral challenge, oral vaccine and parental vaccine induce significant increases in phagocytic antibodies. Promisingly, post immunisation ADMP titres measured in the oral vaccine study were generally higher in individuals who were protected from developing disease after *S*. Paratyphi challenge. It will be interesting to continue the analysis after the VASP study has been completed. Furthermore, a single dose of intramuscular bivalent vaccine induced high levels of ADMP, future efficacy studies are planned for the bivalent vaccine utilising the challenge model, it will be interesting to assess the relationship between ADMP and disease outcome.

Chapter 6. Changes in antibody dependant neutrophil phagocytosis after *S*. Paratyphi A exposure or vaccination

6.1. Introduction

Neutrophils are an abundant white blood cell with a critical role in the clearance of bacterial pathogens. They can be non-specifically activated via binding of pathogen associated molecular pattern (PAMPs) to pattern recognition receptors (PRRs, e.g. LPS to TLR4), or they can respond to specific antigens via antibody binding to FcRs. Neutrophils have a plethora of downstream functions to help clearance of bacteria and manage infection, including phagocytosis, production of reactive oxygen species, and the release of potent proteases, and neutrophil extracellular traps [205].

Similar to monocyte phagocytosis, FcR engagement with the Fc fraction of an antibody activates intracellular processes causing actin rearrangement and uptake of the opsonised pathogen. Within the neutrophil, *Salmonella* reside in the SCV, the process of phagocytosis triggers production of reactive oxygen species (ROS) such as, hydrogen peroxide and superoxide radicals. Further bactericidal activity is elicited by SCV fusion with cytoplasmic granules containing proteases, thus bacteria are destroyed by a concerted effort between ROS and proteolytic and degradative enzymes [206].

Expression of a polysaccharide capsule is a well-known virulence mechanism for evading neutrophil mediated killing, expression of the capsule masks binding to other surface molecules and prevents bacterial recognition by PAMP – PRR binding, complement binding, opsonisation, and subsequent phagocytosis [206]. Vi expression by *S*. Typhi, and expression of very long chain LPS by *S*. Paratyphi A prevents antibody and complement binding to the bacterial surface and promotes survival for

prolonged periods of time in the presence of human serum and within neutrophils [92,162]. The lack of neutrophil recruitment and infiltration in the gut in both *S*. Typhi and *S*. Paratyphi infection shows that preventing neutrophil activation is an important virulence step in systemic *Salmonella* infection. Despite this potent virulence mechanism, antibodies against Vi are capable of mediating protection against *S*. Typhi, as evidence by the moderate protection afforded by T- cell independent Vi CPS subunit vaccines [148,207]. Vaccine induced Vi antibodies are capable of enhancing uptake and ROS production by neutrophils, which correlates with vaccine mediated protection in a human challenge model [140,208].

Antibody dependant neutrophil mediated protection is an important part of the immune response to other encapsulated bacteria. Pneumococcal antibodies induced by conjugate vaccines protect the host by opsonising bacteria and enhancing neutrophil phagocytosis, while the protective threshold differs by specific serotype, opsonophagocytosis data are used to support approval of new pneumococcal vaccines [144,209]. Polyfunctional antibody response induced by meningococcal conjugate vaccine has shown to be important in preventing meningococcal disease against serogroups A, C W and Y, although the regulatory correlate is SBA [210].

The role of *S*. Paratyphi A specific neutrophil phagocytosis and it's relevant contribution to protection from paratyphoid fever has not yet been evaluated.

The aims of this chapter are:

1. Does neutrophil phagocytosis activity against *S*. Paratyphi A change in response to *S*. Paratyphi A challenge and homologous rechallenge? Does baseline ADNP activity at either challenge correlate with protection from paratyphoid infection?
2. Does live attenuated oral *S*. Paratyphi A vaccine (CVD1902) induce changes in ADNP? Is there a correlation between CVD1902 vaccine induced ADNP titres and diagnostic outcome in an *S*. Paratyphi CHIM?
3. Does novel *S*. Typhi – Paratyphi A bivalent conjugate vaccine induce changes in ADNP?

6.2. Methods

The samples included in this analysis are detailed in section **2.1, Table 2-1**. Samples were included from the P1 and PATCH oral challenge studies, the VASP oral vaccine and challenge study, and the phase 1 *S*. Typhi – Paratyphi bivalent study.

The methods for the ADNP assay are described in detail in section **2.7**.

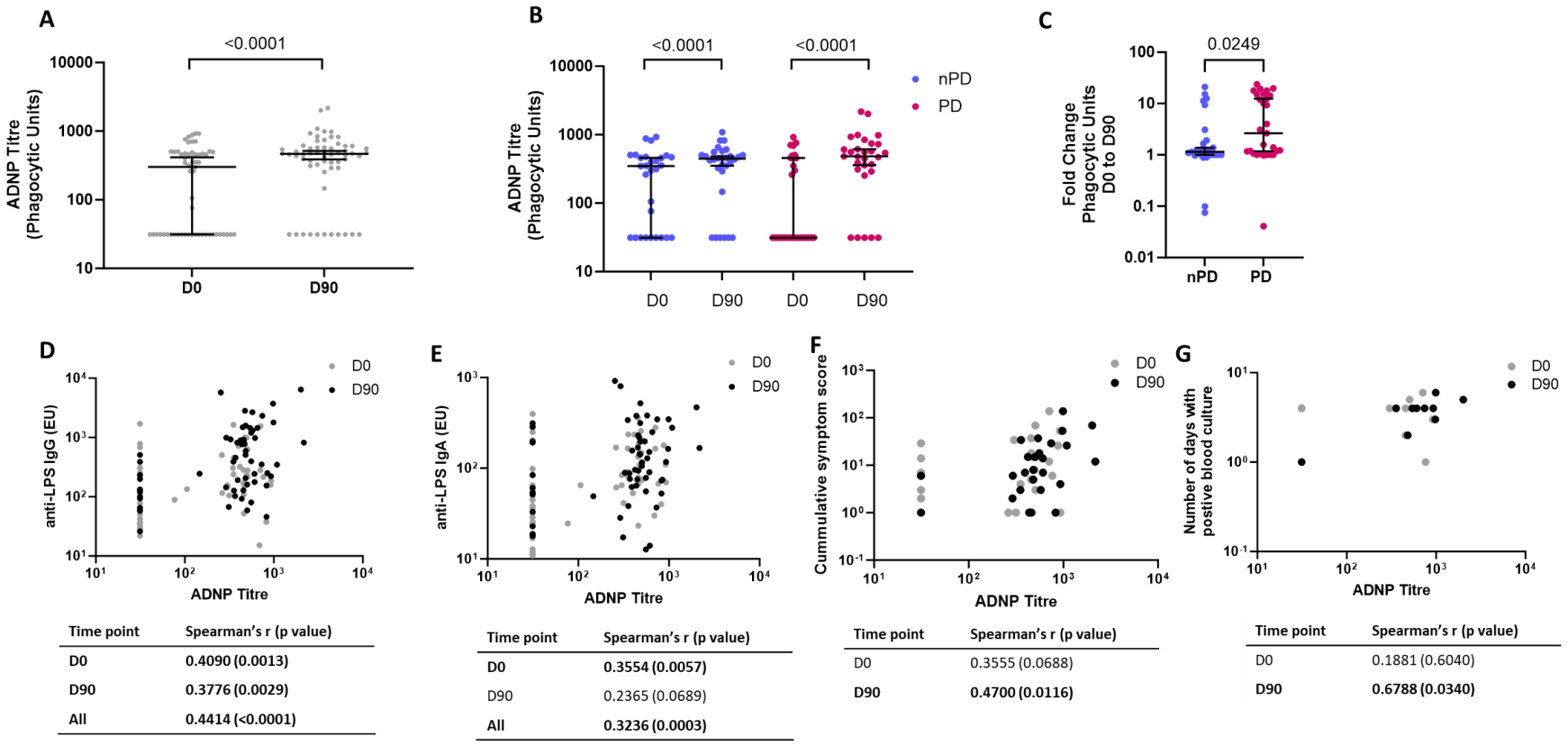
ADNP titres are correlated with data obtained from other assays, the methods for measuring each immune marker are noted in chapter **2.8** (binding antibodies quantified by ELISA).

6.3. Results

6.3.1. Changes in neutrophil phagocytosis in participant in an S. Paratyphi A challenge model

To evaluate the impact of oral challenge on neutrophil phagocytosis, phagocytic uptake of *S*. Paratyphi was measured in healthy volunteers at baseline (D0) and 90 days post challenge (D90) in 62 individuals enrolled in the P1 and PATCH paratyphoid CHIM studies. Samples were selected based on availability, all available samples where participants had consented were included in the analysis. Ninety days after challenge, there was a significant increase in phagocytosis titres (**Figure 6-1**A, p <0.0001). Significant increase in ADNP titres after challenge was independent of challenge outcome (**Figure 6-1** B, nPD; <0.0001, PD; <0.0001, Odds ratio 1.000, p 0.5756). When comparing fold change data (baseline to D90) there was a significantly greater FC in ADNP titres in individuals who developed acute paratyphoid fever after challenge (**Figure 6-1** C, p 0.0249). Furthermore, both symptom severity score and the number of positive blood culture days positively correlated with (D90) ADNP titre, suggesting that individuals that experienced more severe disease were more likely to develop a neutrophil activating serological response (**Figure 6-1** F&G). There were significant correlations between ADNP and LPS specific IgG and IgA, suggesting that both isotypes can contribute to ADNP (**Figure 6-1**

D&E). There was no significant difference in D0 ADNP titres in those who developed infection and those who did not (**Figure 6-1** B, Odds ratio 0.994, p 0.4304).



Time point	Spearman's r (p value)
D0	**0.4090 (0.0013)**
D90	**0.3776 (0.0029)**
All	**0.4414 (<0.0001)**

Time point	Spearman's r (p value)
D0	**0.3554 (0.0057)**
D90	0.2365 (0.0689)
All	**0.3236 (0.0003)**

Time point	Spearman's r (p value)
D0	0.3555 (0.0688)
D90	**0.4700 (0.0116)**

Time point	Spearman's r (p value)
D0	0.1881 (0.6040)
D90	**0.6788 (0.0340)**

Figure 6-1 ADNP titres against S. Paratyphi A in a human challenge model

ADNP titres at baseline (D0) and 90 days (D90) after challenge with *S*. Paratyphi NVGH308 strain. (A) All participants grouped together. (B) split by diagnosis outcome. (C) Fold change in ADNP titre from D0 to D90 split by diagnosis outcome after challenge. (D - G) Correlation between ADNP titre and LPS specific IgG, (D) and IgA titres (E), cumulative symptom score (F), number of days of bacteraemia (G). Correlation assessed using non-parametric spearman's rank correlation coefficient, tabulated underneath each graph. PD; paratyphoid diagnosed, nPD; not paratyphoid diagnosed.

Lines represent median and 95% confidence intervals. Between time point comparisons analysed using Wilcoxon matched−pairs signed rank test, between group comparisons analysed by Mann−Whitney. For panel B comparisons were performed between all time points and groups, only those that reached statistical significance are presented.

6.3.1.1. Changes in ADNP in an *S*. Paratyphi A challenge dose finding study

The effect of the *S*. Paratyphi A oral challenge exposure dose on ADNP titres was assessed by comparing titres between recipients of the low dose (500-1000 CFU) and the high dose (1-5 $x10^3$ CFU). ADNP titres were significantly elevated 90 days after challenge in both dose groups (**Figure 6-2** A low dose; p 0.0186, high dose; p<0.0001). Although there were no significant differences in the titres between dose groups at D90.

When assessed by challenge outcome, ADNP titres were significantly elevated 90 days after challenge irrespective of challenge outcome in the high dose group. However, the observed increase was only significant in individuals who developed acute paratyphoid in the low dose group similar to the dose response seen for the ADMP in chapter 5 (**Figure 6-2** B. Low dose; nPD p 0.0155, PD 0.0312 p high dose; nPD p 0.0094, PD p 0.0010).

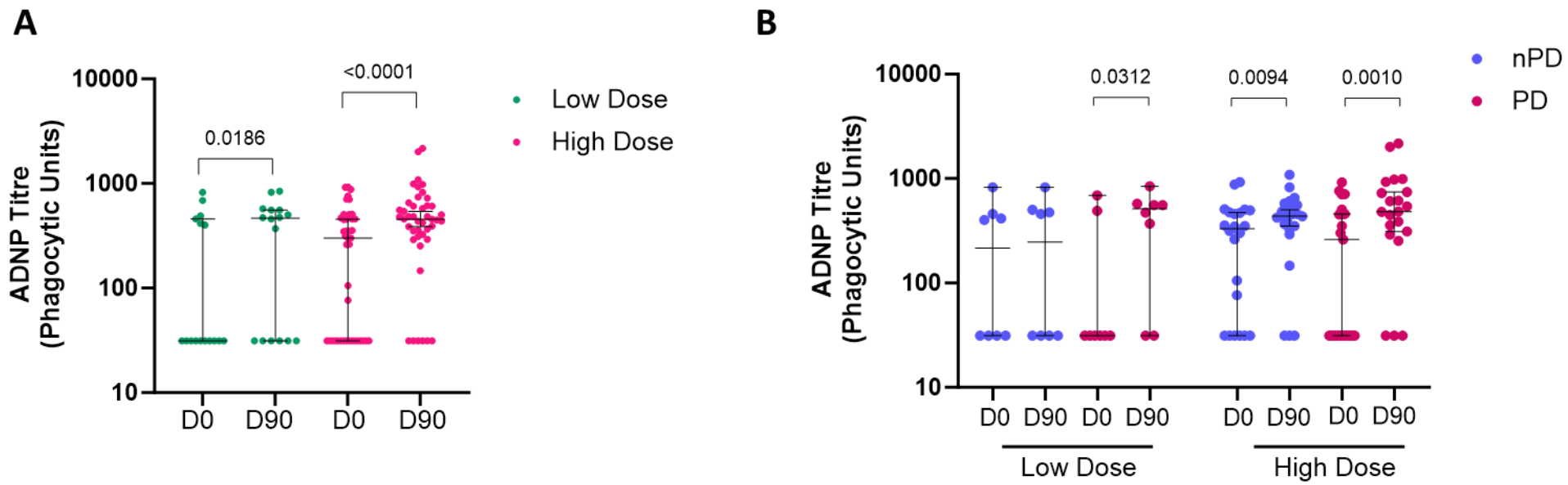


Figure 6-2 Comparison of ADNP titres in individuals challenged with S. Paratyphi at two different doses.

ADNP titres at baseline (D0) and 90 days after oral challenge (D90) with *S*. Paratyphi A NVGH308 strain, split by dose received (A) or dose and diagnosis outcome (B). High dose; 1-5 x 103 CFU, low dose; 500-1000 CFU. PD; paratyphoid diagnosed, nPD; not paratyphoid diagnosed. Between timepoint comparisons analysed using Wilcoxon matched−pairs signed rank test, between group comparisons analysed by Mann−Whitney. Comparisons were performed between all time points and groups, only those that reached statistical significance are presented.

6.3.1.2. Effect of prior exposure to *S*. Paratyphi A and homologous rechallenge

To investigate the effect of prior exposure on ADNP titres, titres at D0 and D90 were compared between 45 naïve and 12 rechallenged volunteers enrolled in the P1 and PATCH *S*. Paratyphi A CHIM studies. Samples were selected based on availability; all available samples where participants had consented were included in the analysis. ADNP titres were significantly elevated at D90 compared with baseline in those who were naïve at the point of challenge only (**Figure 6-3** A, p <0.0001), in contrast, no significant changes in ADNP titre were detected 90 days after rechallenge (**Figure 6-3** A). ADNP titres had significantly waned by the point of rechallenge compared with D90 of the naïve challenge (median interval between challenges was 17 months D90 naïve vs D0 rechallenge p <0.0001). There were no differences in titres upon rechallenge relating to the outcome from the initial challenge (**Figure 6-3** B & C). To determine if ADNP pre-existing antibodies had a protective effect when rechallenged, titres at the point of rechallenge were compared. While there were generally higher ADNP titres in those who developed acute paratyphoid fever after rechallenge, the difference did not reach statistical significance (**Figure 6-3** D, p 0.6667, Odds ratio 1.002, p 0.4119).

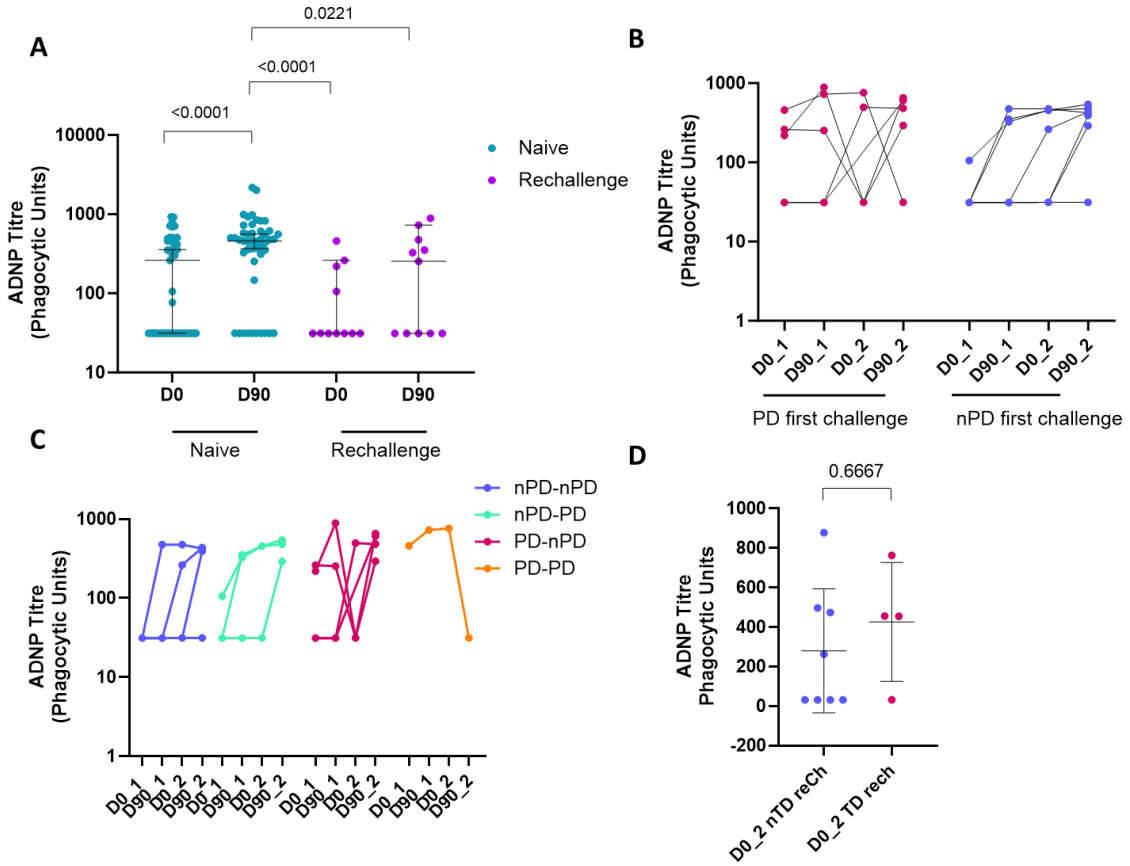


Figure 6-3 Comparison of ADNP titres after homologous rechallenge with S. Paratyphi.

ADNP titres at baseline (D0) and 90 days after oral challenge (D90) with S. Paratyphi A NVGH308 strain. (A) Results plotted by challenge iteration. (B) Timeline of ADMP titres in the rechallenge group only, plotted based on initial challenge outcome. (C) ADNP titres in the rechallenge group only plotted based on challenge outcome of both challenges. PD; paratyphoid diagnosed, nPD; not paratyphoid diagnosed. Time point from the first challenge are denoted as D0_1 and D90_1, time points from the second challenge are denoted as D0_2 and D90_2. Between time point comparisons analysed using Wilcoxon matched−pairs signed rank test, between group comparisons analysed by Mann−Whitney. Bars represent median and 95% confidence interval.

On panel A all groups were compared, p-values are presented only from comparisons that reached statistical significance. No formal comparisons were performed on panel B or C, due to the low numbers of participants in each group these data are for descriptive purposes only.

6.3.2. Changes in neutrophil phagocytosis in participants in an oral *S.* Paratyphi A vaccine and challenge study

ADNP titres where measured at baseline (D-42) and 42 days later (D0) in 27 healthy volunteers enrolled in an oral vaccine study. Participants received either 2 doses of live attenuated oral *S.* Paratyphi A vaccine, CVD1902, or two doses of sodium bicarbonate placebo alternative, with a two week interval. As this study is ongoing at the point of writing so only a subset of individuals have been analysed, and includes vaccine and placebo participants. There was no significant increase in ADNP titre between D-42 and D0 when everyone is grouped together (**Figure 6-4** A, p 0.1511). Changes in ADNP titre after live oral vaccine were compared with those observed after oral challenge, significant changes induced after oral challenge were not mirrored by live oral vaccine, however the post-vaccine titres were not significantly different to those measured post challenge (**Figure 6-4** B, p 0.2073). To determine the impact of ADNP titre on challenge outcome, ADNP data were analysed based group by diagnosis outcome. There was no significant increase in ADNP post vaccine/placebo compared with baseline in those who developed acute paratyphoid fever. There was a significant increase in post-vaccine/placebo titre in individuals who were protected from disease (**Figure 6-4** C, p 0.0479, Odd's ratio 0.9989, p 0.5028), however, there was no significant difference in ADNP activity at the point of challenge when comparing between the diagnosis groups (p 0.1017). Fold change from baseline to pre-challenge was generally higher in participants who did not develop paratyphoid fever, the comparison did not meet statistical significance (**Figure 6-4** D, p 0.1063).

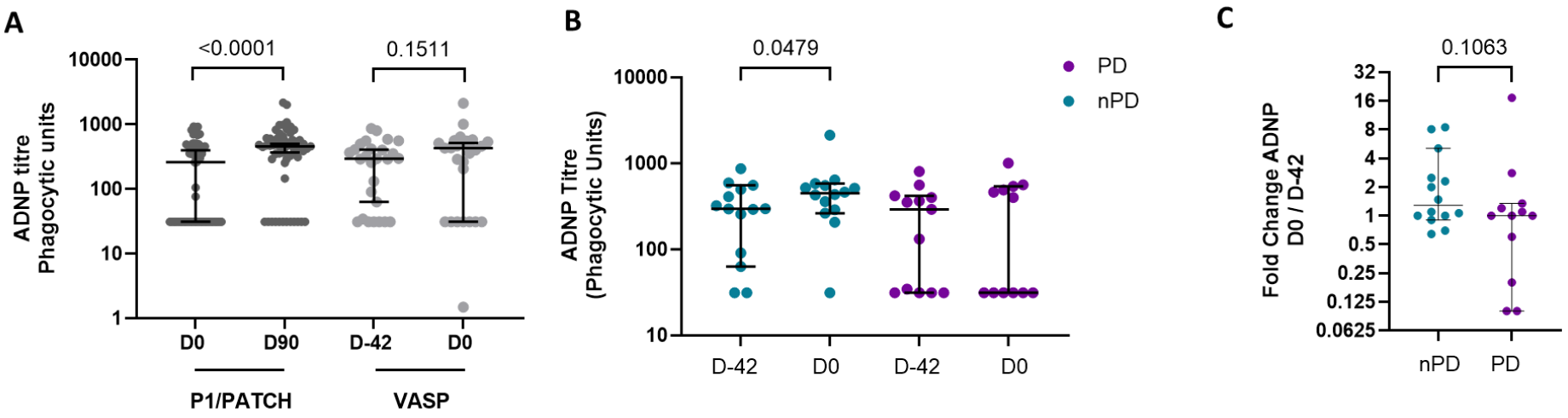


Figure 6-4 Comparison of ADNP titres after oral S. Paratyphi vaccine (VASP study)

ADNP titres at baseline (D-42) and 42 days after vaccination with either an oral S. Paratyphi A vaccine (CVD1902) or placebo (sodium bicarbonate) at the point of challenge with *S.* Paratyphi A NVGH308 strain (D0). (A) all results plotted together, comparison of ADNP titres in vaccinated (VASP) participants with challenge participants (P1/PATCH). (B) ADNP titres in combined blinded VASP participants separated based on challenge outcome. (C) Fold change in ADNP titre from D0 to D90 split by diagnosis outcome after challenge. PD; paratyphoid diagnosed, nPD; not paratyphoid diagnosed. Bars represent median and 95% confidence interval. On panels A and B all groups were compared, p-values are presented only from the baseline to post-vaccine or post-challenge or from comparisons that reached statistical significance.

6.3.3.Changes in neutrophil phagocytosis in participants in a typhoid – paratyphoid bivalent conjugate vaccine study

ADNP titres against *S*. Paratyphi A, NVGH308 strain were quantified at baseline and 29 days after receiving either Typbar-TCV vaccine or Sii-PTCV, bivalent *S*. Typhi - Paratyphi A conjugate vaccine, in participants enrolled in a phase I clinical trial. ADNP titres were significantly elevated above baseline levels in individuals who received Sii-PTCV (**Figure 6-5** A, p 0.0040), while titres remained comparable with baseline levels 29 days post Typbar-TCV vaccine (**Figure 6-5** A, p 0.3816). ADNP titres were significantly elevated titres 29 days post Sii-PTCV compared with 90 days post challenge in a CHIM study (**Figure 6-5** A, p 0.0010). Baseline ADNP titres were also significantly elevated in Indian residents enrolled in the SIIPL Phase I study compared with UK residents enrolled in the P1/PATCH CHIM studies (**Figure 6-5** A, p 0.0421). Fold change (FC) increases in ADNP titres 29 days after Sii-PTCV and 90 days after challenge were significantly higher than after Typbar-TCV (**Figure 6-5** B, Sii-PTCV; p 0.0263, P1/PATCH; p 0.0261). Spearman's rank correlation coefficient assessment showed a strong, positive correlation between ADNP titres and anti-LPS IgG titres in the SIIPL study population using data collected from both D0 and D29 (**Figure 6-5** C).

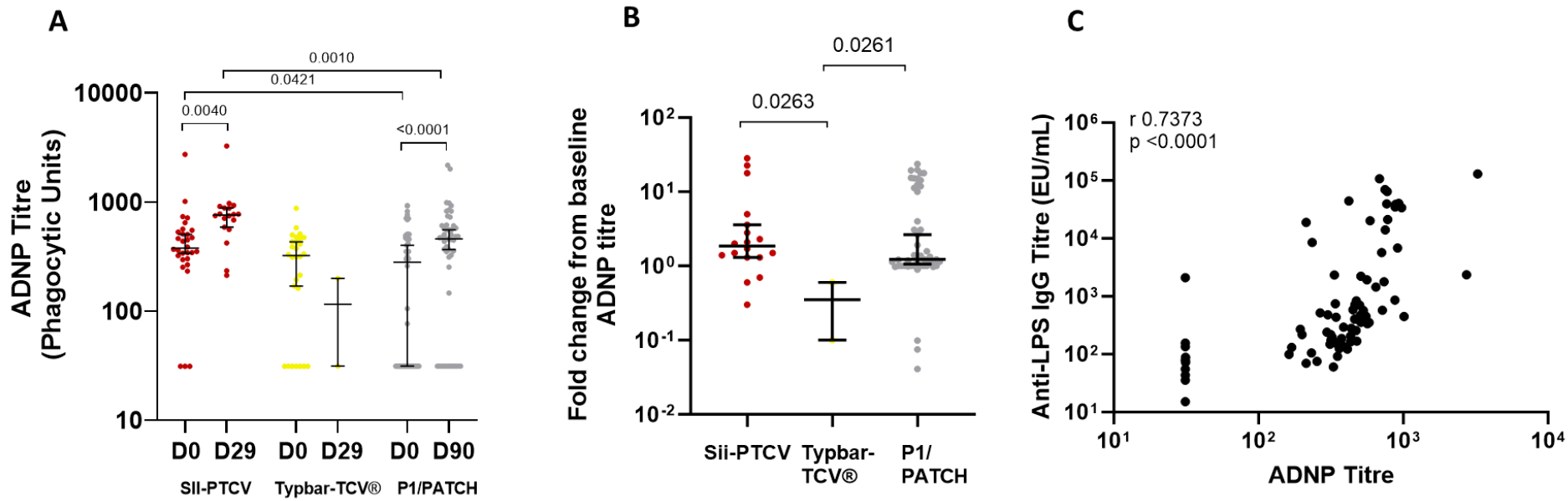


Figure 6-5 Comparison of ADNP titres in S. Paratyphi A challenged individuals or vaccine recipients

ADNP titres against *S*. Paratyphi A challenge strain, NVGH308 measured in individuals who received either Sii-PTCV, Typbar-TCV, or were challenged in the controlled human infection studies (P1/PATCH). (A) ADNP titres measured at baseline, D0, and 29 days after vaccine, or D0 and 90 days post challenge (P1/PATCH). (B) Fold change ADNP from baseline measured at D29 in vaccinees and D90 in challenge study participants. (C) Correlations between ADNP titre and anti-LPS IgG in Sii-PTCV assessed with D0 and D29 combined data. Between timepoint comparisons analysed using Wilcoxon matched−pairs signed rank test between group comparisons analysed by Mann−Whitney U test. Correlations assessed using a spearman's rank correlation coefficient assessment. Bars represent median and 95% confidence interval. On panels A and B all groups were compared, p-values are presented only from comparisons that reached statistical significance

6.4. Discussion

6.4.1.ADNP responses to experimental *S*. Paratyphi A challenge in a CHIM

Experimental challenge with *S*. Paratyphi A leads to significant increases in ADNP titres, measured after 90 days (p <0.0001). This significant increase was observed irrespective the development of clinical disease post challenge, although the magnitude of the response was greater in individuals who developed paratyphoid fever after challenge (as defined by: bacteraemia and/or fever ≥38°C for ≥12 hours, FC D0-D90, nPD Vs PD; p 0.0249). This indicates that exposure to *S*. Paratyphi A in a challenge setting is enough to stimulate the changes in the phagocytic capacity of antibodies, similar to ADMP responses but in contrast to observations of binding antibodies and bactericidal antibodies (chapter 4), where changes post challenge are only significant in the group who developed acute paratyphoid fever [134].

The lack of neutrophil infiltration in the gut in enteric fever coupled with the observation of equal opsonophagocytosis stimulation irrespective of disease status, suggests there is not a huge role for neutrophils or ADNP in mitigating disease at the early, acute phase of infection in paratyphoid naïve adults. This is supported by the fact that prechallenge, D0 ADNP titres do not correlate with symptom severity (data not shown: r 0.3493, p 0.0741). Conversely, opsonophagocytosis measured in human volunteers in a *Shigella* challenge model showed that higher titres at the point of challenge correlated with reduced disease index [178,211].

ADNP titres in challenged individuals significantly correlated with LPS IgG titres, and weakly correlated with LPS specific IgA titres (IgG; p <0.0001, r 0.4181, IgA; p 0.0244, r 0.2062). Both IgG and IgA enhance phagocytosis through binding Fc receptors expressed on the surface of neutrophils (expression on dHL-60 cells shown in section **2.3.2**, **Figure 2-3**B).

ADNP titres increased significantly when participants were challenged with either low or high dose of *S*. Paratyphi A, however in individuals who did not develop clinical disease after low dose exposure, differences in pre and post-challenge titres were not statistically significant (p 0.4375).

This suggests that the ADNP response is influenced by exposure dose and is somewhat dependant on breakthrough infection. Low dose exposure that does not result in systemic infection, does not stimulate changes in ADNP (i.e. bacteria just pass through the digestive system), whereas exposure to a high enough dose even in the absence of acute disease, can stimulate ADNP. Moreover, individuals with a higher symptom severity score have higher ADNP titres at D90 (r 0.4238, p 0.0246). Likewise, in a human challenge model of shigellosis, O antigen specific ADNP titres collected 28 days post challenge correlated with disease severity [179]. Collectively these data suggest that a stronger infection drives functional immunity, although how this impacts exposure driven immunity in endemic setting is not well understood.

In slight contradiction to the above observation of a single exposure being enough to stimulate ADNP titres, homologous rechallenge with *S*. Paratyphi A was not associated with any boosting of ADNP titres (p 0.0625). Hyporesponsiveness (immune tolerance) is a characteristic of repeated exposure to T cell independent, polysaccharide antigens [212]. Expression of LPS on the bacterial surface masks availability of other antigens and therefore the lack of immune boosting might be exposure leads to a T cell independent response against the dominant surface polysaccharide [92]. There was significant waning of phagocytic antibodies after 17 months, at the point of rechallenge (p <0.0001), and higher rechallenge baseline titres did not protect against development of paratyphoid fever (Odds ratio 0.9994, p 0.4304). This is reminiscent of responses to repeated *Shigella* exposure which suggested that O antigen specific functional immunity is limited and short lived [213]. These findings also raise the possibility that the reduced risk of infection after rechallenge may not be due to an adaptive immune response but could be mediated by other factors such as epigenetic changes or effector T cell mediated responses.

6.4.2.ADNP responses to an oral *S*. Paratyphi A vaccine, CVD1902, and *S*. Paratyphi A challenge

ADNP SBA titres were measured in 27 volunteers enrolled in the VASP trial where participants received either two doses of an oral live attenuated *S*. Paratyphi A vaccine CVD1902, or sodium bicarbonate placebo. The primary endpoint of the study was not complete at the time of writing this thesis and data were analysed while still blinded. There were no significant changes in ADNP titre measured post vaccine when everyone was grouped together (p 0.1511). However, when broken down into those who did develop disease, and those who did not, there was a significant increase in ADNP titres in the group who were subsequently protected from clinical infection after challenge (PD; p >0.9999, nPD; p 0.0479). Interestingly, there was no significant difference in the titres at the point of challenge between the two groups (PD D0 vs nPD D0; p 0.1017), and a higher ADNP titre at the point of challenge did not protect against paratyphoid fever (Odds ratio 1.002, p 0.4119). When CVD1902 was administered to human volunteers in a phase 1 trial, there was a strong induction of CD4+ and CD8+ effector and memory cells [214]. These findings suggest that neutrophil phagocytosis might not be the direct mechanism associated with protection but could be a surrogate marker of another mechanism, or perhaps it is indicative of generally protective, (poly)functional immunity.

Vaccine induced local mucosal immune responses may also play a role in mitigating development of disease following exposure to *S*. Paratyphi. Oral Shigella vaccines WRSs2 or WRSs3 stimulate a significant systemic ADNP functional response, despite *Shigella Sonnei* being a non-invasive enteric pathogen. Further investigation of responses to WRSs2 + WRSs3 showed that fecal ADNP correlated closely with systemic ADNP, and was surprisingly mediated primarily by IgG antibodies, with synergistic efforts from IgA [215].

ADNP titres measured after two doses or CVD1902 or placebo were comparable with those generated after challenge despite the much higher dose given for the vaccine ($2x10^{10}$ CFU for vaccine, or 10^3 -10^4 CFU for challenge). Attenuation of the vaccine strain could reduce it's ability to

stimulate ADNP, or this could be a reflection of a technological or biological upper limit of detection, whereby the presence of more bacteria does not stimulate greater functional response after a certain CFU. This also aligns with the dose dependent response described when looking at high and low dose challenge.

The inclusion of placebo participants reduces the statistical power of this analysis. While this interim analysis provides exciting insight into a potential mechanism of protection of oral paratyphoid vaccines, these results should be interpreted with caution until the data can be analysed in an unblinded way.

6.4.3.ADNP responses to a novel *S.* Typhi – Paratyphi bivalent conjugate vaccine

A single dose of bivalent vaccine elicited a significant increase in neutrophil phagocytosis (D0 – D29; p 0.0040). Responses to Sii-PTCV were higher than those made in response to oral *S*. Paratyphi A challenge in the P1/PATCH studies (D29 post vaccine vs D90 post challenge; p 0.0010). This indicates LPS conjugate vaccine can induce ADNP capable antibodies against *S*. Paratyphi bacteria, and is a more potent stimulus compared with exposure to live, whole bacteria.

Assessment of opsonophagocytic activity after vaccination with *Shigella* GMMA vaccines showed that the highest responses were induced when the GMMA blebs expressed O antigen. Although this varied by strain; for *S. Flexneri* 3a neutrophil phagocytosis responses were showed less discrepancy based on O antigen expression, this highlights the importance of the global antigenic stimulus and how antigenic context can influence antibody effector function outcomes [216]. A deep analysis of antigen specific functional responses in a *Shigella* CHIM showed differential ADNP responses depending on the antigen [179]. However, the use of different strains for the ADNP analysis suggests that Sii-PTCV induces cross-reactive immune responses. The ADNP was optimised using the same strain used for challenge; NVGH308 strain, whereas the ATCC9150 strain was the source of purified

LPS used in the manufacture of the Sii-PTCV. These findings suggest that Sii-PTCV elicits functional immunity towards multiple strains which supports it's use for effective control of paratyphoid.

Baseline titres measured in the Indian population were significantly higher than those in UK residents (p 0.0421). The presence of functional antibodies at baseline in the Indian residents participating in this study is most likely due to stimulation through natural exposure or infection in the environment.

Future analyses will investigate the relationship between ADNP and vaccine efficacy using a human challenge model.

6.5. Conclusion

The data presented in this chapter describe the first evaluation of neutrophil phagocytosis in an experimental paratyphoid infection model and in response to two *S*. Paratyphi A vaccine. The observations reported here show that oral challenge and parental vaccine, and to a lesser extent oral vaccine stimulate increases in phagocytic antibodies. Promisingly, post immunisation ADNP titres measured in the oral vaccine study were generally higher in individuals who were protected from developing disease after *S*. Paratyphi A challenge. Furthermore, a single dose of intramuscular bivalent vaccine induced high levels of ADNP against *S*. Paratyphi A. Future efficacy studies are planned for the bivalent vaccine utilising the challenge model, it will be interesting to assess the relationship between ADNP and disease outcome.

Chapter 7. Integrated analysis of immunological correlates of protection

7.1. Introduction

Correlates of protection (CoP) stand as a critical pillar in the assessment and development of vaccines, they are measurable immunological markers that are indicative of an individual's immunity against a particular pathogen or disease.

Historically, identification of CoP has been through meticulous experimentation and observational studies. However, the emergence of novel vaccine platforms, coupled with advancements in immunological assays and technologies, has catalyzed the identification and validation of correlates of protection. Experimental infection studies are valuable tools for determining CoPs and the employment of the data driven platform termed 'system serology' can further assist in deconvolution of the immune responses to vaccines or infection to help identify a protective immune signature [217].

Establishing an absolute correlate (a single immune function that correlates with total protection) from infection is the ultimate goal, however due to the heterogeneous and redundant nature of the immune system it is difficult to achieve this for most pathogens. Defining a relative CoP where the protective threshold is less well defined and individuals may get infected above that threshold is also a useful tool in vaccine development [131]. CoPs fall in to multiple categories; they can be mechanistic, surrogate, or co-correlates. Mechanistic correlates are markers of functional immune responses that statistically correlate with protection, such as the defined relationship between a 1/8 SBA titre and protection against Meningococcal serogroups A, C, W, Y disease [167]. A surrogate correlate is a non-mechanistic immune marker that statistically correlates with protection but does not define the underlying protective mechanism, an example of a surrogate correlate is the use of an antibody titre

of 0.35 mcg/mL to indicate protection from invasive pneumococcal after a conjugate vaccine [144]. Co-correlates are when multiple immune mechanisms act synergistically to provide protection, for example the link between Vi-TCV induced IgA (quantity and avidity) and IgG1 avidity in the protection from infection in a human typhoid challenge model [140].

There is no known CoP against paratyphoid fever currently but identification of an immune correlate would provide insights into the underlying protective immune response following vaccination or natural infection would help predict vaccine efficacy, and inform us on vaccine strategies. In the previous chapters of this thesis I have explored the opportunity for single mechanistic correlates of protection in paratyphoid fever. System serology is a type of analysis that connects data on antibody biophysical and functional properties to help identify which feature, or combinations of features confers protection against infection. This approach has helped elucidate the importance of HIV envelope targeting ADCC (antibody dependant cellular cytotoxicity) and multifunctional IgG3 antibodies in mediating protection after RV144 HIV vaccine, compared with previous expectations that vaccine mediated immunity to HIV was primarily attributable to antibody mediated neutralisation [187]. Application of systems serology has also helped our understanding of the mechanisms underpinning Vi vaccine driven immunity to typhoid fever. In a CHIM study measuring the vaccine efficacy of a Vi polysaccharide (Vi-PS) and a Vi conjugate vaccine (Vi-TCV) Jin *et al* described synergistic polyclonal antibody responses that were protective against typhoid fever. They described distinct immune signatures resulting from each vaccine. Vi-PS induced generalised polyclonal response with protection from infection being associated with increases in IgA, IgM, all subclasses of IgG, and a polyfunctional serological response. Whereas protective immune signatures following Vi-TCV were more distinct and associated with IgA quantity and avidity, IgG1 avidity, and ADNP (antibody dependent neutrophil phagocytosis) [139,141].

Application of systems serology can help our understanding of natural and vaccine mediated correlates of protection in paratyphoid fever. Furthermore, identification of protective immune

features will help us investigate vaccine efficacy and possibly inform us on effective vaccine implementation strategy.

The aims of this chapter are to answer the following questions:

1. Are there any baseline immunological features that can protect from paratyphoid fever in an *S*. Paratyphi A naïve population?
2. Does oral *S*. Paratyphi A challenge induce a specific immune signature?
3. Are there any protective immune signatures or co-correlates that can protect from paratyphoid fever in a homologous rechallenge model, or after oral paratyphoid vaccine?
4. Does novel *S*. Typhi – Paratyphi A bivalent conjugate vaccine induce a specific immune signature?

7.2. Methods

The samples included in this analysis are detailed in section **2.1, Table 2-1**. Samples were included from the P1 and PATCH oral challenge studies, the VASP oral vaccine and challenge study, and the phase 1 *S*. Typhi – Paratyphi A bivalent conjugate vaccine study.

The detailed methods for the individual assays and statistical analysis are described in **Chapter 2 (2.10)**.

7.3. Results

7.3.1. Baseline clustering of immune responses in the naïve *S*. Paratyphi A challenged participants

L-SBA, ADMP, ADNP, and LPS specific binding antibodies were measured at baseline in 45 paratyphoid naive individuals who received oral challenge with *S*. Paratyphi A. In chapters 4-6 I have provided evidence to suggest that no single immune parameter (L-SBA, ADMP, ADNP) correlated with protection from paratyphoid fever. Multivariate analysis approaches can be used to find patterns in the data that are sometimes not obvious when analysing single variables. To determine if

there is an immune signature or number of co-correlates that can protect against paratyphoid infection I performed a principal component analysis (PCA) to assess the clustering of baseline immune markers in the context of diagnosis outcome after challenge. **Figure 7-1** A shows that baseline immune status cannot distinguish between those who go on to develop paratyphoid fever after challenge and those who do not, as demonstrated by the significant overlap of the PD and nPD clusters. The primary contributor to PC1 was LPS specific IgG, and the primary contributor to PC2 was L-SBA. Clustering analysis of the same data depicted by a heat map (**Figure 7-1** B) shows that there is little to no clustering based on diagnosis. Correlation between baseline antibody functions and ELISA titres were assessed by Spearman's rank correlation coefficient. There is a significant positive correlation between ADMP, ADNP, anti-LPS IgG and IgA, but not L-SBA. However, L-SBA showed a significant, but weak negative correlation with ADNP (**Figure 7-2**).

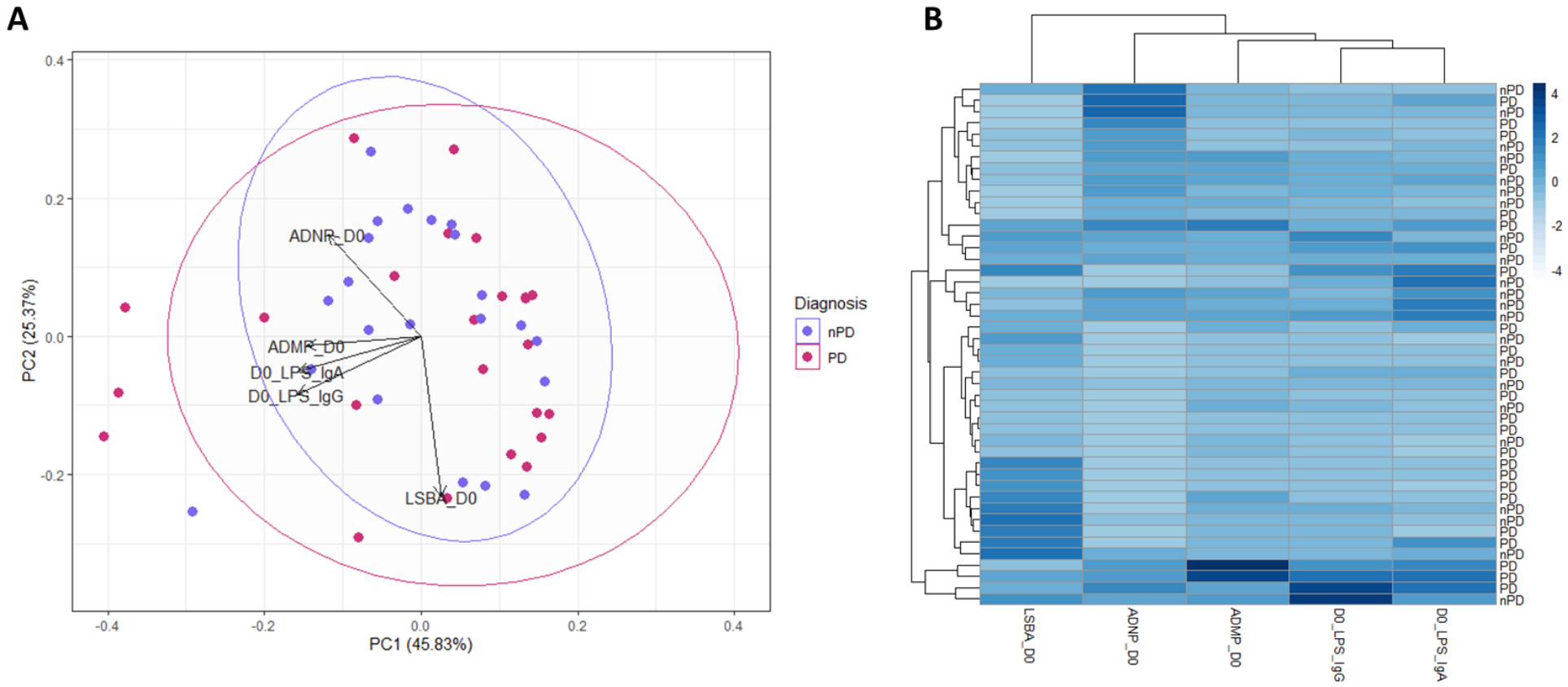


Figure 7-1 Baseline immune markers in paratyphoid naïve participants challenged with S. Paratyphi A

(A) Principal component analysis (PCA) biplot of baseline immune parameters in paratyphoid naïve, UK based individuals measured immediately prior to *S.* Paratyphi A challenge (P1 and PATCH studies) individual PCA scores plotted and colours by diagnosis; PD; paratyphoid diagnosed, nPD; not paratyphoid diagnosed. Black arrows represent the correlation between the assay and the principal component. Elipses represent 95% confident intervals (B) Heat map and dendrograms shows hierarchical clustering of immune parameters measured at baseline P1 and PATCH participants.

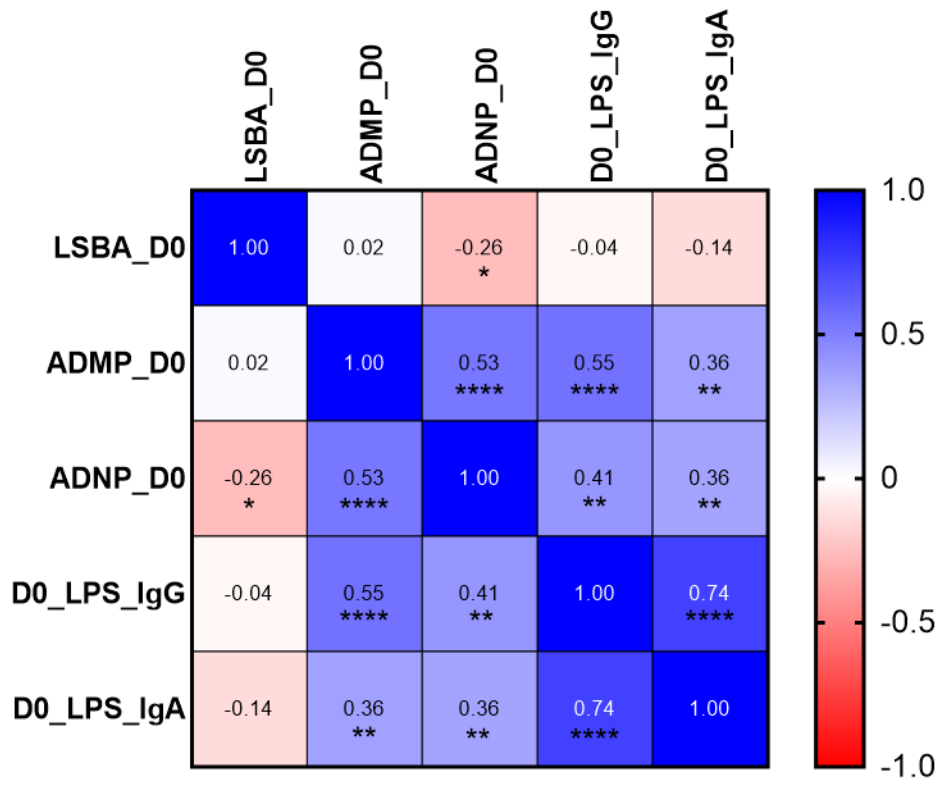


Figure 7-2 Correlation matrix of baseline immune markers in paratyphoid naïve, S. Paratyphi A challenged participants

Heat map showing the Spearman's correlation coefficient between pairs of antibody functions and ELISA titres measured at baseline immediately prior to S. Paratyphi A challenge in participants enrolled in the P1 and PATCH studies. Stars represent associated p values; *p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001; ****p ≤ 0.0001.

7.3.2. Multivariate *S.* Paratyphi A exposure induced immune response clustering

As described in chapters 4-6, exposure to *S*. Paratyphi A in the challenge model induces significant increases in L-SBA, ADMP, and ADNP. Increases in ADMP and ADNP, ninety days after challenge, where significant in both those who developed acute paratyphoid fever and those who did not, while increases in L-SBA titres were only significant in those who developed disease. To determine if the development of disease elicited different immune signatures I performed a clustering analysis. There is significant overlap in between the group that developed paratyphoid fever and those who did not (**Figure 7-3** A). Hierarchical clustering of immune responses at D90 as presented by heat

map shows that the cluster of individuals with the highest responses belong to the group who developed clinical infection, suggesting that infection drives multifaceted immune response. Correlations between pairs of immune parameters were assessed using a Spearman's rank correlation coefficient (**Figure 7-4**). There is a significant correlation between all parameters measured, apart from L-SBA and ADNP and LPS specific IgA suggesting that exposure to *S*. Paratyphi A induces polyfunctional immunity. The lack of correlation between L-SBA and IgA is expected as IgA is not a complement fixing isotype.

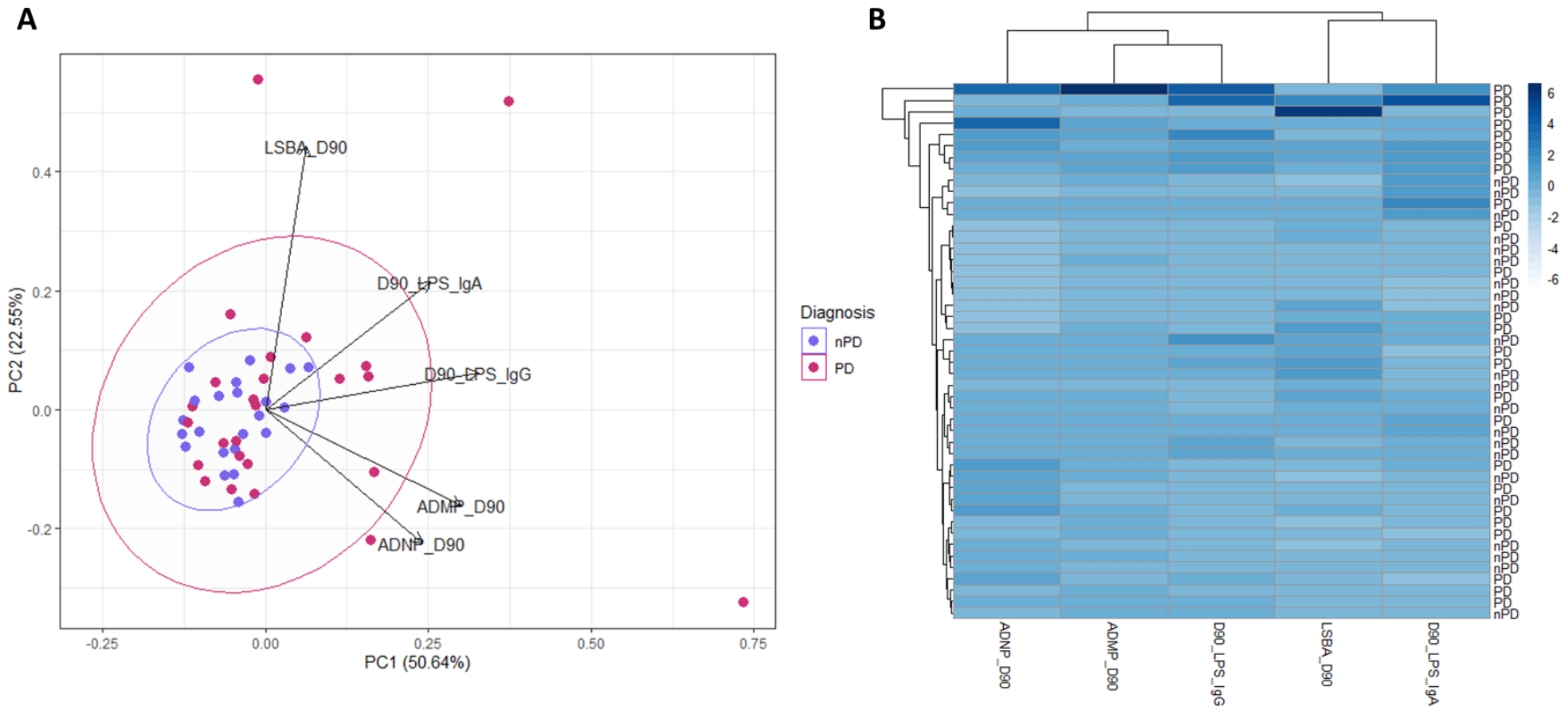


Figure 7-3 Post-challenge immune responses in P1 and PATCH participants exposed to S. Paratyphi A

(A) Principal component analysis (PCA) biplot of immune responses against *S*. Paratyphi A measured P1 and PATCH study enrolled individuals 90 days after challenge. Individual PCA scores plotted and coloured by diagnosis; PD; paratyphoid diagnosed, nPD; not paratyphoid diagnosed. Black arrows represent the correlation between the assays and the principal component. (B) Heat map and dendrograms shows hierarchical clustering of immune responses measured 90 days after *S*. Paratyphi A challenge P1 and PATCH participants.

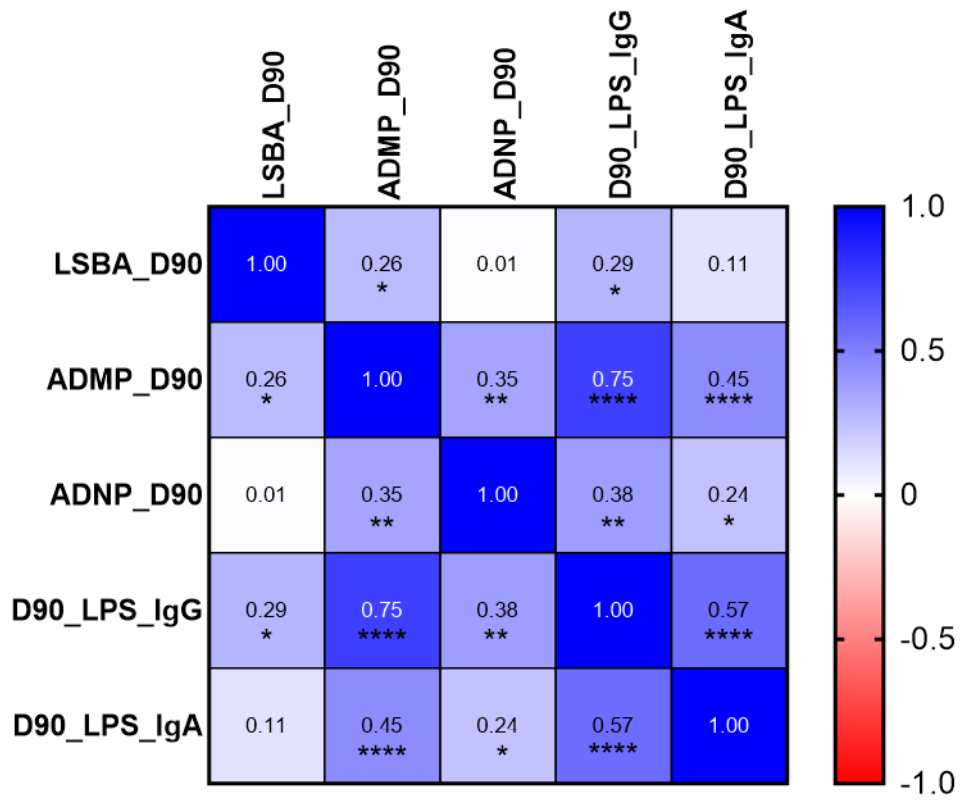


Figure 7-4 Correlation matrix of post-challenge immune responses in S. Paratpyhi A challenged participants

Heat map showing the Spearman's correlation coefficient between pairs of antibody functions and ELISA titres measured 90 days after *S*. Paratyphi A challenge in participants enrolled in the P1 and PATCH studies. Stars represent associated p values; *p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001; ****p ≤ 0.0001.

7.3.3. Integrated analysis of baseline immune features prior to *S*. Paratyphi A homologous rechallenge

L-SBA, ADMP, ADNP, and binding antibodies were measured in 12 individuals enrolled in the PATCH study who were orally challenged with *S*. Paratyphi A twice over the course of 17 months. In chapters 4-6 I demonstrated that no single immune marker correlated with protection from paratyphoid infection upon rechallenge. To identify any immune signatures or co-correlates that could associate with protection I employed a principle component analysis and hierarchical clustering on the data collected at baseline, immediately prior to *S*. Paratyphi A rechallenge. The PCA showed significant overlap in the features in the group that developed paratyphoid fever and those

that don't, suggesting that these immune markers are not able to distinguish between those who developed disease and those that don't (**Figure 7-5** A). The main contributor to PC1 is ADNP and the main variable contributing to PC2 is LPS specific IgG. Results from the hierarchical clustering (**Figure 7-5** B) show that those with the highest assays scores form a cluster at the top of the heat map mostly belong to the group who don't develop fever, but overall there is no distinct clustering pattern separating those who went on to develop enteric fever and those who did not. There are limited correlations between the immune features measured at the rechallenge baseline (**Figure 7-6**). An observation that is likely influenced by the low number of participants and that the assay titres return to baseline levels between the 17 month interval period.

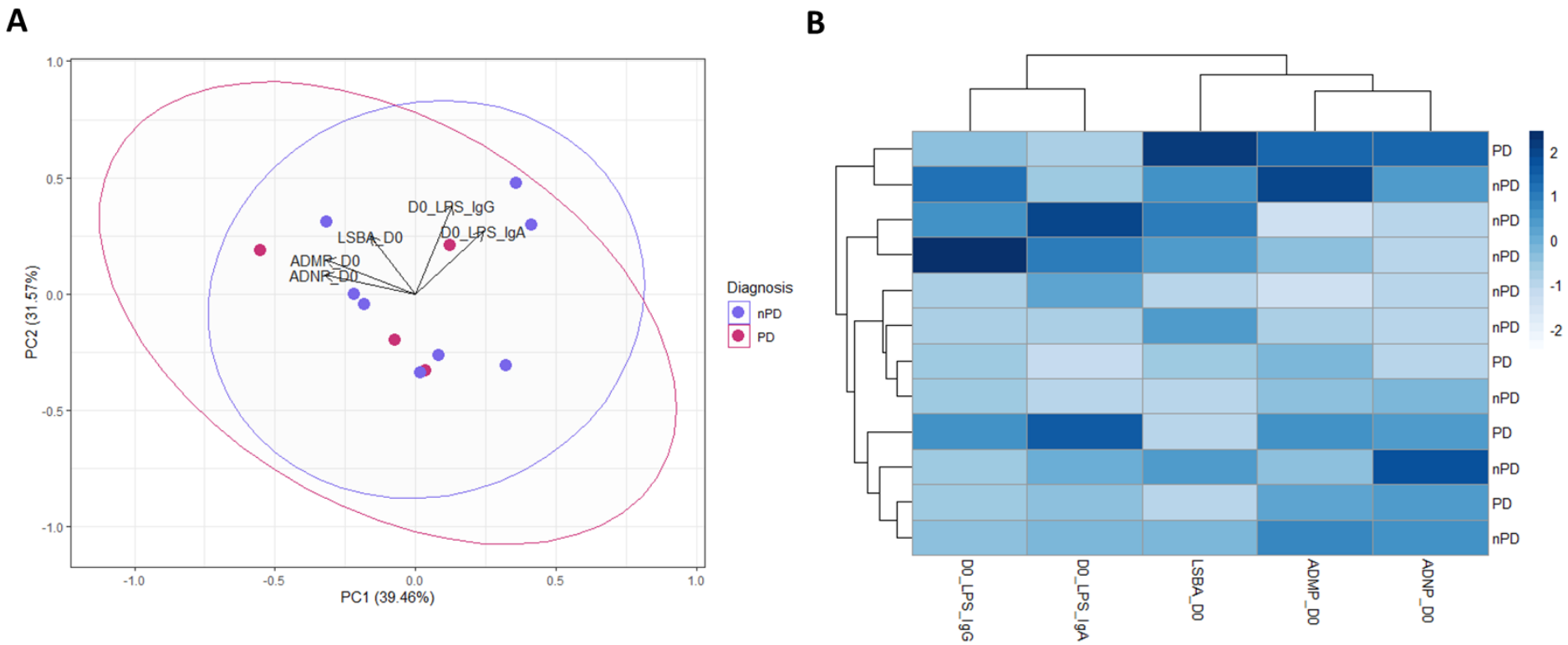


Figure 7-5 Baseline immune markers in participants immediately prior to homologous rechallenge with S. Paratyphi A

(A) Principal component analysis (PCA) biplot of baseline immune features in P1 and PATCH participants measured immediately prior to *S*. Paratyphi A rechallenge. Individual PCA scores plotted and coloured by diagnosis; PD; paratyphoid diagnosed, nPD; not paratyphoid diagnosed. Black arrows represent the correlation between the assay and the principal component. (B) Heat map and dendrograms show hierarchical clustering of immune parameters measured at baseline in PATCH rechallenged participants.

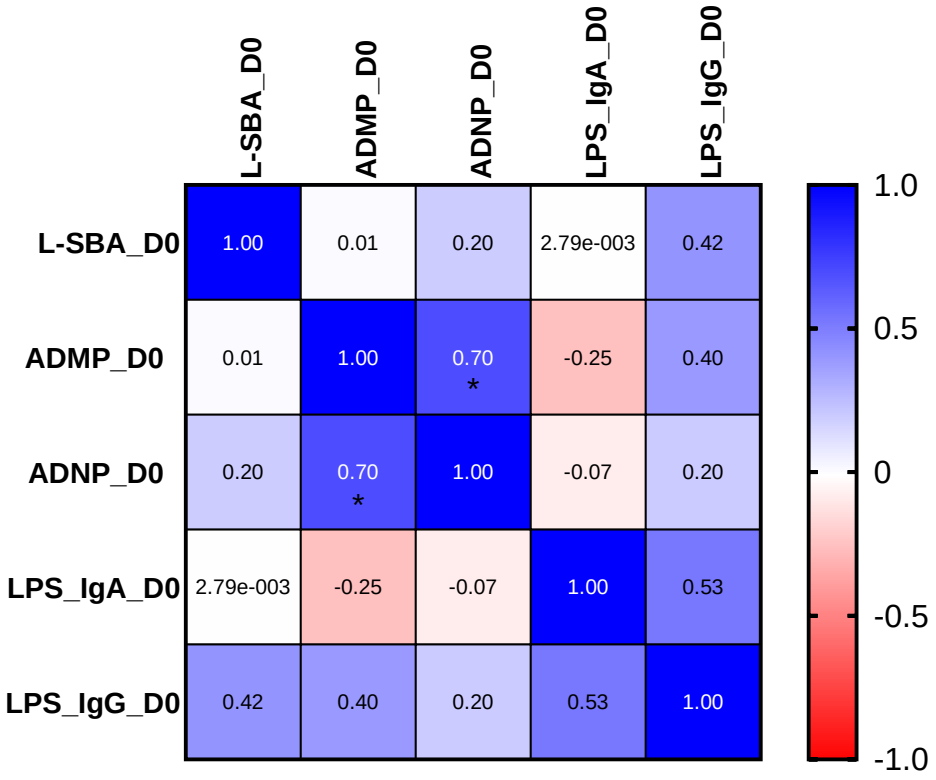


Figure 7-6 Correlation matrix of baseline immune responses prior to S. Paratpyhi A rechallenge

Heat map showing the Spearman's correlation coefficient between pairs of antibody functions and ELISA titres measured immediately prior *S*. Paratyphi A rechallenge in participants enrolled in the PATCH study. Stars represent associated p values; *p ≤ 0.05

7.3.4. Integrated analysis of immune responses induced by two doses of oral vaccine, CVD1902

L-SBA, ADMP and ADNP were measured in 27 individuals at baseline and 42 days after two doses two weeks apart of either oral vaccine CVD1902 or sodium bicarbonate placebo. As described previously in chapter 4-6, ADMP and ADNP are elevated at the post vaccine timepoint in individuals who do not develop paratyphoid fever once challenged. However, L-SBA titres were no different in those who developed paratyphoid fever compared with those who do not. I used PCA and hierarchical clustering analysis to explore the possibility of vaccine induced protective immune signatures and to investigate co-correlation of immune markers. There is significant overlap between the PD and nPD groups (**Figure 7-6** A), however this analysis combines data from those who received

the candidate vaccine and those who received the placebo as I am blinded to the individual vaccine status at the time of writing this thesis. The hierarchical clustering reveals that those with the highest titres from all three assays seem to be subsequently protected from infection (**Figure 7-6** B). However, this is just a trend as overall there is still cluster overlap between those who develop disease and those who do not. Due to the blinded nature of this data, I am unable to attribute these findings to the vaccine. Correlations between the different assays were assessed (**Figure 7-8**), and demonstrate that post-vaccine/placebo ADNP and ADMP strongly correlate, but neither assay correlated with L-SBA titres. Suggesting that there might be a common underlying antibody feature that facilitates phagocytosis, but does not facilitate L-SBA, for example these antibodies could primarily belong to the IgA isotype.

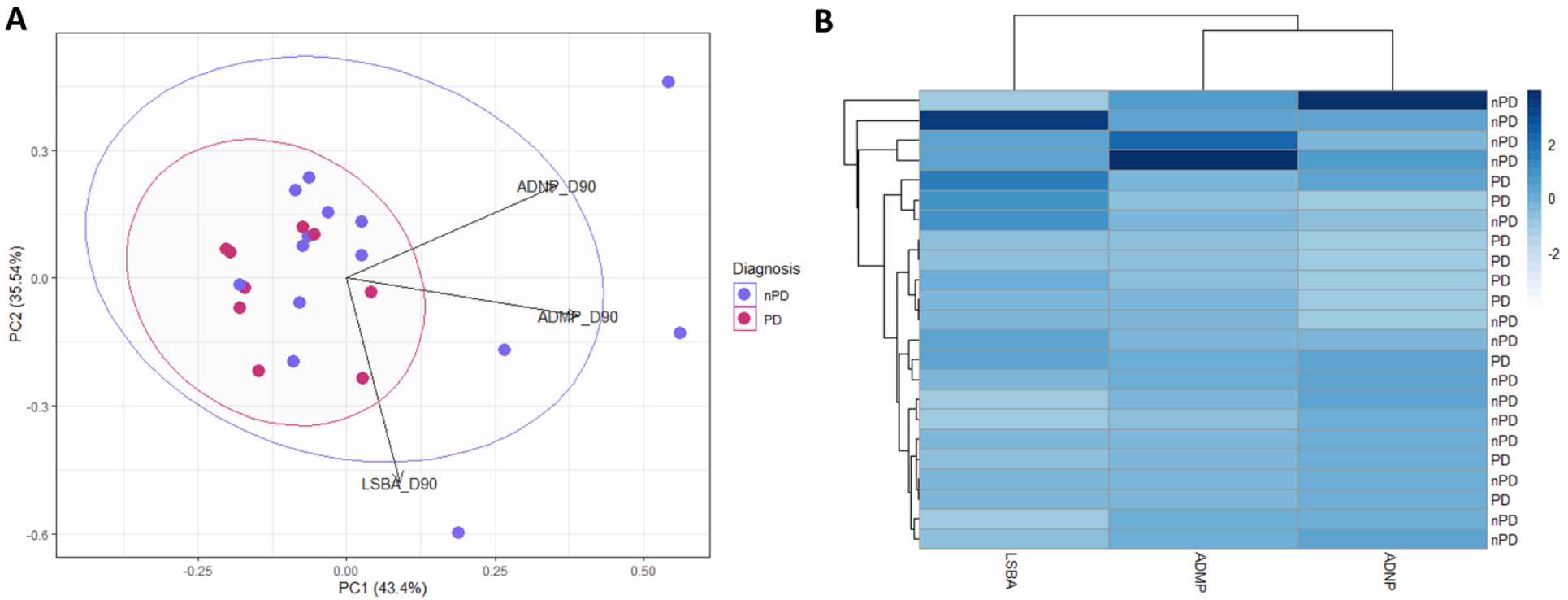


Figure 7-7 Post-vaccine or placebo immune markers in VASP participants immediately prior to challenge with S. Paratyphi A

(A) Principal component analysis (PCA) biplot of baseline immune features in VASP participants measured 28 days after receiving two doses of oral vaccine, CVD1902 or placebo, and immediately prior to *S*. Paratyphi A challenge. Individual PCA scores plotted and coloured by diagnosis; PD; paratyphoid diagnosed, nPD; not paratyphoid diagnosed. Black arrows represent the correlation between the assay and the principal component. (B) Heat map and dendrograms show hierarchical clustering of immune parameters measured at the post-vaccine / pre-challenge time point in VASP participants.

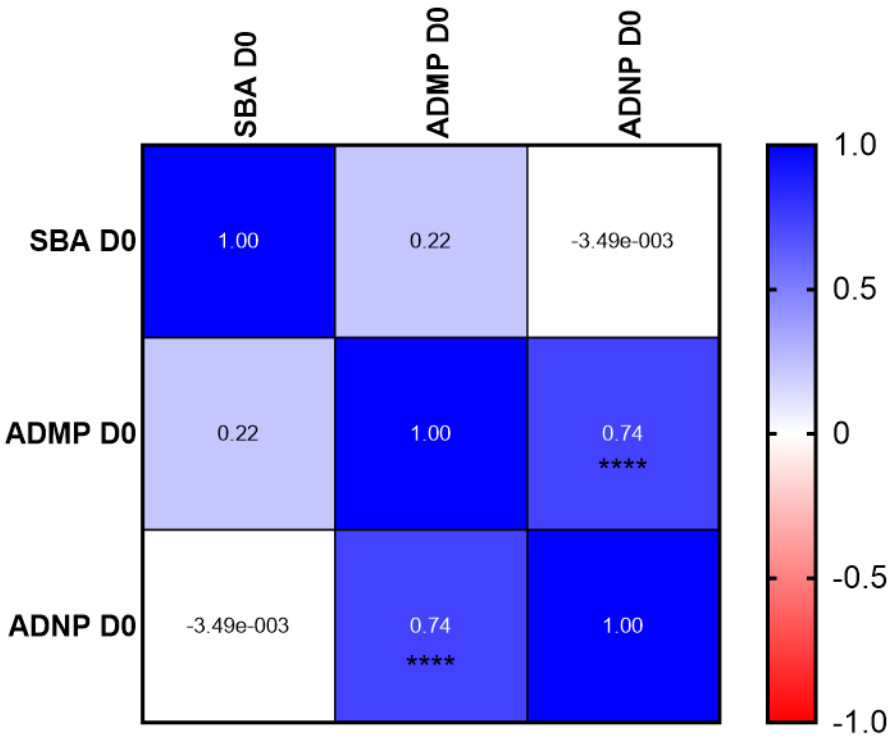


Figure 7-8 Correlation matrix of post-vaccine or placebo immune markers prior to S. Paratyphi A challenge

Heat map showing the Spearman's correlation coefficient between pairs of antibody functions and ELISA titres measured immediately prior *S*. Paratyphi A rechallenge in participants enrolled in the PATCH study. Stars represent associated p values; ****p ≤ 0.0001

7.3.5. Correlation of immune responses induced by bivalent vaccine, Sii-PTCV

In the phase 1 study conducted by SIIPLTD 30 individuals received the novel typhoid-paratyphoid bivalent conjugate vaccine (Sii-PTCV). Twenty nine days after Sii-PTCV there is a significant increase in SBA, ADMP, ADNP, and LPS specific IgG as discussed in chapters 4-6. A heat map of hierarchical clustering by individual shows that those who have the highest ADMP, ADNP, and LPS specific IgG form a distinct cluster while those who have the highest SBA titres form a separate cluster suggesting that these function are mediated by distinct antibody populations (**Figure 7-9** A). When performing a correlation analysis between pairs of the measured immune responses, there was a significant positive correlation between each pair, apart from ADNP and SBA (**Figure 7-9** B).

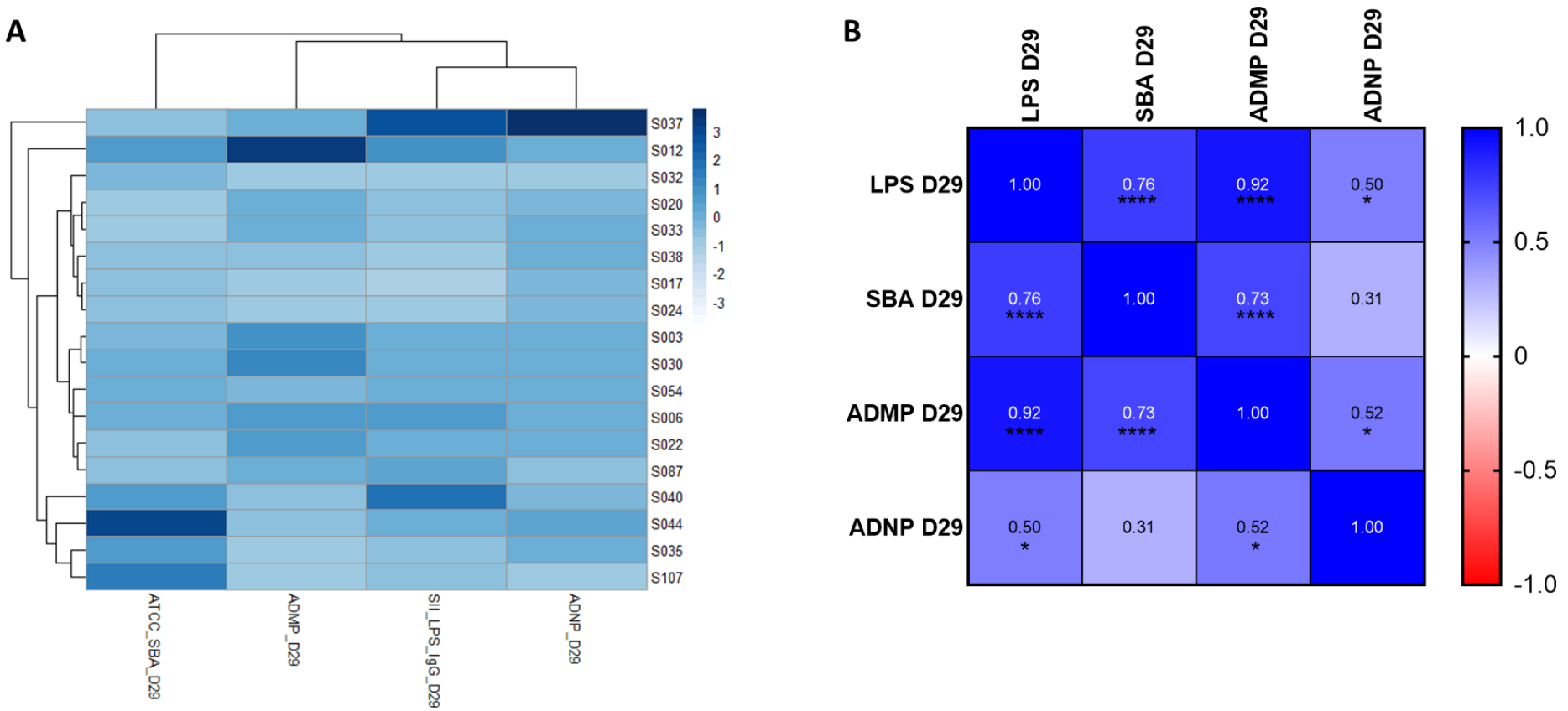


Figure 7-9 Clustering and correlation of immune responses 29 days after receiving Sii-PTCV

(A) Heat map and dendrograms show hierarchical clustering of immune responses in individuals 29 days after receiving a single dose of Sii-PTCV. (B) Heat map showing the Spearman's correlation coefficient between pairs of antibody functions and ELISA titres measured 29 days after receiving Sii-PTCV. Stars represent associated p values; *p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001; ****p ≤ 0.0001.

7.4. Discussion

In this chapter, I have integrated antibody functional and binding data to determine any protective immune signatures in a series of paratyphoid challenge trials, and I have described the described the pattern of responses in participants who received a new typhoid – paratyphoid bivalent conjugate vaccine.

The attack rates in the paratyphoid challenge model are between 40 – 60% [132]. This observation suggests that there is some level of pre-existing immunity to paratyphoid even in a UK population that is paratyphoid naïve (exclusion criteria for the P1 and PATCH studies excluded anyone that has lived in or been a resident in an enteric fever endemic area for > 6 months). Pre-existing immunity to *S*. Paratyphi A infection in the UK population likely comes from a combination of factors, such as physical barriers to infection, non-specific innate immune clearance, and cross protective immunity derived from historical exposure to homolog antigens. None of the single humoral features measured were able to distinguish between those who would go on to develop paratyphoid fever after challenge and those who remained well throughout the challenge period. While attempting to identify immunological CoPs in a paratyphoid naïve, UK population is statistically difficult due to low levels pre-existing immunity. Enteric fever is mainly a problem in immunologicaly naïve children, and therefore perhaps the CHIM study population provides an adequate model of natural infection compared with adults from endemic areas.

PCA analysis and hierarchical clustering revealed divergent immune signatures characterised by either L-SBA, ADMP, and LPS specific IgG and IgA or ADNP, ADMP, and LPS specific IgG and IgA. While neither of these signatures cluster with protection on the heat map, it shows that some functions co-correlate with each other more than other functions. L-SBA and ADNP show the lowest correlation whereas LPS specific IgG and IgA show the highest degree of correlation. There are a myriad of factors that can drive divergent functional responses. One factor could be the binding density required for each function. Activation of C1q, and therefore the complement cascade, as

measured by the SBA requires close binding of at least 2 IgG molecules on the bacterial cell surface, although for other isotypes it differs, e.g. for IgM only a single bound molecule is required because of it's pentameric form [165]. For opsonisation the Fc receptor density also impacts phagocytosis in a dose dependant fashion although the minimum binding density has not been so well defined compared with those relating to C1q activation [218]. This finding could also be due to other underlying antibody biophysical properties that have not been measured yet, such as glycosylation of the Fc region. For example, galactosylation of IgG positively impacts C1q binding and therefore complement cascade activity, whereas sialyation of IgG enhances binding to DC-SIGN on macrophages resulting in upregulation of the inhibitory FcγRIIb and a reduction in inflammation[219]. Divergent signatures based on the L-SBA response could also be attributable to technical variation in the assays. The L-SBA contains complement, but the ADNP and ADMP do not. The addition of complement to the SBA but not the other assays provides an extra variable that could be adding to the differences observed.

Interestingly, this same divergent signature was not present at baseline in rechallenge participants, indicating that a single exposure episode can shape the immune response to *S*. Paratyphi A. In countries where paratyphoid is endemic a single exposure event or multiple exposures to *S*. Paratyphi A could reshape the immune responses and contribute to acquired immunity and the observation of increase age associated decline in paratyphoid incidence.

Ninetey days post challenge the functional and quantitative immune responses were highest in those who developed paratyphoid fever during the 14 days post-challenge follow-up period. Similar to observations made in a shigellosis model where SBA and OPKA titres were higher in those who had experienced more severe disease although S*higella* antigen specific IgG were highest in those with mild disease [178]. On an individual level, increases in anti-LPS IgG, IgA, L-SBA post challenge were only significant in individuals who developed paratyphoid disease whereas increases in ADMP and ADNP were significant regardless of post-challenge infection status. These results suggest potential

differential routes for priming of different immune functions, but also suggest that systemic infection can override this and produce a broad stimulation of a multifunctional response.

Oral vaccine, CVD1902, elicits a multifunctional immune response. Hierarchical clustering showed a cluster of 4 individuals with the highest scores in all three functional assays were protected from infection. The non-exclusive clustering of PD and nPD individuals in the remaining clusters indicates that the vaccine may not provide protection in everyone. It may also indicate that protection in some individuals cannot be explained purely by humoral responses. Oral vaccines are stimulators of humoral and cellular immunity. CVD 1902 has previously been shown to elicit *S*. Paratyphi A specific CD8+ and CD4+ T effector and memory cell responses after just a single dose [65]. Individuals who remain healthy in the challenge model despite moderate to low humoral immune responses may be protected by another immune mechanism e.g T cell mediated clearance or by a physical barrier such as stomach acid.

Furthermore, these findings are confounded by the inclusion of placebo recipients in the analysis, a more accurate analysis can be performed by including more participants and unblinding of the vaccine status.

Bivalent enteric fever conjugate vaccine stimulates significant increases *S*. Paratyphi A humoral immunity. Analysis of the relationship between immune responses elicited by the bivalent shows two key observations. Firstly, that all the immune features measured correlate to some degree. Secondly, hierarchical cluster shows patterns of some immune features. Individuals with the highest antibody quantity, ADMP, and ADNP scores seem to group together while those with the highest SBA activity form a distinct cluster. This clustering pattern is similar to those observed at baseline in participants enrolled in the P1/PATCH studies. A possible explanation for this observation in bivalent conjugate vaccine recipients is that distinct antibody properties influence each feature. Clustering of high antibody quantity with ADNP and ADMP but not SBA suggests that antibody quantity might be a more important driver of phagocytosis compared with SBA. The presence of complement may

partially account for this difference. Bacterial opsonisation and binding of phagocyte Fc receptors is heavily reliant on the presence and properties of antibodies whereas SBA is a complement mediated process, which can be activated as long as there is enough antibody e.g. 2 IgG molecule bound to the bacteria surface. A limitation of some immunoassays is the prozone effect, a feature which is particularly well documented for SBA assays. The prozone effect occurs when antibody levels are so high that the increased binding density actually blocks binding and activation of downstream complement proteins, resulting in a false low reading [220]. The assay QC criteria have been designed to account for this on an individual sample level by repeating samples at a higher dilution if they initially show evidence of the prozone effect. However, in the single correlation between SBA and anti LPS IgG titres there is a plateau in SBA activity at high IgG levels signifying that at high antibody concentrations, the antibody quantity no longer influences the SBA titre (**Figure 4-9 C**). However, this is not the case for the single correlations between anti-LPS IgG and ADMP (**Figure 5-5 C**), or ADNP (**Figure 6-5 C**). Other immunoglobin features, such as isotype and binding avidity/affinity are known to influence antibody effector function. For example, IgM is an excellent activator of the classical complement cascade due to it's pentameric structure, whereas IgG1 is a more potent opsonin and activator phagocytosis [165]. Individual variation in immune response to vaccination is complex, and in this particular case the influence of natural priming due to environmental exposure is unknown. These observations imply that there could be considerable person to person variability in the mechanism of action of *S*. Paratyphi A conjugate vaccines, the influence of this on being able to identify a single correlate or protective immune signature after bivalent conjugate vaccine is unknown. Future analysis of the bivalent conjugate vaccine in the CHIM studies may highlight protective roles for specific humoral immune features at different stages of infection similarly those observed in the typhoid CHIM study. Evidence suggests that Vi vaccine induced IgA protects against establishing infection but Vi specific IgG reduces disease severity [141]. While the ultimate goal of vaccination is to prevent infection, when thinking of effective vaccination strategies reduced disease severity is an important factor to consider. Reduced disease severity can positively influence a

vaccine campaign by reducing forward transmission (i.e. by reducing probability of shedding), reducing the impact of enteric fever on a person's quality of life, and by reducing the economic cost of enteric fever at an individual or population level.

Direct comparisons of data collected between groups is not possible in some cases due to the use of different methodologies (e.g. use of L-SBA or colony counting SBA) and lack of an international standard.

7.4.1.Conclusion

In this chapter, I have applied integrative analysis techniques to identify immune signatures in paratyphoid vaccine and challenge trials. I have described the clustering of immune responses in each dataset. While no protective immune signatures have been discovered, the data described showed some key findings. I have demonstrated the presence of distinct clusters of humoral immune parameters in paratyphoid naïve population that can be changed by a single *S*. Paratyphi A exposure event. I have shown that Sii-PTCV stimulates broad activation of humoral immunity, which can be clustered based on the induction of high SBA responses or high anti-LPS IgG, ADMP, or ADNP. Future studies of the SIIPLTD bivalent vaccine in a paratyphoid experimental challenge model will help determine the role of these divergent immune responses in protection from infection.

Furthermore, this analysis serves as a blueprint for future studies investigating natural and vaccine mediated correlates of protection in paratyphoid fever.

Chapter 8. Discussion

8.1. Summary of findings

8.1.1. Development of standardised laboratory assays

In this thesis, I provide data to demonstrate the steps taken to develop standardised laboratory assays to measure multiple antibody effector functions against *S*. Typhi and *S*. Paratyphi A. I have developed and adapted two SBA assays; a luminescence based protocol from GlaxoSmithKline (GSK), and a colony counting protocol from Serum Institute India Private LTD (SIIPL). I have also developed novel techniques to measure monocyte and neutrophil phagocytosis using a green fluorescent protein expressing *S*. Paratyphi A.

Standardisation of laboratory assays allows comparison of outcomes between independently collected samples (i.e. to compare responses to different vaccines or populations or to compare samples collected at different times). Each of the protocols have been developed using either a commercially available international reference standard (*S*. Typhi L-SBA) or an internal standard (*S*. Paratyphi A SBA protocols). I have demonstrated that the assays exhibit robust reproducibility with comparable measurements across different days. There is good resolution between "negative" and "positive" samples, meaning they can measure a wide range of sample titres.

Application of these protocols to various sample subsets provide insights into immunological changes during enteric fever, and they also highlight which features help protect an individual from infection and identify important targets for vaccine design.

One particular aim of developing these protocols is to help identify potential natural or vaccine mediated serological correlates of protection against paratyphoid fever. Correlates of protection (CoPs) are measurable immune markers that correlate with protection. For many pathogens, conventional measurement of binding antibodies via ELISA does not provide a definitive protective threshold, above which individuals are protected from disease [144]. Antibody Fc effector functions as

mechanistic correlates have proven to be important in many diseases (e.g. streptococcus), and furthermore demonstration of functional responses is often a prerequisite for vaccine licensure [217]. Development and approval of vaccines against paratyphoid is a global priority and development of assays to determine potential protective functional correlates is imperative. The protocols that I have developed provide the beginnings of a cohort of assays which can be utilised, along with other antibody related data such as antibody quantification, avidity or glycosylation measurement, and used to identify serological correlates of protection.

Furthermore, the protocols described here also provide evidence for a "proof of concept" and could be a template for other bacteria (e.g. against different *S*. Paratyphi strains or bacterial species)

8.1.2. Changes in bactericidal antibodies after Vi vaccination and *S*. Typhi challenge

This is the first study to measure and compare bactericidal antibodies in response to Vi vaccination in a human challenge model. Vaccination with either Vi plain polysaccharide or Vi conjugate vaccine induces a significant increase in SBA, although higher titres at the point of challenge do not associate with protection. While other studies have described a role for SBA in protecting from infection from other encapsulated bacteria (*N. meningitidis* serogroups A, C, W, Y), and in mitigating symptom severity in an oral typhoid vaccine challenge study [163,207]. *N. meningitides* pathogenesis includes a definite blood borne phase in which the majority of bacteria are extracellular and so high levels of serum bactericidal antibodies pre-infection can protect against disease [221]. In contrast, during the systemic phase of infection typhoidal *Salmonella* bacteria can reside and replicate intracellularly where they are protected from bactericidal antibodies and therefore bactericidal antibodies less relevant to preventing infection but could work in mitigating disease severity during the later stages of infection.

It is relatively unsurprising that there were no significant correlations between SBA and protection in this model because in a multivariate study on the same cohort of Vi vaccinated and challenged

individuals, IgG1 and IgA significantly associated with protection whereas SBA more closely correlated with IgG2 and IgM [140,168]. In contrast to oral typhoid vaccine induced SBA, no link was found between Vi vaccine bactericidal antibodies and disease severity. While antibody depletion assays demonstrated that the majority of the SBA seen in these samples was due to Vi specific antibodies, Vi expression *in vivo* is highly regulated and the lack of Vi capsule on extracellular bacteria could explain why bactericidal Vi antibodies do not appear to have an important role in acute disease. Furthermore, the facultative intracellular nature of *S*. Typhi means the window for complement deposition and opsonisation during these early stages is small, and therefore SBA might not be an important factor for mitigating infection but might be beneficial at a later stage of infection when bacteria are extracellular, i.e. SBA limits infection rather than prevents it. However, this stage of infection is not captured by the challenge model.

Other antibody effector functions and biophysical properties in response to Vi vaccination have been measured elsewhere [139,141]. Analysis of SBA provided here adds to this body of work and further cements the observation that Vi vaccines induce a polyfunctional serological response which may contribute to their measured efficacy.

8.1.3. Description of antibody effector functions in a paratyphoid challenge - rechallenge model

This is the first study to characterise antibody effector functions in a paratyphoid human challenge model. In the study, I show that oral challenge with *S*. Paratyphi A causes significant changes the antibody effector function profile, characterised by increased L-SBA, ADMP and ADNP.

Both neutrophil and monocyte phagocytosis were significantly elevated regardless of the development of paratyphoid fever. L-SBA titres were only significantly stimulated in the group who had acute paratyphoid fever, similar to infection driven changes in binding antibody described previously by Gibani *et al*[134]. These differences may indicate differential engagement of antibody effector functions throughout the various phases of the infection life cycle. The location of the

immune stimulus has marked impact on the outcome: antibody effector functions can differ depending on many factors such as antibody isotype, subclass, Fc region glycosylation status, which can all be influenced by the context of immune stimulation. In this model, we see that ADNP and ADMP increase in all exposed participants, therefore development of *S*. Paratyphi A specific phagocytosis enhancing antibodies might occur at the mucosal surface. Whereas stimulation of bactericidal antibodies is dependent on systemic infection, and therefore stimulation of a bactericidal antibodies might primarily occur at a later stage of the infection cycle once the infection has spread to the reticuloendothelial and circulatory systems. While not an absolute requirement, development of systemic infection does impact the ADNP and ADMP response. Higher fold change titres and absolute D90 titres were measured in those who developed paratyphoid fever, as well as noting a positive correlation between symptom severity and D90 phagocytic titres.

Stimulation of all functional parameters occurred regardless of exposure dose, this is an interesting observation since natural exposure is likely to happen with a wide range of doses. *Salmonella* are difficult to culture from the environment and therefore the environmental burden is hard to ascertain, but these data suggest that immune stimulation can occur at a different doses.

The challenge-rechallenge study was set up to investigate the effect of prior exposure on recurring exposure. In endemic settings, the incidence of enteric fever decreases with age and it's hypothesised that this is due to recurring natural exposure boosting immunity to infection [222]. In a study assessing risk factors associated with enteric fever, a reported previous episode of enteric fever is significantly associated with a reduced risk of getting enteric fever again [30]. In this study, rechallenged participants had a non-significant reduced risk of developing paratyphoid after their second challenge. However, there was significant waning of all measured serological activities after 17 months (median interval), and none of the immune features measured at baseline correlated with protection from paratyphoid fever after rechallenge. Interestingly, rechallenge did not induce any significant boosting. Challenge-rechallenge studies of other enteric bacteria demonstrate that a

single exposure can associate with high levels of protection on reexposure, for example a single exposure to *Vibrio Cholerae* was associated with 100% protection from reinfection [223]. However, the interval between challenges in these studies were shorter, at 4-12 months. Prolonged interval between challenges and waning of immune responses in the paratyphoid model hinders our ability to determine infection derived correlates of protection. Furthermore, this paratyphoid rechallenge study investigated the protective effects of a single re-exposure event, however kinetics of exposure in endemic settings may differ considerably. There are a myriad of risk factors associated with paratyphoid, such as consumption of contaminated food prepared at home or purchased from street vendors, and therefore is it easy to see the exposure frequency, and therefore immunological boosting might occur more frequently in endemic settings than can be modelled in a challenge study [30].

Baseline titres at the point of challenge or rechallenge did not associate with protection from infection for any of the effector functions measured. The fact that no single function correlated with protection might be indicative of significant redundancy in the immune response against *S*. Paratyphi A. It could also signify that protective immunity is mediated by an immune feature not measured here. Lack of significant correlations could also be because these associations have been analysed in UK residents, where baseline immunological activity is likely due to low affinity, cross-reactive antibodies, and therefore is not indicative of a mature and effective humoral response. Although correlates of protection have been identified in naive, non-immunised populations before. For example, the identification of *Haemophilus influenzae* type b (Hib) correlate of protection of an antibody concentration > 0.15µg/mL, and the observation of naturally acquired SBA titres >1/8 protecting from meningococcal serogroup A, C, W, Y disease. However, unlike this attempt to identify CoPs against paratyphoid in a UK population, these associations are primarily observed when the pathogen is in circulation within the population and therefore where some exposure driven, immune stimulation occurs [129,147].

8.1.4. Description of antibody effector functions after live attenuated oral *S.* Paratyphi A vaccine (CVD1902)

In this first-of its kind study, I investigated functional humoral responses to the live attenuated oral *S*. Paratyphi A vaccine; CVD1902. Due to the ongoing clinical trial the samples were analysed whilst still blinded and therefore some of the participants included in the analysis have not received the oral vaccine, but instead have received a sodium bicarbonate placebo. Despite this, there were significant increases in L-SBA and ADMP post-vaccine/placebo. Interestingly, post-vaccine/placebo increases in ADMP and ADNP were observed in those who subsequently did not develop paratyphoid after exposure in the challenge model, suggesting that phagocytic enhancing antibodies contribute to mitigating systemic paratyphoid infection.

Oral vaccines are an effective intervention for preventing infection from enteric (*Vibrio cholerae*) and non-enteric (polio virus) pathogens alike and have shown to be potent inducers of humoral immunity. They are a particularly attractive option for enteric pathogens due to stimulation local mucosal immunity [224]. Assessment of immunological responses after oral vaccines against other enteric pathogens has helped identify potential correlates of protection. For example, a human challenge model for cholera showed that increased vibriocidal antibodies following oral cholera vaccine (CVD-103HgR) associated with protection from mild to severe disease [225]. Oral *Shigella* vaccines generate significant increases in SBA and opsonophagocitic antibodies, which have separately been shown to correlate with reduced symptom severity in a shigellosis challenge model [178,215].

Studies have shown that live attenuated oral typhoid vaccine, Ty21a, elicits cross-reactive, functional immune responses against *S*. Paratyphi A, however most field studies suggest that Ty21a does not appear to confer significant cross protection to *S*. Paratyphi A infection [126,143,197]. The early, interim analysis of functional immunogenicity in the VASP trial warrants further study.

Oral vaccines offer some potential advantages over single antigen parenteral vaccines, as they contain multiple antigenic stimuli including T dependant and independent antigens, they can induce local mucosal immunity, long lived immunological memory, and can be self adjuvanting [226]. However, across multiple pathogens there is an observation of impaired efficacy of oral vaccines in developing countries compared with efficacy rates measured in high-income countries. The reasons for this are unknown but many causes have been theorised, such as the impact of malnutrition, influence of the microbiota, enteropathy [227]. While an inverse pattern is observed for oral typhoid vaccine, Ty21a, which has an estimated protective efficacy of 71% after 1 year in Chile but only 35% in a UK CHIM study [62,137]. Vaccine efficacy and immunogenicity studies for oral paratyphoid vaccines should be carried out in developing countries where paratyphoid is endemic to illustrate an accurate efficacy in the populations that need the vaccine most.

8.1.5. Description of antibody effector functions after *S*. Typhi - Paratyphi A bivalent conjugate vaccine (Sii-PTCV)

In this unique study, I assessed functional serological responses to *S*. Paratyphi A after novel *S*. Typhi – Paratyphi A bivalent conjugate vaccine. Sii-PTCV induces significant increase in functional antibodies as measured by L-SBA, ADMP, and ADNP. In lieu of a known correlate of protection, the WHO recommends licensure of new vaccines be based on demonstrating induction of functional antibodies, supported by evidence of significant specific binding antibody [228]. As there is currently no licensed vaccine for *S*. Paratyphi A, these results in addition to those reported by Kulkarni *et al* from the same study, which show significant induction of binding antibodies against both typhoidal serovars, make this a promising discovery and support the use of this vaccine for use against enteric fever [80].

Conjugate vaccines are highly efficacious against infection with other Gram-negative bacteria, and they are responsible for the “virtual elimination” of bacterial meningitis caused by Hib, meningococcus bacteria [229]. They offer a significant advantage over polysaccharide vaccines, which

do not engage T cell help and therefore have limited efficacy in infants who lack mature marginal zone B cells. They are also potent stimulators of immunological memory and can provide longer lived protection compared with conventional polysaccharide vaccines [230].

A number of studies have investigated the relationship between conjugate vaccines and functional mechanisms of protection. The relationship between SBA and protection from meningococcal disease has long been established, SBA is often referred to as the "gold standard" correlate for measuring vaccine effectiveness, and is always required to support licensure of new meningococcal vaccines [231]. Despite some established absolute correlates of protection, singular absolute correlates for some pathogens are less easy to define and relative correlates are used to describe the relationship between an immune response and certain level of protection, for example the hemagglutination-inhibition antibody titer of 1/40 demarking a 50-70% protection from influenza infection [232].

Vi conjugate vaccines (Vi-TCV) provide significant protection from typhoid fever, extensive studies of serological responses after Vi-TCV could not find an absolute correlate of protection. Instead the studies showed that Vi-TCV mediated protection was multifactorial and was associated with both higher fold change in IgA and higher Vi IgG1 avidity [139]. *S*. Typhi specific immunology is more extensively studied compared with *S*. Paratyphi A, but due to the similarity between the bacteria it often serves as a blueprint for understanding of *S*. Paratyphi A immunology. These findings suggest that further studies into paratyphoid conjugate vaccine mediated correlates should include analysis of antibody biophysical properties as well as effector functions.

The observation of higher baseline functional immunity in Indian residents compared with UK residents further supports the theory that natural exposure boosts immunity. Interestingly, a range of titres for each effector function were observed in both populations indicating intrinsic heterogeneity.

There was no challenge aspect to this phase 1 trial and thus no direct correlations with protection from infection could be performed. These findings show that Sii-PTCV is immunogenic against *S*. Paratyphi A, and antibody effector functions are worthy of investigating in the upcoming Sii-PTCV CHIM study.

	SBA	ADMP	ADNP
All challenged participants	•Significant increase in all challenged D0 correlates with ADMP •D90 correlated with IgG LPS and ADNP	•Significant increase in all challenged •Correlates with IgG and IgA LPS, ADNP and L-SBA (D0 only) •Significant correlation between D90 ADMP and symptom severity, but not diagnosis	•Significant increase in all challenged •Correlates with LPS IgG, IgA (D0 only), ADMP and L-SBA (D0 only) •D90 ADNP correlates with symptom severity and duration of bacteraemia but not diagnosis outcome
PD vs nPD	•Increase is only significant in PD group •Higher baseline titres do not correlate with protection	•Significant increase irrespective of challenge outcome, but higher D90 titres in PD group •Higher FC (D0-D90) in PD group •Higher baseline titres do not correlate with protection	•Significant increase irrespective of challenge outcome, but higher D90 titres in PD group •Higher FC (D0-D90) in PD group •Higher baseline titres do not correlate with protection
Low dose Vs high dose	•Significant increase in L-SBA after exposure to high and low dose challenge •Higher D90 absolute L-SBA titre in high dose challenge group	•Exposure to both doses gives significant increase in ADMP •Not significant in low dose nPD group	•Exposure to both doses gives significant increase in ADMP •Not significant in low dose nPD group
Re-challenge	•Significant waning of L-SBA by rechallenge timepoint •No boosting of titres after rechallenge •No association between D0 rechallenge titre and PD on rechallenge	•Significant waning of ADMP by rechallenge timepoint •No boosting of titres after rechallenge •No association between D0 rechallenge titre and PD on rechallenge	•Significant waning of ADMP by rechallenge timepoint •No boosting of titres after rechallenge •No association between D0 rechallenge titre and PD on rechallenge
CVD1902 vaccinated (Oral vaccine)	•Significant increase in ADMP in all vaccine / placebo •Only significant in group that developed PD after challenge •no correlation D0 titres and protection using logistic regression	•Significant increase in ADMP in all vaccine / placebo •Only significant in group that did not develop PD after challenge •Absolute ADMP titre and D-42-D0 FC higher in protected individuals, but no correlation with protection using logistic regression	•No significant increase post vaccine/placebo when all grouped together •Increase is significant in nPD group, although absolute titres at D0 are no different between PD and nPD groups •D0 ADNP does not correlate with protection
Sii-PTCV vaccinated (Bivalent vaccine)	•Significant increase 29 days post vaccine, which is higher than post challenge titres •Remains elevated at D181 compared with baseline, some waning compared with D29 •Correlates with LPS IgG	•Significant increase 29 days post vaccine, which is higher than post challenge titres •Baseline levels higher in Indian population compared with UK population •Correlates with LPS IgG	•Significant increase 29 days post vaccine, which is higher than post challenge titres •Baseline levels higher in Indian population compared with UK population •Correlates with LPS IgG

- High concordance of titres between different strains and techniques (L-SBA and counting SBA)

Figure 8-1 Summary of findings

Description of analysis performed and a brief summary of findings

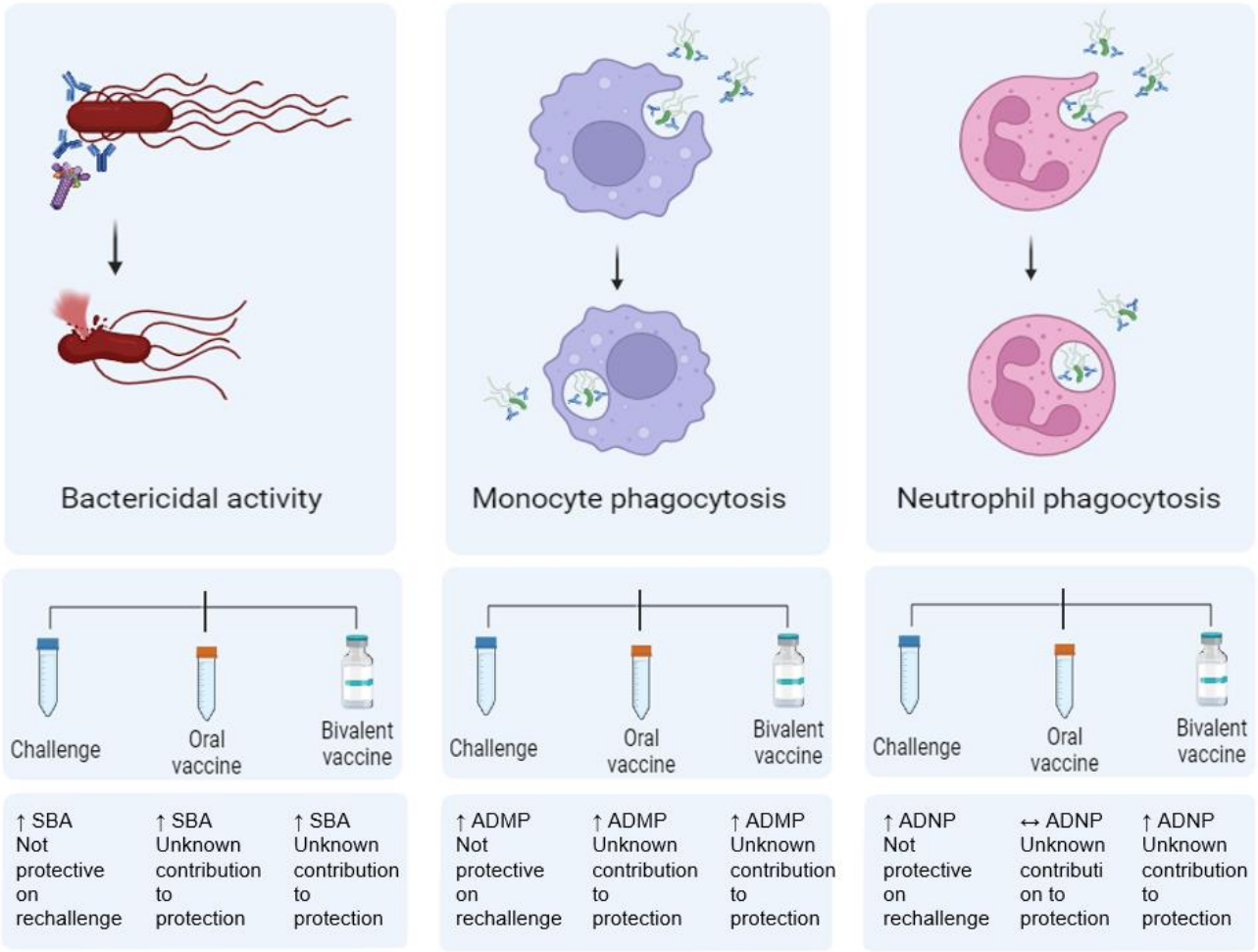


Figure 8-2 Diagrammatic summary of findings

Summary of main findings from applying the optimised assays to various datasets

8.2. Limitations

Two undeniable limitations of the data presented here have already been mentioned. Analysis of a subset of VASP study samples while still being blinded restricts the ability to make meaningful interpretations from this data. Some interesting changes in *S*. Paratyphi A specific immune responses have been observed in response to VASP vaccine/placebo but until the study has been unblinded interpretation of these findings within the context of the challenge study has been done with the assumption that these changes are driven CVD1902 rather than placebo.

No assessments of protective correlates can be made from the Sii-PTCV study due the fact that this is a phase 1 safety and immunogenicity trial. Therefore the immunogenicity findings reported here are purely descriptive, we rely on future studies to define any humoral responses that correlate with protection.

8.2.1. Limitations of the controlled human challenge model

The enteric fever controlled human challenge models aim to mimic natural infection. While the controlled aspect of the trials provides advantages compared with natural infection kinetics, e.g. known dose and timing of exposure, there are some disadvantages to the challenge model. The most relevant ones are described below:

8.2.1.1. Sampling limitations

The challenge model has been developed with a priority on participant welfare. In the early stages of infection immune activity is primarily in the gut. It would be unethical to sample the gut associated lymphoid tissue in the immediate follow-up after exposure to *Salmonella* as the biopsy procedure to obtain the sample damages the intestine, and thus we are limited to interpreting intestinal immunological responses by using systemic samples as a proxy. Although sometimes saliva and stool can be used, these techniques have their own disadvantages and can be difficult to standardise.

Some of the sample sizes presented in this thesis are small (PATCH naïve; n= 15 rechallenged participants; n= 12), especially when making comparisons within groups based on the clinical diagnosis outcome. The sample sizes were selected with the primary endpoint in mind to detect clinically meaningful differences while minimising the number of participants required to go through challenge. Small numbers for the naïve challenge group have been somewhat mitigated by grouping P1 and PATCH naïve challenged groups where appropriate.

All challenged participants were followed up for 14 days after exposure and were given a two week course of antibiotics upon meeting the diagnosis criteria (bacteraemia and/or fever ≥38°C for ≥12 hours). Any participants who did not meet the predefined composite diagnostic criteria commenced antibiotics on day 14 post-challenge. Longitudinal post-challenge samples were collected at predefined time points (28, 90, 180, and 365 days after exposure). Samples from day 28 post challenge could not be used in the functional assays with live bacteria because antibiotics present in the serum would kill the bacteria. While we do not know the peak time point for assessing immune responses to experimental challenge, the D90 time point was used. No investigations were performed to determine if this was the optimal time point to measure antibody effector functions post-challenge but this this something that could be performed on future studies.

8.2.1.2. Limitations of the CHIM study population

The challenge model has been set up in a UK population, pre-existing immunity in a non-endemic setting is likely lower than that seen in populations where typhoidal *Salmonella* serovars are in circulation. Meaning we are underpowered to find statistically meaningful natural correlates of protection in this naïve population. The transfer of the CHIM to endemic regions could curtail this and provide valuable samples for assessing immunological correlates to natural infection.

The CHIM participant population age is 18-55 years, how well this replicates natural infection in children, where the highest burden of enteric fever lies is not known.

8.2.1.3. Diagnostic criteria limitations

Use of microbiological data as a diagnostic for enteric fever is more stringent than diagnostic criteria employed in endemic settings, where blood culture samples for diagnostic purposes would only be taken from individuals who had already developed febrile symptoms. The majority (80%) of the individuals who were diagnosed with acute paratyphoid fever in the PATCH study were diagnosed based on positive blood culture without fever. The impact of these different diagnostic criteria on the findings reported here is unknown, due to low numbers of symptom based diagnoses no statistical analysis can be reliably performed. This further illustrates the value of samples from endemic regions for investigating natural correlates of protection.

8.2.2. Laboratory assay limitations

While every effort has been made to develop standardised assays that are biologically representative, there are some inherent limitations that should be acknowledged when interpreting these data.

Use of a limited number of bacterial strains for this analysis may not be representative of other strains that are in circulation. While NVGH308 is a wild type strain isolated from a paratyphoid fever patient in Nepal, and *S*. Paratyphi A demonstrates limited genomic variation [233]. A successful vaccine would need to be immunogenic against multiple *S*. Paratyphi A strains, including those that are antimicrobial resistant. Immunogenicity data generated with a panel of currently circulating strains could further support the regulatory approval of the first paratyphoid vaccines.

Cells lines were used in lieu of donor cells or participant matched cells. The THP-1 and HL60 cell lines are well characterised and studies have shown that phagocytic uptake by THP-1 and differentiated HL60s are representative of that seen by monocytes and neutrophils respectively [155,234]. However, no other cell lines were investigated in the context of this thesis.

8.3. Future work

The projects presented in this thesis lay the groundwork for some future research exploring immunity to enteric fever and advancing our understanding of immunological correlates of protection.

8.3.1. Assessment of vaccine efficacy and immunological CoPs in the paratyphoid CHIM

As described throughout this thesis one major limitation of investigating vaccine induced CoPs after CVD 1902 is that the VASP study was ongoing at the time of writing. Future work measuring functional immunity of additional samples and unblinding of the vaccine status of the participants will allow for a comprehensive assessment of CVD1902 driven immunity. Additional correlations between immunological features and clinical outcomes beyond the binary diagnosis status (e.g. shedding, disease severity etc) would also help our understanding of oral vaccine induced immunity.

Future studies of bivalent Sii-PTCV will include a CHIM study assessing the immunogenicity and efficacy of this novel vaccine. I have used samples from the phase I trial to demonstrate immunogenicity, samples collected in the phase II CHIM trial can be used to investigate Sii-PTCV induced immunological CoPs. Characterisation of protective immune responses in the unique, controlled setting of the paratyphoid challenge model will further our understanding of vaccine mediated protection against enteric fever. Simultaneously to the CHIM study, a phase III trial in Indian residents will assess the safety and immunogenicity of the Sii-PTCV vaccine. Comparison of immune responses in UK CHIM participants and Indian participants will allow direct a head to head evaluation of vaccine responses in paratyphoid naïve individuals compared with individuals who reside in a paratyphoid endemic region.

8.3.2. Development of assays to assess other immunological features

Data presented in this thesis is on a limited number of immunological features. Assays to measure other Fc mediated functions and properties (e.g. antibody dependant NK cell activation, ADNK, or glycosylation) or T cell mediated immunity have not been assessed in these datasets yet. Evaluation of ADNK activation after Vi-TCV revealed a diverse role for this cell type in experimental typhoid infection, cytotoxic activity of NK cells (measured by CD107a degranulation) is associated with protection but proinflammatory activity (measured by MIP-1β release) is associated with typhoid infection. Furthermore, few studies have investigated T cell immunity in paratyphoid infection. Samples collected in the clinical trials described throughout this thesis offer a valuable opportunity to study T cell immunity in the context of paratyphoid vaccine and infection.

Additionally, the immunoassays described in this thesis are based on serum antibody activity. While serum antibodies are thought to be a good representative of humoral immunity, future analysis evaluating immune responses in the salvia and stool may provide a valuable insight into local mucosal immunity.

Development and application of such a wide variety of assays would complement the data presented in this thesis and help identify any vaccine induced protective immune signatures.

8.4. Conclusions

The primary aim of this thesis was to fill knowledge gaps in our understanding of immune mechanisms in the context of enteric fever infection and vaccination, with an overarching aim to obtain information on potential correlates of protection. While I have not been able to define any correlates of protection, development and application of standardised assays has improved our understanding of enteric fever immunity. The findings presented in this thesis contribute to the growing data on enteric fever vaccines, and form one of the first comprehensive studies on antibody effector functions against *S*. Paratyphi A.

Currently there are no licensed vaccines against paratyphoid fever although many candidate vaccines are in development and are at various stages of testing. Demonstration of vaccine induced functional immunity is a fundamental part of vaccine approval and regulation. It is hoped that the data generated in this thesis on vaccine induced functional immunity will support the approval of the novel *S*. Typhi – Paratyphi A conjugate vaccine (Sii-PTCV). In addition, it is hoped that the assays developed throughout this project will be used to improve our understanding infection-induced immunity, to help assess future paratyphoid vaccine immunogenicity, and to help identify potential correlates of protection.

The widespread use of cost effective, efficacious vaccines against both typhoid and paratyphoid are just one factor in a multiple factorial strategy to reduce the burden of enteric fever incidence. For successful elimination of enteric fever, further advances are in the treatment of disease including development of sensitive and specific diagnostics, as well as implementation of effective intervention through improved water and sanitation hygiene are needed.

With the noted increase in paratyphoid fever incidence, increased rates of antimicrobial resistance, and the exponential threat of climate change to public health, the time for effective intervention is now.

Bibliography

1. Maskey, A. P. *et al.* Salmonella enterica serovar Paratyphi A and S. enterica serovar Typhi cause indistinguishable clinical syndromes in Kathmandu, Nepal. *Clin. Infect. Dis.* **42**, 1247–1253 (2006).

2. Neupane, D. P., Dulal, H. P. & Song, J. Enteric Fever Diagnosis: Current Challenges and Future Directions. *Pathogens* **10**, 410 (2021).

3. Parry, C. M., Hien, T. T., Dougan, G., White, N. J. & Farrar, J. J. Typhoid Fever. *N. Engl. J. Med.* **347**, 1770–1782 (2002).

4. Crump, J. A. & Kirk, M. D. Estimating the Burden of Febrile Illnesses. *PLoS Negl. Trop. Dis.* **9**, (2015).

5. Hancuh, M. *et al.* Typhoid Fever Surveillance, Incidence Estimates, and Progress Toward Typhoid Conjugate Vaccine Introduction — Worldwide, 2018–2022. *MMWR. Morb. Mortal. Wkly. Rep.* **72**, 171–176 (2023).

6. Stanaway, J. D. *et al.* The global burden of typhoid and paratyphoid fevers: a systematic analysis for the Global Burden of Disease Study 2017. *Lancet Infect. Dis.* **19**, 369–381 (2019).

7. Crump, J. A. & Mintz, E. D. Global trends in typhoid and paratyphoid fever. *Clinical Infectious Diseases* vol. 50 241–246 (2010).

8. The Surveillance for Enteric Fever in Asia Project (SEAP), Severe Typhoid Fever Surveillance in Africa (SETA), Surveillance of Enteric Fever in India (SEFI), and Strategic Typhoid Alliance Across Africa and Asia (STRATAA) Population-based Enteric Fever Studies: A Review of Methodological Similarities and Differences - PubMed. https://pubmed.ncbi.nlm.nih.gov/32725221/.

9. Antillón, M. *et al.* The burden of typhoid fever in low- and middle-income countries: A meta-regression approach. *PLoS Negl. Trop. Dis.* **11**, (2017).

10. Mogasale, V. *et al.* Burden of typhoid fever in low-income and middle-income countries: a systematic, literature-based update with risk-factor adjustment. *Lancet. Glob. Heal.* **2**, e570–e580 (2014).

11. Buckle, G. C., Walker, C. L. F. & Black, R. E. Typhoid fever and paratyphoid fever: Systematic review to estimate global morbidity and mortality for 2010. *J. Glob. Health* **2**, 1–9 (2012).

12. Kim, S. *et al.* Spatial and Temporal Patterns of Typhoid and Paratyphoid Fever Outbreaks: A Worldwide Review, 1990-2018. *Clinical Infectious Diseases* vol. 69 S499–S509 (2019).

13. Marks, F. *et al.* Incidence of invasive salmonella disease in sub-Saharan Africa: a multicentre population-based surveillance study. *Lancet Glob. Heal.* **5**, e310–e323 (2017).

14. Meiring, J. E. *et al.* Burden of enteric fever at three urban sites in Africa and Asia: a multicentre population-based study. *Lancet Glob. Heal.* **9**, e1688–e1696 (2021).

15. Darton, T. C. *et al.* The STRATAA study protocol: A programme to assess the burden of enteric fever in Bangladesh, Malawi and Nepal using prospective population census, passive surveillance, serological studies and healthcare utilisation surveys. *BMJ Open* **7**, 1–9 (2017).

16. Zellweger, R. M. *et al.* A 23-year retrospective investigation of Salmonella Typhi and Salmonella Paratyphi isolated in a tertiary Kathmandu hospital. *PLoS Negl. Trop. Dis.* **11**, e0006051 (2017).

17. Vlieghe, E. *et al.* Increase in salmonella enterica serovar paratyphi a infections in Phnom Penh, Cambodia, january 2011 to august 2013. *Eurosurveillance* **18**, 20592 (2013).

18. Sood, S. *et al.* Paratyphoid fever in India: An emerging problem. *Emerg. Infect. Dis.* **5**, 483–484 (1999).

19. Arndt, M. B. *et al.* Estimating the Burden of Paratyphoid A in Asia and Africa. *PLoS Negl. Trop. Dis.* **8**, (2014).

20. Jin, Y. Enteric fever in south China: Guangxi province. *J. Infect. Dev. Ctries.* **2**, 283–288 (2008).

21. Breiman, R. F. *et al.* Population-Based Incidence of Typhoid Fever in an Urban Informal Settlement and a Rural Area in Kenya: Implications for Typhoid Vaccine Use in Africa. *PLoS One* **7**, e29119 (2012).

22. Akinyemi, K. O., Bamiro, B. S. & Coker, A. O. Salmonellosis in Lagos, Nigeria: Incidence of Plasmodium falciparum-associated Co-infection, Patterns of Antimicrobial Resistance, and Emergence of Reduced Susceptibility to Fluoroquinolones. *J. Health. Popul. Nutr.* **25**, 351 (2007).

23. Ibrahim, Y. K. E., Adedare, T. A. & Ehinmidu, J. O. Antibiotic sensitivity profiles of salmonella organisms isolated from presumptive typhoid patients in Zaria, northern Nigeria. *Afr. J. Med. Med. Sci.* **34**, 109–14 (2005).

24. Garrett, D. O. *et al.* Incidence of typhoid and paratyphoid fever in Bangladesh, Nepal, and Pakistan: results of the Surveillance for Enteric Fever in Asia Project. *Lancet Glob. Heal.* **10**, e978–e988 (2022).

25. Dave, J. & Sefton, A. Enteric fever and its impact on returning travellers. *Int. Health* **7**, 163–168 (2015).

26. Herdman, M. T. *et al.* Increasingly limited options for the treatment of enteric fever in travellers returning to England, 2014-2019: A cross-sectional analytical study. *J. Med. Microbiol.* **70**, 001359 (2021).

27. Gauld, J. S. *et al.* Spatial and genomic data to characterize endemic typhoid transmission. *Clin. Infect. Dis.* (2021) doi:10.1093/CID/CIAB745.

28. Vanderslott, S., Phillips, M. T., Pitzer, V. E. & Kirchhelle, C. Water and Filth: Reevaluating the First Era of Sanitary Typhoid Intervention (1840–1940). *Clin. Infect. Dis.* **69**, S377–S384 (2019).

29. Pitzer, V. E. *et al.* Predicting the Impact of Vaccination on the Transmission Dynamics of Typhoid in South Asia: A Mathematical Modeling Study. *PLoS Negl. Trop. Dis.* **8**, e2642 (2014).

30. Karkey, A. *et al.* Differential Epidemiology of Salmonella Typhi and Paratyphi A in Kathmandu, Nepal: A Matched Case Control Investigation in a Highly Endemic Enteric Fever Setting. *PLoS Negl. Trop. Dis.* **7**, (2013).

31. Watson, C. H. & Edmunds, W. J. A review of typhoid fever transmission dynamic models and economic evaluations of vaccination. *Vaccine* **33 Suppl 3**, C42-54 (2015).

32. Baker, S., Blohmke, C. J., Maes, M., Johnston, P. I. & Darton, T. C. The Current Status of Enteric Fever Diagnostics and Implications for Disease Control. *Clin. Infect. Dis.* **71**, S64–S70 (2020).

33. Wang, S. K. *et al.* Study on blood cultures and bacteria counts in the blood of paratyphoid fever A patients. *Eur. J. Clin. Microbiol. Infect. Dis.* **28**, 1259–1261 (2009).

34. Wain, J. *et al.* Quantitation of Bacteria in Blood of Typhoid Fever Patients and Relationship between Counts and Clinical Features, Transmissibility, and Antibiotic Resistance. *J. Clin. Microbiol.* **36**, 1683–1687 (1998).

35. Antillon, M., Saad, N. J., Baker, S., Pollard, A. J. & Pitzer, V. E. The Relationship between Blood Sample Volume and Diagnostic Sensitivity of Blood Culture for Typhoid and Paratyphoid Fever: A Systematic Review and Meta-Analysis. *Journal of Infectious Diseases* vol. 218 S255–S267 (2018).

36. Crump, J. A., Sjölund-Karlsson, M., Gordon, M. A. & Parry, C. M. Epidemiology, clinical presentation, laboratory diagnosis, antimicrobial resistance, and antimicrobial management of invasive Salmonella infections. *Clinical Microbiology Reviews* vol. 28 901–937 (2015).

37. Strobel, A. G. *et al.* Assessment of Rapid Diagnostic Tests for Typhoid Diagnosis and Assessment of Febrile Illness Outbreaks in Fiji. *Am. J. Trop. Med. Hyg.* **106**, 543–549 (2021).

38. Blohmke, C. J. *et al.* Diagnostic host gene signature for distinguishing enteric fever from other febrile diseases. *EMBO Mol. Med.* **11**, (2019).

39. Baker, S., Favorov, M. & Dougan, G. Searching for the elusive typhoid diagnostic. *BMC Infect. Dis.* **10**, 1–8 (2010).

40. O'Reilly, P. J., Pant, D., Shakya, M., Basnyat, B. & Pollard, A. J. Progress in the overall understanding of typhoid fever: implications for vaccine development. *Expert Rev. Vaccines* **19**, 367–382 (2020).

41. Wong, V. K. *et al.* Phylogeographical analysis of the dominant multidrug-resistant H58 clade of Salmonella Typhi identifies inter- and intracontinental transmission events. *Nat. Genet. 2015 476* **47**, 632–639 (2015).

42. Khanam, F., Ross, A. G., McMillan, N. A. J. & Qadri, F. Towards Typhoid Fever Elimination. *Int. J. Infect. Dis.* (2022) doi:10.1016/J.IJID.2022.03.036.

43. Klemm, E. J. *et al.* Emergence of an extensively drug-resistant Salmonella enterica serovar typhi clone harboring a promiscuous plasmid encoding resistance to fluoroquinolones and third-generation cephalosporins. *MBio* **9**, (2018).

44. Kamal, R. *et al.* Identification of Multiple Variant Extensively Drug-Resistant Typhoid Infections across Pakistan. *Am. J. Trop. Med. Hyg.* (2023) doi:10.4269/AJTMH.22-0071.

45. Zuckerman, J. N., Hatz, C. & Kantele, A. Review of current typhoid fever vaccines, cross-protection against paratyphoid fever, and the European guidelines. *Expert Review of Vaccines* vol. 16 1029–1043 (2017).

46. Parry, C. M. *et al.* Randomized controlled comparison of ofloxacin, azithromycin, and an

ofloxacin-azithromycin combination for treatment of multidrug-resistant and nalidixic acid-resistant typhoid fever. *Antimicrob. Agents Chemother.* **51**, 819–825 (2007).

47. Sajib, M. S. I. *et al.* Trends in antimicrobial resistance amongst Salmonella Paratyphi A isolates in Bangladesh: 1999–2021. *PLoS Negl. Trop. Dis.* **17**, e0011723 (2023).

48. Prabhurajan, R., Selvam, R. M. E. & Padmavathy, K. Unusual antibiotic resistance pattern among blood culture isolates of Salmonella Paratyphi A. *J. Infect. Dev. Ctries.* **15**, 579–583 (2021).

49. Dutta, S. *et al.* Antimicrobial resistance, virulence profiles and molecular subtypes of Salmonella enterica serovars typhi and paratyphi A blood isolates from Kolkata, India during 2009-2013. *PLoS One* **9**, (2014).

50. Levine, M. M., Ferreccio, C., Black, R. E., Tacket, C. O. & Germanier, R. Progress in Vaccines Against Typhoid Fever. *Clin. Infect. Dis.* **11**, S552–S567 (1989).

51. Path, F. R. C. & Biol, M. I. CURRENT SURVEY On vaccines and vaccination: typhoid-paratyphoid fevers WALLY VELLA. *Postgrad. Med. J.* **48**, 98–106 (1972).

52. Drysdale, A. A comparative study of phenolized and alcoholized T.A.B vaccines. doi:10.1017/S0022172400013644.

53. Ivanoff, B., Levine, M. M. & Lambert, P. H. Vaccination against typhoid fever: present status. *Bull. World Health Organ.* **72**, 957 (1994).

54. Wahdan, M. H., Serie, C., Cerisier, Y., Sallam, S. & Germanier, R. A Controlled Field Trial of Live Salmonella typhi Strain Ty 21a Oral Vaccine Against Typhoid: Three-Year Results. *J. Infect. Dis.* **145**, 292–295 (1982).

55. Levine, M. M., Black, R. E., Ferreccio, C. & Germanier, R. LARGE-SCALE FIELD TRIAL OF TY21A LIVE ORAL TYPHOID VACCINE IN ENTERIC-COATED CAPSULE FORMULATION. *Lancet* **329**,

1049–1052 (1987).

56. Levine, M. M. *et al.* Duration of efficacy of Ty21a, attenuated Salmonella typhi live oral vaccine. *Vaccine* **17**, (1999).

57. Martin, L. B. Vaccines for typhoid fever and other salmonelloses. *Curr. Opin. Infect. Dis.* **25**, 489–499 (2012).

58. Hindle, Z. *et al.* Characterization of Salmonella enterica derivatives harboring defined aroC and Salmonella pathogenicity island 2 type III secretion system (ssaV) mutations by immunization of healthy volunteers. *Infect. Immun.* **70**, 3457–3467 (2002).

59. Kirkpatrick, B. O. *et al.* The novel oral typhoid vaccine M01ZH09 is well tolerated and highly immunogenic in 2 vaccine presentations. *J. Infect. Dis.* **192**, 360–366 (2005).

60. Tran, T. H. *et al.* A Randomised Trial Evaluating the Safety and Immunogenicity of the Novel Single Oral Dose Typhoid Vaccine M01ZH09 in Healthy Vietnamese Children. *PLoS One* **5**, e11778 (2010).

61. Kirkpatrick, B. D. *et al.* Evaluation of Salmonella enterica serovar Typhi (Ty2 aroC-ssaV-) M01ZH09, with a defined mutation in the Salmonella pathogenicity island 2, as a live, oral typhoid vaccine in human volunteers. *Vaccine* **24**, 116–123 (2006).

62. Darton, T. C. *et al.* Using a Human Challenge Model of Infection to Measure Vaccine Efficacy: A Randomised, Controlled Trial Comparing the Typhoid Vaccines M01ZH09 with Placebo and Ty21a. *PLoS Negl. Trop. Dis.* **10**, e0004926 (2016).

63. Tacket, C. O. & Levine, M. M. CVD 908, CVD 908-htrA, and CVD 909 Live Oral Typhoid Vaccines: A Logical Progression. *Clin. Infect. Dis.* **45**, S20–S23 (2007).

64. Tacket, C. O., Pasetti, M. F., Sztein, M. B., Livio, S. & Levine, M. M. Immune Responses to an Oral Typhoid Vaccine Strain That Is Modified to Constitutively Express Vi Capsular

Polysaccharide. *J. Infect. Dis.* **190**, 565–570 (2004).

65. Wahid, R., Kotloff, K. L., Levine, M. M. & Sztein, M. B. Cell mediated immune responses elicited in volunteers following immunization with candidate live oral Salmonella enterica serovar Paratyphi A attenuated vaccine strain CVD 1902. *Clin. Immunol.* **201**, 61–69 (2019).

66. McCann, N. *et al.* Accelerating clinical development of a live attenuated vaccine against Salmonella Paratyphi A (VASP): study protocol for an observer-participant-blind randomised control trial of a novel oral vaccine using a human challenge model of Salmonella Paratyphi A infection in healthy adult volunteers. *BMJ Open* **13**, (2023).

67. Milligan, R., Paul, M., Richardson, M. & Neuberger, A. Vaccines for preventing typhoid fever. *Cochrane Database of Systematic Reviews* vol. 2018 (2018).

68. Fraser, A., Paul, M., Goldberg, E., Acosta, C. J. & Leibovici, L. Typhoid fever vaccines: Systematic review and meta-analysis of randomised controlled trials. *Vaccine* **25**, 7848–7857 (2007).

69. Wang, Z. G., Zhou, W. Z. & Shi, J. Efficacy and side effects following immunization with Salmonella typhi Vi capsular polysaccharide vaccine. *Zhonghua Liu Xing Bing Xue Za Zhi* **18**, 26–29 (1997).

70. Rijkers, G. T., Sanders, E. A. M., Breukels, M. A. & Zegers, B. J. M. Infant B cell responses to polysaccharide determinants. *Vaccine* **16**, 1396–1400 (1998).

71. Froeschle, J. E. & Decker, M. D. Duration of Vi antibodies in participants vaccinated with Typhim Vi (Typhoid Vi polysaccharide vaccine) in an area not endemic for typhoid fever. *Vaccine* **28**, 1451–1453 (2010).

72. Szu, S. C., Stone, A. L., Robbins, J. D., Schneerson, R. & Robbins, J. B. Vi capsular polysaccharide-protein conjugates for prevention of typhoid fever. Preparation, characterization, and immunogenicity in laboratory animals. *J. Exp. Med.* **166**, 1510–1524

(1987).

73. Kossaczka, Z. *et al.* Safety and Immunogenicity of Vi Conjugate Vaccines for Typhoid Fever in Adults, Teenagers, and 2- to 4-Year-Old Children in Vietnam. *Infect. Immun.* **67**, 5806–5810 (1999).

74. Lin, F. Y. C. *et al.* The Efficacy of a Salmonella typhi Vi Conjugate Vaccine in Two-to-Five-Year-Old Children . *N. Engl. J. Med.* **344**, 1263–1269 (2001).

75. Voysey, M. & Pollard, A. J. Seroefficacy of VI polysaccharide–tetanus toxoid typhoid conjugate vaccine (typbar TCV). *Clin. Infect. Dis.* **67**, 18–24 (2018).

76. Jin, C. *et al.* Efficacy and immunogenicity of a Vi-tetanus toxoid conjugate vaccine in the prevention of typhoid fever using a controlled human infection model of Salmonella Typhi: a randomised controlled, phase 2b trial. *Lancet* **390**, 2472–2480 (2017).

77. Shakya, M. *et al.* Efficacy of typhoid conjugate vaccine in Nepal: final results of a phase 3, randomised, controlled trial. *Lancet Glob. Heal.* **9**, e1561–e1568 (2021).

78. Patel, P. D. *et al.* Safety and Efficacy of a Typhoid Conjugate Vaccine in Malawian Children. *N. Engl. J. Med.* **385**, 1104–1115 (2021).

79. Konadu, E. Y. *et al.* Phase 1 and phase 2 studies of Salmonella enterica serovar paratyphi A O-specific polysaccharide-tetanus toxoid conjugates in adults, teenagers, and 2- to 4-year-old children in Vietnam. *Infect. Immun.* **68**, 1529–1534 (2000).

80. Kulkarni, P. S. *et al.* Safety and Immunogenicity of a Bivalent Paratyphoid A-Typhoid Conjugate Vaccine in Healthy Indian Adults: A Phase I, Randomized, Active Controlled Study. doi:10.2139/SSRN.4546789.

81. MacLennan, C. A., Stanaway, J., Grow, S., Vannice, K. & Steele, A. D. Salmonella Combination Vaccines: Moving Beyond Typhoid. *Open Forum Infect. Dis.* **10**, S58–S66 (2023).

82. Sabbagh, S. C., Forest, C. G., Lepage, C., Leclerc, J. M. & Daigle, F. So similar, yet so different: uncovering distinctive features in the genomes of Salmonella enterica serovars Typhimurium and Typhi. *FEMS Microbiol. Lett.* **305**, 1–13 (2010).

83. Chart, H. *et al.* Serodiagnosis of Salmonella enterica serovar Typhi and S. enterica serovars Paratyphi A, B and C human infections. *J. Med. Microbiol.* **56**, 1161–1166 (2007).

84. Wang, B. X., Butler, D. S., Hamblin, M. & Monack, D. M. One species, different diseases: the unique molecular mechanisms that underlie the pathogenesis of typhoidal Salmonella infections. *Curr. Opin. Microbiol.* **72**, 102262 (2023).

85. McClelland, M. *et al.* Comparison of genome degradation in Paratyphi A and Typhi, human-restricted serovars of Salmonella enterica that cause typhoid. *Nat. Genet.* **36**, 1268–1274 (2004).

86. Brenneman, K. E., Willingham, C., Kong, W., Curtiss, R. & Roland, K. L. Low-pH Rescue of Acid-Sensitive Salmonella enterica Serovar Typhi Strains by a Rhamnose-Regulated Arginine Decarboxylase System. *J. Bacteriol.* **195**, 3062 (2013).

87. Rychlik, I. & Barrow, P. A. Salmonella stress management and its relevance to behaviour during intestinal colonisation and infection. *FEMS Microbiol. Rev.* **29**, 1021–1040 (2005).

88. Townsend, S. M. *et al.* Salmonella enterica Serovar Typhi Possesses a Unique Repertoire of Fimbrial Gene Sequences. *Infect. Immun.* **69**, 2894 (2001).

89. Gal-Mor, O., Boyle, E. C. & Grassl, G. A. Same species, different diseases: How and why typhoidal and non-typhoidal Salmonella enterica serovars differ. *Frontiers in Microbiology* vol. 5 391 (2014).

90. Winter, S. E., Winter, M. G., Poon, V., Keestra, A. M. & Sterzenbach, T. Salmonella enterica Serovar Typhi Conceals the Invasion-Associated Type Three Secretion System from the Innate Immune System by Gene Regulation. *PLoS Pathog* **10**, 1004207 (2014).

91. Haneda, T. *et al.* The capsule-encoding viaB locus reduces intestinal inflammation by a Salmonella pathogenicity island 1-independent mechanism. *Infect. Immun.* **77**, 2932–2942 (2009).

92. Hiyoshi, H. *et al.* Mechanisms to Evade the Phagocyte Respiratory Burst Arose by Convergent Evolution in Typhoidal Salmonella Serovars. *CellReports* **22**, 1787–1797 (2018).

93. Mylona, E. *et al.* Very long O-antigen chains of Salmonella Paratyphi A inhibit inflammasome activation and pyroptotic cell death. *Cell. Microbiol.* cmi.13306 (2020) doi:10.1111/cmi.13306.

94. Hume, P. J., Singh, V., Davidson, A. C. & Koronakis, V. Swiss Army Pathogen: The Salmonella Entry Toolkit. *Front. Cell. Infect. Microbiol.* **7**, (2017).

95. Nikolaus, T. *et al.* SseBCD proteins are secreted by the type III secretion system of Salmonella pathogenicity island 2 and function as a translocon. *J. Bacteriol.* **183**, 6036–6045 (2001).

96. Figueira, R. & Holden, D. W. Functions of the Salmonella pathogenicity island 2 (SPI-2) type III secretion system effectors. *Microbiology* **158**, 1147–1161 (2012).

97. Garai, P., Gnanadhas, D. P. & Chakravortty, D. Salmonella enterica serovars Typhimurium and Typhi as model organisms. *Virulence* **3**, 377–388 (2012).

98. Dalebroux, Z. D. & Miller, S. I. Salmonellae PhoPQ regulation of the outer membrane to resist innate immunity. *Curr. Opin. Microbiol.* **17**, 106 (2014).

99. Watson, K. G. & Holden, D. W. Dynamics of growth and dissemination of Salmonella in vivo. *Cell. Microbiol.* **12**, 1389–1397 (2010).

100. Darton, T. C. *et al.* Blood culture-PCR to optimise typhoid fever diagnosis after controlled human infection identifies frequent asymptomatic cases and evidence of primary bacteraemia. *J. Infect.* **74**, 358–366 (2017).

101. Zhang, L. F. *et al.* The Vi Capsular Polysaccharide of Salmonella Typhi Promotes Macrophage Phagocytosis by Binding the Human C-Type Lectin DC-SIGN. *MBio* **13**, (2022).

102. Zhang, L. F. *et al.* The Vi Capsular Polysaccharide of Salmonella Typhi Promotes Macrophage Phagocytosis by Binding the Human C-Type Lectin DC-SIGN. *MBio* (2022) doi:10.1128/MBIO.02733-22.

103. Voedisch, S. *et al.* Mesenteric lymph nodes confine dendritic cell-mediated dissemination of Salmonella enterica serovar Typhimurium and limit systemic disease in mice. *Infect. Immun.* **77**, 3170–3180 (2009).

104. Vazquez-Terres, A. *et al.* Extraintestinal dissemination of Salmonella by CD18-expressing phagocytes. *Nat. 1999 4016755* **401**, 804–808 (1999).

105. Brown, S. P. *et al.* Intracellular Demography and the Dynamics of Salmonella enterica Infections. *PLOS Biol.* **4**, e349 (2006).

106. Sheppard, M. *et al.* Dynamics of bacterial growth and distribution within the liver during Salmonella infection. *Cell. Microbiol.* **5**, 593–600 (2003).

107. Gibani, M. M. *et al.* The impact of vaccination and prior exposure on stool shedding of salmonella typhi and salmonella paratyphi in 6 controlled human infection studies. *Clin. Infect. Dis.* **68**, 1265–1273 (2019).

108. Gunn, J. S. *et al.* Salmonella chronic carriage: epidemiology, diagnosis, and gallbladder persistence. *Trends Microbiol.* **22**, 648–655 (2014).

109. Dongol, S. *et al.* The Microbiological and Clinical Characteristics of Invasive Salmonella in Gallbladders from Cholecystectomy Patients in Kathmandu, Nepal. *PLoS One* **7**, e47342 (2012).

110. González, J. F., Hitt, R., Laipply, B. & Gunn, J. S. The Effect of the Gallbladder Environment

during Chronic Infection on Salmonella Persister Cell Formation. *Microorganisms* **10**, 2276 (2022).

111. Dunstan, S. J. *et al.* Genes of the class II and class III major histocompatibility complex are associated with typhoid fever in Vietnam. *J. Infect. Dis.* **183**, 261–268 (2001).

112. Crump, J. A. *et al.* Invasive bacterial and fungal infections among hospitalized HIV-infected and HIV-uninfected children and infants in northern Tanzania. *Trop. Med. Int. Heal.* **16**, 830–837 (2011).

113. Bhuiyan, M. S. *et al.* Cellular and Cytokine Responses to Salmonella enterica Serotype Typhi Proteins in Patients with Typhoid Fever in Bangladesh. *Am. J. Trop. Med. Hyg.* **90**, 1024–1030 (2014).

114. Sheikh, A. *et al.* Interferon-γ and Proliferation Responses to Salmonella enterica Serotype Typhi Proteins in Patients with S. Typhi Bacteremia in Dhaka, Bangladesh. *PLoS Negl. Trop. Dis.* **5**, (2011).

115. Blohmke, C. J. *et al.* Interferon-driven alterations of the host's amino acid metabolism in the pathogenesis of typhoid fever. *J. Exp. Med.* **213**, 1061–1077 (2016).

116. McArthur, M. A. & Sztein, M. B. Heterogeneity of Multifunctional IL-17A Producing S. Typhi-Specific CD8+ T Cells in Volunteers following Ty21a Typhoid Immunization. *PLoS One* **7**, e38408 (2012).

117. Lundin, B. S., Johansson, C. & Svennerholm, A. M. Oral immunization with a Salmonella enterica serovar typhi vaccine induces specific circulating mucosa-homing CD4+ and CD8+ T cells in humans. *Infect. Immun.* **70**, 5622–5627 (2002).

118. Fresnay, S. *et al.* Salmonella Typhi-specific multifunctional CD8+ T cells play a dominant role in protection from typhoid fever in humans. *J. Transl. Med.* **14**, 1–14 (2016).

119. Fresnay, S. *et al.* Importance of Salmonella typhi-responsive CD8+ T cell immunity in a human typhoid fever challenge model. *Front. Immunol.* **8**, 208 (2017).

120. McArthur, M. A. *et al.* Activation of Salmonella Typhi-Specific Regulatory T Cells in Typhoid Disease in a Wild-Type S. Typhi Challenge Model. *PLOS Pathog.* **11**, e1004914 (2015).

121. Salerno-Goncalves, R. *et al.* Challenge of humans with wild-type Salmonella enterica serovar typhi elicits changes in the activation and homing characteristics of mucosal-associated invariant T cells. *Front. Immunol.* **8**, 398 (2017).

122. Salerno-Gonçalves, R. *et al.* Mucosal-Associated Invariant T cells exhibit distinct functional signatures associated with protection against typhoid fever. *Cell. Immunol.* **378**, 104572 (2022).

123. Salerno-Gonçalves, R. *et al.* Mucosal-Associated Invariant T cells exhibit distinct functional signatures associated with protection against typhoid fever. *Cell. Immunol.* **378**, 104572 (2022).

124. Toapanta, F. R. *et al.* Oral Challenge with Wild-Type Salmonella Typhi Induces Distinct Changes in B Cell Subsets in Individuals Who Develop Typhoid Disease. (2016) doi:10.1371/journal.pntd.0004766.

125. Gibani, M. Investigating Enteric Fever – Application of Controlled Human Infection Models to Understand Enteric Fever Pathogenesis and Immunity. (University of Oxford, 2018).

126. Wahid, R., Simon, R., Zafar, S. J., Levine, M. M. & Sztein, M. B. Live Oral Typhoid Vaccine Ty21a Induces Cross-Reactive Humoral Immune Responses against Salmonella enterica Serovar Paratyphi A and S. Paratyphi B in Humans. *Clin. Vaccine Immunol.* **19**, 825 (2012).

127. Cross, D. L. *et al.* Vi-Vaccinations Induce Heterogeneous Plasma Cell Responses That Associate With Protection From Typhoid Fever. *Front. Immunol.* **11**, (2020).

128. Kantele, A., Pakkanen, S. H., Karttunen, R. & Kantele, J. M. Head-to-Head Comparison of Humoral Immune Responses to Vi Capsular Polysaccharide and Salmonella Typhi Ty21a Typhoid Vaccines-A Randomized Trial. *PLoS One* **8**, 6–8 (2013).

129. Plotkin, S. A. Correlates of protection induced by vaccination. *Clinical and Vaccine Immunology* vol. 17 1055–1065 (2010).

130. Breukels, M. A., Jol-Van der Zijde, E. M., Van Tol, M. J. D. & Rijkers, G. T. Concentration and avidity of anti-Haemophilus influenzae type b (Hib) antibodies in serum samples obtained from patients for whom Hib vaccination failed. *Clin. Infect. Dis.* **34**, 191–197 (2002).

131. Plotkin, S. A. Vaccines: Correlates of Vaccine-Induced Immunity. *Clin. Infect. Dis.* **47**, 401–409 (2008).

132. Dobinson, H. C. *et al.* Evaluation of the Clinical and Microbiological Response to Salmonella Paratyphi A Infection in the First Paratyphoid Human Challenge Model. *Clin. Infect. Dis.* **64**, 1066–1073 (2017).

133. Waddington, C. S. *et al.* An outpatient, ambulant-design, controlled human infection model using escalating doses of salmonella typhi challenge delivered in sodium bicarbonate solution. *Clin. Infect. Dis.* **58**, 1230–1240 (2014).

134. Gibani, M. M. *et al.* Homologous and heterologous re-challenge with Salmonella Typhi and Salmonella Paratyphi A in a randomised controlled human infection model. *PLoS Negl. Trop. Dis.* **14**, e0008783 (2020).

135. Pulickal, A. S. *et al.* Kinetics of the natural, humoral immune response to Salmonella enterica serovar Typhi in Kathmandu, Nepal. *Clin. Vaccine Immunol.* **16**, 1413–1419 (2009).

136. Aiemjoy, K., Seidman, J. C., Charles, R. C. & Andrews, J. R. Seroepidemiology for Enteric Fever: Emerging Approaches and Opportunities. *Open Forum Infect. Dis.* **10**, S21 (2023).

137. Levine, M. M., Black, R. E., Ferreccio, C. & Germanier, R. LARGE-SCALE FIELD TRIAL OF TY21A LIVE ORAL TYPHOID VACCINE IN ENTERIC-COATED CAPSULE FORMULATION. *Lancet* **329**, 1049–1052 (1987).

138. Lindow, J. C., Fimlaid, K. A., Bunn, J. Y. & Kirkpatrick, B. D. Antibodies in action: Role of human opsonins in killing Salmonella enterica serovar Typhi. *Infect. Immun.* **79**, 3188–3194 (2011).

139. Dahora, L. C. *et al.* IgA and IgG1 Specific to Vi Polysaccharide of Salmonella Typhi Correlate With Protection Status in a Typhoid Fever Controlled Human Infection Model. *Front. Immunol.* **10**, (2019).

140. Jin, C. *et al.* Vi-specific serological correlates of protection for typhoid fever. (2020) doi:10.1084/jem.20201116.

141. Jin, C. *et al.* Vi-specific serological correlates of protection for typhoid fever. *J. Exp. Med.* **218**, (2021).

142. Pakkanen, S. H., Kantele, J. M. & Kantele, A. Cross-Reactive Immune Response Induced by the Vi Capsular Polysaccharide Typhoid Vaccine Against Salmonella Paratyphi Strains. *Scand. J. Immunol.* **79**, 222–229 (2014).

143. Wahid, R. *et al.* Live oral Salmonella enterica serovar typhi vaccines Ty21a and CVD 909 induce opsonophagocytic functional antibodies in humans that cross-react with S. Paratyphi a and S. Paratyphi B. *Clin. Vaccine Immunol.* **21**, 427–434 (2014).

144. Plotkin, S. A. Updates on immunologic correlates of vaccine-induced protection. *Vaccine* **38**, 2250–2257 (2020).

145. Heath, P. T. *et al.* Antibody Concentration and Clinical Protection After Hib Conjugate Vaccination in the United Kingdom. *JAMA* **284**, 2334–2340 (2000).

146. Kayhty, H., Peltola, H., Karanko, V. & Makela, P. H. The Protective Level of Serum Antibodies

to the Capsular Polysaccharide of Haemophilus influenzae Type b. *J. Infect. Dis.* **147**, 1100–1100 (1983).

147. Borrow, R., Andrews, N., Goldblatt, D. & Miller, E. Serological basis for use of meningococcal serogroup C conjugate vaccines in the United Kingdom: Reevaluation of correlates of protection. *Infect. Immun.* **69**, 1568–1573 (2001).

148. Jin, C. *et al.* Efficacy and immunogenicity of a Vi-tetanus toxoid conjugate vaccine in the prevention of typhoid fever using a controlled human infection model of Salmonella Typhi: a randomised controlled, phase 2b trial. *Lancet* **390**, 2472–2480 (2017).

149. McCullagh, D. *et al.* Understanding paratyphoid infection: study protocol for the development of a human model of Salmonella enterica serovar Paratyphi A challenge in healthy adult volunteers. *BMJ Open* **5**, 7481 (2015).

150. Salerno-Gonçalves, R., Galen, J. E., Levine, M. M., Fasano, A. & Sztein, M. B. Manipulation of Salmonella typhi gene expression impacts innate cell responses in the human intestinal mucosa. *Front. Immunol.* **9**, 2543 (2018).

151. Hindle, Z. *et al.* Characterization of Salmonella enterica Derivatives Harboring Defined aroC and Salmonella Pathogenicity Island 2 Type III Secretion System (ssaV) Mutations by Immunization of Healthy Volunteers. *Infect. Immun.* **70**, 3457 (2002).

152. Germanier, R. & Fuerer, E. Isolation and Characterization of Gal E Mutant Ty 21a of Salmonella typhi: A Candidate Strain for a Live, Oral Typhoid Vaccine. *J. Infect. Dis.* **131**, 553–558 (1975).

153. Dunstan, S. J., Simmons, C. P. & Strugnell, R. A. In vitro and in vivo stability of recombinant plasmids in a vaccine strain of Salmonella enterica var. Typhimurium. *FEMS Immunol. Med. Microbiol.* **37**, 111–119 (2003).

154. Ackerman, M. E. *et al.* A robust, high-throughput assay to determine the phagocytic activity

of clinical antibody samples. *J. Immunol. Methods* **366**, 8–19 (2011).

155. Manda-Handzlik, A. *et al.* The influence of agents differentiating HL-60 cells toward granulocyte-like cells on their ability to release neutrophil extracellular traps. *Immunol. Cell Biol.* **96**, 413–425 (2018).

156. Martinez, J. E. *et al.* A flow cytometric opsonophagocytic assay for measurement of functional antibodies elicited after vaccination with the 23-valent pneumococcal polysaccharide vaccine. *Clin. Diagn. Lab. Immunol.* **6**, 581–586 (1999).

157. Worley, M. J. *et al.* Neutrophils mediate HIV-specific antibody-dependent phagocytosis and ADCC. *J. Immunol. Methods* **457**, 41–52 (2018).

158. Guo, Y. *et al.* Differentiation of HL-60 cells in serum-free hematopoietic cell media enhances the production of neutrophil extracellular traps. *Exp. Ther. Med.* **21**, 1–1 (2021).

159. Bentley, T. *et al.* Persistence of Antibody After a Vi-Tetanus Toxoid Conjugate Vaccine and Effect of Boosting With a Plain Polysaccharide Vaccine on Vi Antibody and Antigen-Specific B Cells. *Front. Trop. Dis.* **0**, 24 (2021).

160. Necchi, F., Saul, A. & Rondini, S. Development of a high-throughput method to evaluate serum bactericidal activity using bacterial ATP measurement as survival readout. *PLoS One* **12**, 1–15 (2017).

161. Necchi, F., Saul, A. & Rondini, S. Setup of luminescence-based serum bactericidal assay against Salmonella Paratyphi A. *J. Immunol. Methods* **461**, 117–121 (2018).

162. Hart, P. J. *et al.* Differential killing of Salmonella enterica serovar Typhi by antibodies targeting VI and lipopolysaccharide O:9 antigen. *PLoS One* **11**, 1–17 (2016).

163. Juel, H. B. *et al.* Salmonella Typhi bactericidal antibodies reduce disease severity but do not protect against typhoid fever in a controlled human infection model. *Front. Immunol.* **8**, 1–11

(2018).

164. Chanput, W., Mes, J. J. & Wichers, H. J. THP-1 cell line: An in vitro cell model for immune modulation approach. *Int. Immunopharmacol.* **23**, 37–45 (2014).

165. Murphy, K. Travers, P. Walport, M. *Janeway's Immunobiology*. (Garland Science, 2008).

166. Goldschneider, I., Gotschlich, E. C. & Artenstein, M. S. Human immunity to the meningococcus. I. The role of humoral antibodies. *J. Exp. Med.* **129**, 1307–1326 (1969).

167. Balmer, P., Beeslaar, J., Findlow, J. & Srivastava, A. Understanding immunogenicity assessments for meningococcal serogroup B vaccines. *Postgraduate Medicine* vol. 132 184–191 (2020).

168. Dahora, Lindsay C., Celina Jin , Rachel L. Spreng, F. F., Mathura, Ryan., Kelly E. Seaton., Lu Zhang., Jennifer Hill ., Jones E, Alam, S. Munir. S. Moses Dennison, A. J. P. & Tomaras, and G. D. IgA and IgG1 Specific to Vi Polysaccharide of Salmonella Typhi Correlate With Protection Status in a Typhoid Fever Controlled Human Infection Model. *Front. Immunol.* **10**, (2019).

169. Das, S., Chowdhury, R., Pal, A., Okamoto, K. & Das, S. Salmonella Typhi outer membrane protein STIV is a potential candidate for vaccine development against typhoid and paratyphoid fever. *Immunobiology* **224**, 371–382 (2019).

170. Zhang, F. *et al.* A Bivalent MAPS Vaccine Induces Protective Antibody Responses against Salmonella Typhi and Paratyphi A. *Vaccines 2023, Vol. 11, Page 91* **11**, 91 (2022).

171. Boyd, M. A. *et al.* Serum bactericidal assays to evaluate typhoidal and nontyphoidal Salmonella vaccines. *Clin. Vaccine Immunol.* **21**, 712–721 (2014).

172. Ahmadi, H. *et al.* Immunological evaluation of Vi capsular polysaccharide of Salmonella enterica subsp. Typhi vaccine by serum bactericidal assay. *J. Med. Microbiol.* **62**, 283–286 (2013).

173. VALIM, Y. M. L. & LACHMANN, P. J. The effect of antibody isotype and antigenic epitope density on the complement-fixing activity of immune complexes: a systematic study using chimaeric anti-NIP antibodies with human Fc regions. *Clin. Exp. Immunol.* **84**, 1–8 (1991).

174. de Jong, H. K., Parry, C. M., van der Poll, T. & Wiersinga, W. J. Host–Pathogen Interaction in Invasive Salmonellosis. *PLoS Pathog.* **8**, (2012).

175. Hu, X. *et al.* Vi capsular polysaccharide: Synthesis, virulence, and application. *Crit. Rev. Microbiol.* **43**, 440–452 (2017).

176. Yang, J. S., An, S. J., Jang, M. S., Song, M. & Han, S. H. IgM specific to lipopolysaccharide of Vibrio cholerae is a surrogate antibody isotype responsible for serum vibriocidal activity. *PLoS One* **14**, 1–16 (2019).

177. Harris, J. B. Cholera: Immunity and prospects in vaccine development. *J. Infect. Dis.* **218**, S141–S146 (2018).

178. Shimanovich, A. A. *et al.* Functional and antigen-specific serum antibody levels as correlates of protection against shigellosis in a controlled human challenge study. *Clin. Vaccine Immunol.* **24**, 1–13 (2017).

179. Bernshtein, B. *et al.* Systems approach to define humoral correlates of immunity to Shigella. *Cell Rep.* **40**, 111216 (2022).

180. Sow, S. O. *et al.* Randomized, placebo-controlled, double-blind phase 2 trial comparing the reactogenicity and immunogenicity of a single standard dose to those of a high dose of CVD 103-HgR live attenuated oral cholera vaccine, with shanchol inactivated oral vaccine as an open-label immunologic comparator. *Clin. Vaccine Immunol.* **24**, (2017).

181. Gat, O. *et al.* Cell-associated flagella enhance the protection conferred by mucosally-administered attenuated Salmonella paratyphi A vaccines. *PLoS Negl. Trop. Dis.* **5**, (2011).

182. Shalaby, W. S. W. Development of oral vaccines to stimulate mucosal and systemic immunity: Barriers and novel strategies. *Clin. Immunol. Immunopathol.* **74**, 127–134 (1995).

183. Zhu, Q. & Berzofsky, J. A. Oral vaccines: Directed safe passage to the front line of defense. *Gut Microbes* **4**, 246 (2013).

184. Steffen, U. *et al.* IgA subclasses have different effector functions associated with distinct glycosylation profiles. *Nat. Commun.* **11**, (2020).

185. Toh, Z. Q. *et al.* Evaluating functional immunity following encapsulated bacterial infection and vaccination. *Vaccines* **9**, 677 (2021).

186. Kamen, L. *et al.* A novel method for determining antibody-dependent cellular phagocytosis. *J. Immunol. Methods* **468**, 55–60 (2019).

187. Arnold, K. B. & Chung, A. W. Prospects from systems serology research. *Immunology* **153**, 279–289 (2018).

188. Tay, M. Z., Wiehe, K. & Pollara, J. Antibody dependent cellular phagocytosis in antiviral immune responses. *Front. Immunol.* **10**, 332 (2019).

189. Chung, A. W. Multifaceted roles of antibody Fc effector functions: from protection to pathology. *Immunology and Cell Biology* vol. 98 251–252 (2020).

190. Duchemin, M., Tudor, D., Cottignies-Calamarte, A. & Bomsel, M. Antibody-Dependent Cellular Phagocytosis of HIV-1-Infected Cells Is Efficiently Triggered by IgA Targeting HIV-1 Envelope Subunit gp41. *Front. Immunol.* **11**, (2020).

191. GR, P., JF, G., J, C., SA, R. & SJ, F. The Role of Macrophages in Staphylococcus aureus Infection. *Front. Immunol.* **11**, (2021).

192. Kassa, M. W., Hasang, W. & Rogerson, S. J. Antibody-Dependent THP-1 Cell-Mediated Phagocytosis of Plasmodium falciparum-Infected Erythrocytes. *Methods Mol. Biol.* **2470**, 617–

628 (2022).

193. Ackerman, Margaret E. Nimmerjahn, F. *Antibody Fc: Chapter 2. Antibody-Dependent Cellular Phagocytosis and Its ... - Stefan S. Weber, Annette Oxenius - Google Books*.

194. Bowman, K. A., Kaplonek, P. & McNamara, R. P. Understanding Fc function for rational vaccine design against pathogens. *MBio* (2023) doi:10.1128/MBIO.03036-23.

195. Fisher, R. A., Gollan, B. & Helaine, S. Persistent bacterial infections and persister cells. *Nat. Rev. Microbiol. 2017 158* **15**, 453–464 (2017).

196. Kozarsky, P. E. & Keystone, J. S. The effect of oral and parenteral typhoid vaccination. *Arch. Intern. Med.* **150**, 2422 (1990).

197. Simanjuntak, C. H. *et al.* Oral immunisation against typhoid fever in Indonesia with Ty21a vaccine. *Lancet (London, England)* **338**, 1055–1059 (1991).

198. Baker, S., Blohmke, C. J., Maes, M., Johnston, P. I. & Darton, T. C. The current status of enteric fever diagnostics and implications for disease control. *Clin. Infect. Dis.* **71**, S64–S70 (2020).

199. Jones, C., Mermelstein, N., Kincaid-Smith, P., Powellt, H. & Roberton, D. Quantitation of human serum polymeric IgA, IgA1 and IgA2 immunoglobulin by enzyme immunoassay. *Clin. Exp. Immunol.* **72**, 344 (1988).

200. Scott, N. A. & Mann, E. R. Regulation of mononuclear phagocyte function by the microbiota at mucosal sites. *Immunology* **159**, 26 (2020).

201. Pakkanen, S. H., Kantele, J. M. & Kantele, A. Cross-reactive gut-directed immune response against Salmonella enterica serovar Paratyphi A and B in typhoid fever and after oral Ty21a typhoid vaccination. *Vaccine* **30**, 6047–6053 (2012).

202. Tagliabue, A. *et al.* IgA-driven T cell-mediated anti-bacterial immunity in man after live oral Ty 21a vaccine. *J. Immunol.* **137**, 1504–1510 (1986).

203. Heil, T. L., Volkmann, K. R., Wataha, J. C. & Lockwood, P. E. Human peripheral blood monocytes versus THP-1 monocytes for in vitro biocompatibility testing of dental material components. *J. Oral Rehabil.* **29**, 401–407 (2002).

204. Hirayama, D., Iida, T. & Nakase, H. The Phagocytic Function of Macrophage-Enforcing Innate Immunity and Tissue Homeostasis. *Int. J. Mol. Sci. 2018, Vol. 19, Page 92* **19**, 92 (2017).

205. Wang, Y. & Jönsson, F. Expression, role, and regulation of neutrophil Fcγ receptors. *Front. Immunol.* **10**, 474374 (2019).

206. Kobayashi, S. D., Malachowa, N. & DeLeo, F. R. Neutrophils and Bacterial Immune Evasion. *J. Innate Immun.* **10**, 432–441 (2018).

207. Klugman, K. P. *et al.* Protective activity of Vi capsular polysaccharide vaccine against typhoid fever. *Lancet* **330**, 1165–1169 (1987).

208. Johnson, M. *et al.* Association of Antibody-Dependent Neutrophil Phagocytosis With Distinct Antibody Glycosylation Profiles Following Typhoid Vaccination. *Front. Trop. Dis.* **2**, 25 (2021).

209. Jódar, L. *et al.* Serological criteria for evaluation and licensure of new pneumococcal conjugate vaccine formulations for use in infants. *Vaccine* **21**, 3265–3272 (2003).

210. Martinez, J. *et al.* Opsonophagocytosis of Fluorescent Polystyrene Beads Coupled to Neisseria meningitidis Serogroup A, C, Y, or W135 Polysaccharide Correlates with Serum Bactericidal Activity. *Clin. Diagn. Lab. Immunol.* **9**, 485 (2002).

211. Kotloff, K. L. *et al.* Evaluation of the safety, immunogenicity, and efficacy in healthy adults of four doses of live oral hybrid Escherichia coli-Shigella flexneri 2a vaccine strain EcSf2a-2. *Vaccine* **13**, 495–502 (1995).

212. Poolman, J. & Borrow, R. Expert Review of Vaccines Hyporesponsiveness and its clinical implications after vaccination with polysaccharide or glycoconjugate vaccines. (2014)

doi:10.1586/erv.11.8.

213. Bernshtein, B. *et al.* Systems approach to define humoral correlates of immunity to Shigella. *Cell Rep.* **40**, 111216 (2022).

214. Wahid, R., Kotloff, K. L., Levine, M. M. & Sztein, M. B. Cell mediated immune responses elicited in volunteers following immunization with candidate live oral Salmonella enterica serovar Paratyphi A attenuated vaccine strain CVD 1902. *Clin. Immunol.* **201**, 61–69 (2019).

215. Shrivastava, S. *et al.* Oral immunization with Shigella sonnei WRSs2 and WRSs3 vaccine strains elicits systemic and mucosal antibodies with functional anti-microbial activity. *mSphere* (2023) doi:10.1128/MSPHERE.00419-23.

216. Mancini, F. *et al.* Exploring the Role of GMMA Components in the Immunogenicity of a 4-Valent Vaccine against Shigella. *Int. J. Mol. Sci.* **24**, 2742 (2023).

217. Arnold, K. B. & Chung, A. W. Prospects from systems serology research. *Immunology* **153**, 279–289 (2018).

218. Hendrickx-Rodriguez, S., Falvo, M. R., O' Brien, E. T. & Superfine, R. Effects of Opsonin Density and type on the Phagocytosis of Beads. *Biophys. J.* **112**, 90a-91a (2017).

219. Irvine, E. B. & Alter, G. Understanding the role of antibody glycosylation through the lens of severe viral and bacterial diseases. *Glycobiology* **30**, 241–253 (2020).

220. Jacobs, J. F. M., Van Der Molen, R. G., Bossuyt, X. & Damoiseaux, J. Antigen excess in modern immunoassays: To anticipate on the unexpected. *Autoimmun. Rev.* **14**, 160–167 (2015).

221. Tunkel, A. R. & Scheld, W. M. Pathogenesis and pathophysiology of bacterial meningitis. *Clin. Microbiol. Rev.* **6**, 118 (1993).

222. Pulickal, A. S. *et al.* Kinetics of the natural, humoral immune response to Salmonella enterica serovar Typhi in Kathmandu, Nepal. *Clin. Vaccine Immunol.* **16**, 1413–1419 (2009).

223. Cash, R. A. *et al.* Response of Man to Infection with Vibrio cholerae. II. Protection from Illness Afforded by Previous Disease and Vaccine. *J. Infect. Dis.* **130**, 325–333 (1974).

224. Moor, K. & Slack, E. What Makes A Bacterial Oral Vaccine a Strong Inducer of High-Affinity IgA Responses? *Antibodies 2015, Vol. 4, Pages 295-313* **4**, 295–313 (2015).

225. Chen, W. H. *et al.* Single-dose Live Oral Cholera Vaccine CVD 103-HgR Protects Against Human Experimental Infection With Vibrio cholerae O1 El Tor. *Clin. Infect. Dis.* **62**, 1329–1335 (2016).

226. Kwong, K. W. Y. *et al.* Oral Vaccines: A Better Future of Immunization. *Vaccines 2023, Vol. 11, Page 1232* **11**, 1232 (2023).

227. Parker, E. P. K. *et al.* Causes of impaired oral vaccine efficacy in developing countries. *Future Microbiol.* **13**, 97–118 (2018).

228. WHO technical report series, N. 1004. *WHO guidelines on clinical evaluation of vaccines: regulatory expectations*. (2017).

229. Rappuoli, R., De Gregorio, E. & Costantino, P. On the mechanisms of conjugate vaccines. *Proc. Natl. Acad. Sci. U. S. A.* **116**, 14 (2019).

230. Pollard, A. J. & Bijker, E. M. A guide to vaccinology: from basic principles to new developments. *Nature Reviews Immunology* (2020) doi:10.1038/s41577-020-00479-7.

231. McIntosh, E. D. G., Bröker, M., Wassil, J., Welsch, J. A. & Borrow, R. Serum bactericidal antibody assays - The role of complement in infection and immunity. *Vaccine* **33**, 4414–21 (2015).

232. Co, M. D. T. *et al.* Relationship of Preexisting Influenza Hemagglutination Inhibition, Complement-Dependent Lytic, and Antibody-Dependent Cellular Cytotoxicity Antibodies to the Development of Clinical Illness in a Prospective Study of A(H1N1)pdm09 Influenza in Children. *Viral Immunol.* **27**, 375 (2014).

233. Hooda, Y., Tanmoy, A. M., Saha, S. K. & Saha, S. Genomic Surveillance of Salmonella Paratyphi A: Neglected No More? *Open Forum Infect. Dis.* **10**, S53–S57 (2023).

234. Boero, E., Vezzani, G., Micoli, F., Pizza, M. & Rossi, O. Functional assays to evaluate antibody-mediated responses against Shigella: a review. *Front. Cell. Infect. Microbiol.* **13**, 1171213 (2023).