Investigating Novel Mechanisms of Immunity against Enteric Fever



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Abstract

Approximately 14.9 million cases of enteric fever are caused by *Salmonella* enterica serovar Typhi (*S.* Typhi) and *S.* Paratyphi, with several strains becoming resistant to frontline antibiotics. Consequently, effective vaccination campaigns are needed now more than ever to help tackle disease. In 2018, a Vi-tetanus toxoid conjugate vaccine (Vi-TCV), manufactured by Bharat Biotech and targeting the Vi polysaccharide capsule of *S.* Typhi, was awarded WHO pre-qualification and has since demonstrated promising field efficacy. Given that *S.* Paratyphi can also cause enteric fever, bivalent conjugate vaccines against *S.* Typhi and *S.* Paratyphi are in clinical development. Correlates of protection can be used to evaluate vaccine immunogenicity in target populations and bolster confidence in candidate vaccines. There have been several attempts to establish a protective antibody threshold after typhoid vaccination, but none have yet been successful. In addition, our understanding of genetic risk factors for disease, and vaccine responsiveness is also limited. Therefore, I use a systems serology approach and functional genomics to investigate correlates of protection against *S.* Typhi and further characterise protective immune responses.

Over the past five years, awareness of the role antibody-mediated Fc-mediated effector functions may play in vaccine-mediated immunity has been increasing. In natural typhoidal Salmonella infections, bacteria can evade IgM mediated neutrophil respiratory burst and phagocytosis through expression of the Vi-capsule. Vi-TCV vaccination, however, results in the production of immunoglobulin isotypes that target the Vi-capsule. Therefore, it was postulated that typhoid vaccination may be able to overcome this response, resulting in protection against infection. As a result, I developed assays to assess Fc-mediated neutrophil effector functions such as antibody-dependent neutrophil phagocytosis (ADNP) and the oxidative burst (ADNOB). Next, I associated levels of these Fc-functions with the likelihood of developing clinically detectable typhoid fever in a human challenge model.

Here, I find that ADNOB measured in serum from Vi-TCV and Vi-PS vaccinated participants was significantly higher in those who were not diagnosed with typhoid fever in the human challenge model, despite infection ($P < 0.01$). In participants who were diagnosed with typhoid fever, Higher ADNOB levels 28 days post-vaccination also correlated with a lower bacterial load in blood culture, and a longer time to diagnosis and ($P < 0.05$). Encouragingly, I also observed comparable protective ADNP and ADNOB levels in plasma 28-days following Vi-TCV vaccination in Nepalese children, a key demographic for Vi-TCV vaccination campaigns. This study therefore represents the first evaluation of Fc-mediated neutrophil responses in a target typhoid vaccine cohort.

As relatively little is known about the mechanisms underlying Fc-mediated effector functions after vaccination, I integrated ADNP and ADNOB data with numerous antibody-based measurements, including isotype- and subclass-specific titres, Vi-avidity, Fc-receptor binding, and Fc-glycosylation, as well as age. In particular, glycosylation of the Fc- region has been shown to modify downstream cellular activity; however, it was unknown how Fc-glycosylation could affect ADNP following vaccination. Lower levels of Vi-IgG Fc-sialylation and galactosylation were associated with elevated ADNP activity in Nepalese children following Vi-conjugate vaccination ($P < 0.05$). In addition, I investigated the effect of age and previous *S.* Typhi exposure on ADNP after vaccination and found that the fold change in ADNP from baseline was comparable across all age groups, thereby supporting the expansion of Vi-TCV vaccines to school and infant immunisation programmes.

Immune responses to vaccination are influenced by both environmental and genetic factors. Human challenge models account for environmental factors, such as infectious dose, providing suitable settings for analysing the effect of genetic variation. Several studies have suggested that humoral responses to vaccination have a strong genetic component, however, the impact of genetic variation has not yet been examined in the context of typhoid-vaccination. As Vi-based vaccines are administered to different populations, the impact of genetic variation on vaccine immunogenicity could be used to inform vaccine deployment. Subsequently, I performed a GWAS of Vi-IgA titres following Vi-TCV vaccination in the UK. On chromosome 1, I discovered one SNP with genome-wide significance of $P < 5 \times 10^{-8}$ and several SNPs with suggestive significance $P < 1 \times 10^{-4}$. Using public databases, RNA-sequencing, and pathway analysis; I highlight variants in genes including BAFF and APRIL that were associated with higher IgA titres following vaccination, aswell as gene expression ($P < 0.001$).

Moreover, I use this analysis pipeline to investigate genetic determinants of clinical outcome following *S.* Typhi challenge. No SNPs of genome-wide significance were identified; however, SNPs of suggestive significance revealed a number of novel inflammatory pathways, including platelet response genes. There are a small number of genome-wide association studies (GWAS) on enteric fever, but none have been examined for pathway-based analysis and functional mapping. I therefore demonstrate the applicability of a functional genomics approach that can highlight relevant pathways and genes, thereby enhancing our knowledge of the immune response to *S.* Typhi infection and vaccination. Taken together, this thesis describes the first in depth evaluation of Fc-mediated neutrophil functions and the heritability of humoral responses following typhoid conjugate vaccination.

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It takes a village to complete a PhD, and I am beyond grateful for my village. First and foremost, I would like to thank my mum who has supported me every step of the way, and whose ambition and kindness has been a constant source of inspiration. Thanks to my supervisors, Andy and Irina, for believing in me and this project - I couldn't have done it without you both. I'd like to thank everyone on the enteric team, who have been some of the best co-workers and friends. In particular, Lizzy, who has been there from CL3 assay optimisation, COVID supply drop-off's, and not to mention the occasional holiday. A big thank you to Debbie and Ruth, a.k.a. "Team Gene". Not only did they somehow make blood clot mashing fun, but they also helped produce such exciting data. To Amber Barton for all her GWAS help and advice, as well as Daniel for his insightful comments and feedback.

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Last but not least, I'd like to thank my family and friends. I would not be here if it weren't for you. Dad, you provided essential football games and Budgens trips. Mum, you scooped me when I needed to be scooped; and Maeve you always knew when I needed a sci-fi movie night. Thank you to Elsie for contributing constant entertainment and pets, and to Pouchie for being the best friend anyone could have asked for. Thank you, Alexandra, for always making me smile and waiting for me whilst I dealt with the Queen. Thanks to all my favourite aunties, uncle, and cousins for loving me along the way. To Sandra for instilling a love of muddy fields, and thanks to my football and softball friends for giving me something worth running for. I also couldn't have imagined my time at Oxford without Hannah, Cara and Simon who were the best housemates and friends. Finally, thanks to all my wonderful school pals who were there for me even when I couldn't see them.

Declaration of contributions

Human Challenge Study Oversight

Data from previous *S.* Typhi and *S.* Paratyphi human challenge studies conducted at the Oxford Vaccine Group under the direction of Sir Prof Andrew Pollard were consolidated for genome-wide association analyses. Dr. Tom Darton supervised the typhoid dose-finding trial, while Drs. Claire Waddington and Tom Darton supervised the typhoid oral vaccine study. Dr. Malick Giabani led the typhoid-toxin knock-out trial, and the typhoid/paratyphoid re-challenge investigation. Dr. Celina Jin led the Vi-vaccine study, VAST. Lizzy Jones and Sonu Shresta were also involved across trials as senior research assistants, and numerous OVG laboratory and clinical staff members assisted with sample processing, immunological analyses, and study management.

Humoral Responses to typhoid-vaccination:

For the VAST study, sample preparation and Vi-IgG and Vi-IgA ELISA's were performed by the OVG laboratory team. I developed the ADNOB bead-based method in conjunction with optimizing the ANDP assay using VAST sera. I then performed ANDP and ADNOB assays on all serum samples from participants vaccinated in the VAST trial. Measures of Vi-isotype specific antibody titres and wider Fc-functions were performed by Dr Celina Jin and Dr Jenny Hill inthe Ragon Institute, with oversight from Prof. Galit Alter. Vi-avidity measurements were also performed by Lindsay Dahora at the Ragon. These data are published inthe New England Journal of Medicine. DOI: 10.1084/jem.20201116. The pairwise correlation analysesof these collective data with other Fc-measures was performed by myself and detailed in chapter 3.

The wider TyVAC Nepal immunogenicity and efficacy analysis was led by Mila Shakya, with oversight from Prof Kathleen Neuzil, Prof Shrijana Shrestha, Prof Buddha Basnyat, Prof Andrew J Pollard and Dr. Merryn Voysey. Sample collection was conducted by the clinical team at Patan Hospital, Lalitpur, Nepal, in addition to local community clinics. Dr. Jenny Clarke oversaw Vi-based ELISA's with assistance from Masy Marinou and Molly Guscott. Susan Tonks was influential in the study management between Nepal and Oxford sites. Funding for the Fc-glycosylation project was secured by Dr. Lisa Stockdale, who also purified Vi-specific plasma and prepared samples for downstream analyses. Prof Manfred Wuhrer oversaw the LC-MS and data pre-processing at the Center for Proteomics and Metabolomics at the Leiden University Medical Center. Noortje de Haan, Jan Nouta, and Carolien Koeleman were instrumental in LC-MS performance.

I myself optmised and ran the ADNP assays on Vi-TCV vaccinated plasma samples and subsequently performed the analysis of Fc-glycosylation profiles and ADNP. Drs Lisa Stockdale, Jenny Hill, and Andrew Pollard supervised these sections of my DPhil, outlined in chapter 3. This data is published at Frontiers in Tropical Diseases 10.3389/fitd.2021.742804

Genomics and Transcriptomics:

The collection of samples across enteric human challenge studies was conducted by the OVG clinical staff. RNA and DNA extraction was performed by the OVG laboratory teams for the P1, TyGER, and T1 studies. DNA extraction for the VAST and PATCH typhoid and paratyphoid challenge studies was conducted by myself, Deborah Cross and Ruth Drury. Genotyping for the VAST and PATCH samples was performed at the Tayside Centre for Genomic Analysis, Scotland. All other genotyping was performed at the Wellcome Trust Centre for Human Genetics, Oxford. RNA-sequencing for the TyGER study was performed at the BDI, China, and VAST study samples were sequenced at the Wellcome Sanger institute. Initial alignment of raw-count data to the reference genome were performed by Drs. Amber Barton, Daniel O'Conner and Irina Chelysheva.

Pre-processing, quality control, and integration of genomic and transcriptomic data across enteric studies was performed by myself, as well as all subsequent downstream analyses including GWAS, eQTL, and functional mapping, with supervision from Drs. Irina Chelysheva and Andrew Pollard. These methods are published at https://github.com/mari-lynne.

Impact of the COVID-19 pandemic:

Prior to the pandemic, my thesis had a strong laboratory component. Upon the announcement of the lockdown, all laboratory work related to typhoid fever was halted. This caused significant disruption to my work, and I had yet to perform the Nepal-cohort ADNP analysis. Moreover, upon the re-opening of the University, the Oxford Vaccine Group was in the unique and critical position of running the Astrazeneca COVID-19 vaccine trials. Laboratory activity related to these trials understandably took priority over non-COVID work. However, for myself, this resulted in continued disruption to the use of lab space and equipment, and uncertainty for several months.

In the interim, I switched my focus to researching genetics, leveraging data I could access remotely and had yet to be examined. After running a preliminary analysis, my colleagues and I (see Team Gene), extracted DNA after hours to accommodate the COVID-19 vaccine trial work. These samples were then sent to the University of Aberdeen for further genotyping. However, an additional lockdown, stricter in Scotland, resulted in a 6-month delay in receiving those data. The relaxation of lockdown restrictions also resulted in several COVID infections which I battled with alongside writing this thesis.

Overall, while this thesis is not what I had planned, I am grateful for the time I acquired to learn bioinformatics analyses and investigate genetic factors involved in our immune response to infection and vaccination. Finally, while my PhD may have faced extended disruption, ultimately it was the roll-out of the Oxford-AZ vaccine that enabled myself and many others to go back to work, and I look forward to using my newfound skills in the future.

List of Publications

Johnson, Mari, Lisa Stockdale, Noortje de Haan, Manfred Wuhrer, Jan Nouta, et al. 2021. "Association of Antibody-Dependent Neutrophil Phagocytosis With Distinct Antibody Glycosylation Profiles Following Typhoid Vaccination." *Frontiers in Tropical Diseases* 2 (September). DOI: 10.3389/fitd.2021.742804

Stockdale, Lisa. K., Noortje de Haan, Jennifer Hill, Mari. Johnson, Adriana Tomic, Manfred Wuhrer, Elizabeth. Jones, et al. 2022. "Distinct Glycosylation and Functional Profile of Typhoid Vaccine-Induced Antibodies in a UK Challenge Study and Nepalese Children." *Frontiers in Analytical Science* 2 (November). DOI: 10.3389/frans.2022.1005558

Jin C, Hill J, Gunn BM, Yu WH, Dahora LC, Jones E, Johnson M, Gibani MM, Spreng RL, Alam SM, Nebykova A, Juel HB, Dennison SM, Seaton KE, Fallon JK, Tomaras GD, Alter G, Pollard AJ. "Vi-specific serological correlates of protection for typhoid fever". *Journal of Experimental Medicine*. 2021 Feb DOI: 10.1084/jem.20201116 PMID: 3318

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List of Abbreviations

Abbreviation	Definition
ACK buffer	Ammonium-Chloride-Potassium buffer
BCR	B cell receptor
BSA	Bovine serum albumin
CD	Cluster of differentiation
CFU	Colony forming units
D0	Day 0 - Day of challenge
D 7	Day 7 post-challenge
DC	Dendritic cell
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
eQTL	Expression quantitative trait loci
ER	Endoplasmic reticulum
Fab	Fragment-antibody-binding
FBS	Fetal bovine serum
Fc	Fragment-crystallisable
FcR	Fc-Receptor
FITC	Fluorescein isothiocyanate
FSC	Forward scatter
GFP	Green fluorescent protein
G0	Agalactosylated glycoform
G1	Single-galactose glycoform
G2	Di-galactosylated glycoform
H	H-antigen
HLA	Human leukocyte antigen
IBD	Inflammatory Bowel Disease
IFN	Interferon
IL	Interleukin
iNTS	Invasive Non-typhoidal *Salmonella* Disease
KO	Knock-out
LAMP	Loop-mediated isothermal amplification
LB	Lysogeny broth
LPS	Lipopolysaccharide
MAIT cell	Mucosal associated invariant T cell

MAPK	Mitogen-activated protein kinase
M-cell	Microfold cell
MCP	Macrophage chemoattractant protein
MFI	Median Fluorescence Intensity
MHC	Major histocompatibility complex
MOI	Multiplicity of infection
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NES	Normalised Enrichment Score
Neu5Ac	N-Acetylneuraminic acid
NK cell	Natural killer cell
NO	Nitric oxide
NOS	Nitric oxide synthase
O	O-antigen
OVG	Oxford Vaccine Group
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline
PE	Phycoerythrin
qPCR	Quantitative polymerase chain reaction
RBC	Red blood cell
RDT	Rapid-detection test
RNA-seq	RNA-sequencing
ROS	Reactive oxygen species
SCV	Salmonella-containing vacuole
S. Paratyphi A	Salmonella enterica serovar Paratyphi A
S. Typhi	Salmonella enterica serovar Typhi
S. Typhimurium	Salmonella enterica serovar Typhimurium
Sip	Salmonella Invasion Protein
SNP	Single nucleotide polymorphism
Sop	Salmonella Outer Protein
SPI	Salmonella Pathogenicity Island
Spv	Salmonella plasmid virulence
SSC	Side scatter
TCM	Central memory T cells
TCR	T cell receptor
TD	Typhoid diagnosis
TEM	Effector memory T cells
TEMRA	CD45RA+ effector memory T cells
TH1	Type 1 T helper cell
TLR	Toll like receptor
TN	Toxin-negative
TNF	Tumour-necrosis factor
TREG	Regulatory T cell

TRM	Tissue-resident memory T cells
TSAP	Typhoid Surveillance in Africa
TyVAC	Typhoid Vaccine Acceleration Consortium
VAST	Vaccines Against *Salmonella* Typhi
Vi capsule	Virulence capsule
Vi-PS	Vi capsular polysaccharide vaccine
Vi-TCV	Vi Polysaccharide-Tetanus Toxoid Conjugate Vaccine
WASH	Water, sanitation, hygiene
WHO	World Health Organisation
WT	Wild-type
AHR	Aryl Hydrocarbon Receptor
C1q	Complement component 1q
C3b	Complement component 3b
C4b	Complement component 4b
CCL3	C-C Motif Chemokine Ligand 3
CD40L	Cluster of differentiation 40 Ligand
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
GRO-α	Protein product of CXCL1
RpoS	RNA polymerase sigma factor
T3SS	Type 3 secretion system
SPI-1	Salmonella pathogenicity island 1
STIV	Salmonella invasion protein
MLN	Mesenteric lymph node
Ig	Immunoglobulin
sIgA	Secretory Immunoglobulin A
pIgAr	Poly IgA receptor
CHIM	Controlled human infection model
TAB	Typhoid, Paratyphoid A and B
UDP	enzyme uridine 5'-diphosphate
STIV	*S.* Typhi invasin protein
OMVs	*Salmonella* outer membrane vesicles
MenB	Meningitis B
MenACWY	Meningococcal group A, C, W-135 and Y conjugate vaccine
SBA	Serum bactericidal assay
ADNP	Antibody dependent neutrophil phagocytosis
ADNOB	Antibody dependent neutrophil oxidative burst
ADCD	Antibody dependent complement deposition
ADCC	Antibody dependent cellular cytotoxicity
ADMP	Antibody dependent monocyte phagocytosis
OPKA	Opsonophagocytosis killing assays

GlcNAc	2 N-acetylglucosamine
RA	Rheumatoid arthritis
SLE	Systemic lupus erythematosus
mAb	Monoclonal antibody
Asp	Asparagine
Strep. pneumonia	Streptococcus pneumonia
HIV	Human Immunodeficiency Virus
SARS	Severe Acute Respiratory Syndrome
CGD	Chronic Granulomatous Disorder
SCID	Severe-Combined Immunodeficiency
FUMA	Functional Mapping and Annotation
1KG	1000 Genomes Project
WGS	Whole Genome Sequencing
SV	Structural Variations
NGS	Next Generation Signaling
CNV	Copy Number Variant
HCV	Hepatitis C Virus
IFNλ3	Interferon Lambda 3
IFNAR1/2	Interferon Alpha and Beta Receptor Subunit 1 and 2
STAT1/2	Signal Transducer and Activator of Transcription 1/2
GTEx	Genotype-Tissue Expression Project
scRNA	Single-cell RNA data
mTB	Mycobacterium Tuberculosis
LC-MS	Liquid Chromatography-Mass Spectrometry
D28	28 days Post-Vaccination
PAGE	Population Architecture Using Genomics and Epidemiology (PAGE) Consortium
PAMP	Pathogen Associated Molecular Pattern
TWEAK	TNF-related Weak Inducer of Apoptosis
BAFF	B-cell Activating Factor

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Introduction

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1.1 An Overview of Enteric Fever

Enteric fever is a serious bacterial infection caused by *Salmonella* enterica serovars Typhi and Paratyphi A, B and C, and is a major health concern in developing countries where access to clean water and adequate sanitation is limited. Infections are characterized by febrile illness and sustained bacteremia, which when left untreated can result in a mortality rate of 10-20% (Buckle et al., 2012). Despite the availability of licensced vaccines and treatments, enteric fever remains a major global health problem, with an estimated 12-16 million cases and 135,000 deaths occurring annually (Stanaway et al., 2019). Therefore, understanding the epidemiology, pathogenesis, diagnosis, treatment, and prevention of enteric fever is critical for developing effective public health strategies to control this disease.

Salmonella enterica Pathophysiology

Salmonella enterica (S. enterica) are a highly diverse and pervasive species of bacteria that are responsible for a major burden of human gastrointestinal illness worldwide. *S. enterica* are Gram-negative, anaerobic, rod-shaped bacteria which utilise motile flagella that propel the bacteria through the gut. A member of the Enterobacteriaceae family, *S. enterica* are phenotypically similar to other Gram-negative enteropathogenic bacteria such as *Shigella and E. coli*. Divergence of *Salmonella* and *E. coli* from a common ancestor was estimated to have occurred 100-140 million years ago *(*Vernikos et al 2007). While pathogenic strains of these bacteria may share common type III systems, they have evolved distinct toxins and immune evasion mechanisms (Coburn et al., 2007).

Salmonella enterica has since evolved into six subspecies, of which only subspecies *enterica* can infect mammals, and consists of approximately 2500 serovars. These are classified based on surface antigen presentation, including changes in lipopolysaccharide (LPS), flagellin proteins, and the expression of a polysaccharide Vi-capsule. (Chattaway et al., 2021; Grimont & Weill, 2007) (Table 1.1).

The high level of serovar diversity can be attributed to the bacteria's ability to rapidly adapt to varying environments and selective pressures through increased genetic diversity, which may arise as a result of point mutations and horizontal gene transfer. Additionally, *S. enterica* has the capability to infect a wide range of hosts, each with unique immune systems, anatomical structures, and microbiota, thus selecting for different serovars. The emergence of novel serovars with unique surface antigens, coupled with phase variation, enables the bacteria to evade host immune responses and cause disease in new hosts (Andino et al, 2015; Mastroeni et al., 2009).

***Salmonella* serovar**	**LPS (O)**	**Flagellum (H)**	**Vi**
Paratyphi A	1, 2, 12	a	-
Paratyphi B	1, 4, 5, 12	b	-
Paratyphi C	6, 7	c	+
Typhi	9, 12	d	+
Typhimurium	1,4, (5), 12	i	+

Table 1.1: Antigens include the outermembrane protien LPS (O), H proteins which make up the flagellum, and the Vi-polysachharide capsule. H–proteins are categorised by phase expression a-i.

The majority of *S. enterica* serovars, such as Enteritidis, cause mild gastrointestinal disease, and are responsible for a major burden of foodborne illnesses worldwide (Andino et al, 2015). However some serovars have evolved to cause invasive, systemic disease. These include the human-specific typhoidal *Salmonella* serovars, *S.* Typhi and *S.* Paratyphi A, B, and C, which cause typhoid fever and paratyphoid fever respectively, and can be collectively referred to as enteric fever. Typhoidal *Salmonella* are pathogens that have co-evolved with humans over tens of thousands of years, generating complex mechanisms for invasion and immune evasion (Hiyoshi et al., 2018).

Transmission of *Salmonella* occurs through the use of contaminated food and water; hence disease prevalence is greatest in locations with limited access to clean water, and is the most common cause of blood-stream infections on the South Asian continent (Lozano et al., 2012). Following ingestion, *Salmonella* invade and replicate inside phagocytic cells, promoting their spread to secondary lymphoid tissues and sites (Mastroeni et al., 2009). The release of inflammatory mediators and cytokines results in symptoms which may include fever, headache, abdominal pain, diarrhoea or constipation. If left untreated, severe consequences such as intestinal perforation and sepsis may develop (Birkhold et al., 2020). Compared with *S.* Typhi infections, there is some evidence that *S.* Paratyphi may result in less severe symptoms when studied under controlled settings. However, in clinical settings symptoms are mostly indistinguishable, and a lack of serovar specific diagnostic test limits these observations (Dobinson et al., 2017; Maskey et al., 2006).

Salmonella enterica serovar Typhimurium (*S.* Typhimurium) is genetically similar to typhoidal *Salmonella*, and typically causes infections localised at the gut mucosa, resulting in self-resolving gastroenteritis (Galán, 2021). However, several strains of *S.* Typhimurium can disseminate when infecting immunocompromised individuals, causing invasive non- typhoidal Salmonellosis (iNTS). These infections therefore present a problem in areas with a high rate of

immune impairment due to HIV, nutritional deficiencies, or frequent malaria infections (Jong et al., 2012; Marks et al., 2017).

Effective conjugate vaccines for protection against *S.* Typhi were approved by the WHO in 2018, and their efficacy and immunogenicity have since been investigated in a number of regions endemic for typhoid fever, including Nepal, Malawi, and Bangladesh (Patel et al., 2021; Qadri et al., 2021; Shakya et al., 2019). Due to the widespread geographical distribution of typhoid fever, it is anticipated that numerous vaccinations will be required to meet the expanding demand for protection against antimicrobial resistance (Birger et al., 2022; Pitzer et al., 2014). In addition, *S.* Paratyphi and iNTS vaccines will be essential for reducing the overall burden of enteric disease.

Developing and evaluating vaccines requires detailed knowledge of *Salmonella* pathophysiology and the immune responses that may be protective or detrimental to infection. Historically, the majority of research into thepathogenesis of typhoidal *Salmonella* was conducted using mouse models of invasive *S.* Typhimurium (Larock et al., 2015). The revival of a human challenge model ofenteric fever over the past decade however, has led to comprehensive analyses ofhost-responses against typhoidal *Salmonella* infection. The primary objectives of this thesis are to characterise protective genetic, transcriptomic, and immunological responses to typhoidal *Salmonella* infection and vaccination.

1.1.1 Diagnostics for enteric fever

The gold standard of diagnostics for enteric fever, is the detection of bacterial isolates collected from blood culture or bone marrow biopsies. However, current blood culture methods have low sensitivity rates, detecting between 40–60% presumptive enteric fever cases (Mogasale et al., 2016). Bone marrow isolation, whilst more sensitive, is rarely performed due to the invasive nature of the procedure. In low resource settings, both these procedures may be difficult to perform, and diagnosis therefore often depends on clinical observations and diagnostic algorithms (Baker et al., 2020). This approach is problematic due to the overlapping symptoms of enteric fever with other febrile illnesses such as influenza, dengue and malaria, and most likely will not be able to distinguish between cases of paratyphoid and typhoid (K. Wangdi et al., 2019).

Recent advances in PCR-based methods using a blood culture enrichment step have reported comparable diagnostic sensitivity to standard culture techniques that detect typhoidal *Salmonella* (Neupane et al., 2021). However, the set-up of a PCR detection system is labour intensive and may not always be available in resource limited settings. Loop-mediated isothermal amplification (LAMP) is a simple, cost-effective alternative to PCR that does not require a thermocycler, and can be distributed in small, portable device (Fan et al., 2015). In laboratory settings, LAMP screenings could accurately detect *S.* Paratyphi serovars, with its sensitivity ranging from 100- 87%, comparable with current blood culture techniques (Rojak et al., 2019). Moreover, when detecting *S.* Typhi and *S.* Paratyphi amongst suspected enteric fever cases in New Delhi (n=55), LAMP detected *Salmonella* infections with 100% sensitivity and specificity (Kaur et al., 2019). This study also integrated a pre-enrichment step, using magnetic-nanoparticles conjugated to anti-*Salmonella* polyclonal antibodies, enabling the technique to potentially work with smaller blood volumes.

The Widal test was once widely used to diagnose enteric fever and uses the agglutination of serum with mixtures of LPS, flagellar and Vi antigens obtained from heat-killed *S.* Typhi and *S.* Paratyphi. However, cross-reactivity with other *Salmonellae*, and the difficulty distinguishing between current and past infections, limits its use in endemic settings (Olopoenia & King, 2000; Parry et al., 1999). Over the past decade, serological testing has progressed with rapid detection tests (RDTs) such as TUBEX, a rapid colourimetric based kit which detects *S.* Typhi anti-O9 IgM antibodies, and *S.* Paratyphi anti-O2 antibodies. In clinical settings, TUBEX assays reported 60.2% sensitivity and 89.9% specificity when detecting typhoid fever (Islam et al., 2016). In addition, protein arrays identifying antigens such as HlyE and Ctdb, specific to typhoidal *Salmonella*, were tested in a saliva-based laminar flow kit, with further field testing required to assess the tests true accuracy (Chin Kai Ling et al., 2016; Franklin et al., 2020).

Until improved diagnostics are widely implemented, investigations of enteric fever epidemiology rely on cases confirmed by blood cultures or bone marrow biopsies. However, due to the resource-intensive nature of these methodologies, valid data is generally reliant on costly hospital research and monitoring programmes (Darton et al., 2017). Therefore, enhancing serological testing could enhance enteric fever surveillance, thereby facilitating the adoption of intervention strategies (Imran Khan et al., 2020; Keddy, 2022).

1.2 Epidemiology of Typhoidal *Salmonella*

1.2.1 Modelling the global incidence and case fatality rates of enteric fever

Given the limitations of current enteric fever diagnostics and reporting, generalised mixed-effects linear modelling can be used to predict morbidity and mortality rates for disease (Heesterbeek et al., 2015). To estimate the population burden of enteric fever, these methods integrate data from qualified surveillance studies, death registries, whilst modelling the effects of established risk factors for disease. As typhoidal *Salmonella* are transmitted by contaminated food and water; these risk factors include the regional access to clean water and the quality of sanitation and hygiene systems (WASH) (Gauld et al., 2020; Hussein Gasem et al., 2001).

Access to water and sanitation systems can be stratified even within the same town or city based on household wealth and social class, therefore these factors are also accounted for when estimating the impact of WASH on disease incidence (Ako et al., 2009; Bennett et al., 2018). In addition, the occurrence of monsoons and flooding has been associated with an increase in enteric fever cases, particularly in low-income urban areas, where the overflow of sewage systems can contaminate drinking water (Akullian et al., 2015; Karkey et al., 2010; Mirembe et al., 2019; Saad et al., 2018). Together with population health-seeking behaviours, these risk factors can be used to model enteric fever disease in global regions and areas with limited surveillance data.

Current estimates of global enteric fever incidence and mortality

Stanaway et al. (2019) used one such non-linear mixed effects model (DisMod-MR) to estimate enteric fever morbidity and death both worldwide and within continental regions. Covariates included the amount of exposure to unsafe water, the source of water supply, and the proportion of the population dwelling inside the monsoon belt of the Indian Ocean, blood culture sensitivity and under-reportingrates were also considered. Using historical hospital observation data, researchers

assessed typical fatality rates and predicted a mortality rate of 1% for typhoid fever when adequate medical treatment was administered. However, when calculating the burden of disease, discrepancies in access to medical treatment and the emergence of antibiotic-resistant strains have not yet been adjusted for. Furthermore, data from death registries and hospital observations may struggle to account for rural incidence and there is consequently a level of uncertainty surrounding these estimates. Therefore, it is likely that the number of deaths estimated by global modelling studies is an underestimate.

Using this approach, a high prevalence of typhoidal *Salmonella* disease was identified across several continental regions (Fig 1.1). South Asia was anticipated to have the highest number of typhoid and paratyphoid infections of any global region, with an incidence of 5492 (4807 to 6254) cases of enteric fever per 100,000 people, which was similar with previously reported findings (Crump et al., 2004). Globally, they estimated that in 2017, there were an estimated 10.9 million cases of typhoid fever and 116,000 deaths. In the same year, there were an estimated 3.4 million cases of paratyphoid fever and 19,000 deaths (Stanaway et al., 2019).

Mogasale et al. (2014) also assessed international typhoid fever incidence via a non-linear mixed effects model, and highlighted a high burden of typhoid fever amongst Central and sub-Saharan Africa, comparable to that estimated across South Asia. The authors in this study were able to validate their model using data from the Typhoid Surveillance in Africa Programme (TSAP) but were unable to offer a distinct breakdown of the incidence of paratyphoid (Antillón et al., 2017; Mogasale et al., 2014). Through a systemic literature review, Marchello et al. (2020) likewise identified overlapping confidence ranges for the incidence of typhoid fever across the Asian and African continents. Furthermore, when sequencing for antimicrobial resistance, the multi-drug resistant (MDR) H58 genotype clade was identified within 40% of isolates reported in Nepal, India, Pakistan.

(Dyson et al.,2019). Of concern, the extremely-drug resistant (XDR) H58 clade is currently the predominant strain of *S.* Typhi circulating in Pakistan; with the potential to spread to other regions if outbreaks and transmission are not contained (Wong et al., 2015).

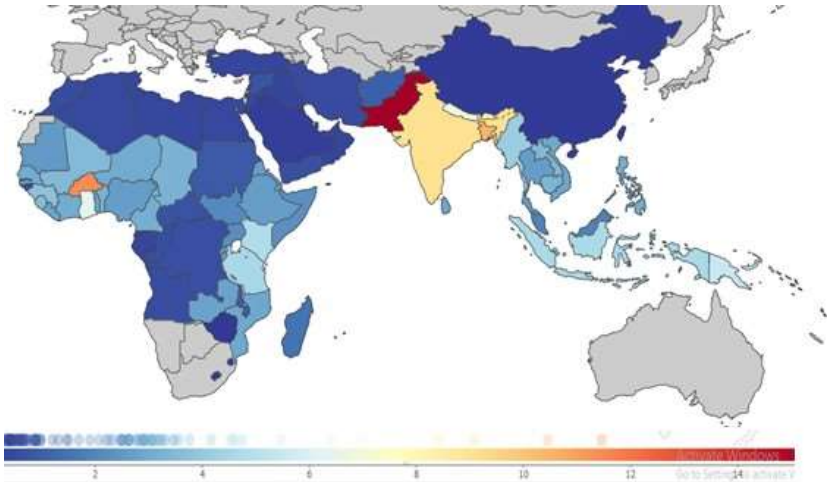


Figure 1.1: Worldwide mortality due to enteric fever Data from the Global Burden of Disease study (2019) was used for visualising the deaths caused by S. Typhi and S. Paratyphi per 100, 000 persons annually across the globe, in adults aged less than 20 years. 0.2 deaths per 100, 000 was used as the minimum threshold.

Epidemiology of *S.* Paratyphi infections

In concordance with observational data, paratyphoid fever was estimated to predominately occur in South and Central Asia, with cases rarely reported across the African continent (Stanaway et al., 2019). Globally, *S.* Paratyphi serovar A is the most prevalent cause of paratyphoid fever, while serovars B and C are seldom detected (Bhan et al., 2005). In several regions across China, *S.* Paratyphi is the most common cause of enteric fever when identified in clinical isolates (Teh et al., 2014). Notably in Guangxi Province, where *S.* Paratyphi was responsible for 90% of all cases of enteric fever (Dong et al., 2010; Jin, 2008). As with *S.* Typhi, there are growing reports of antimicrobial resistance in *S.* Paratyphi infections, particularly in Dhaka and Kathmandu, where 100% of isolates were resistant to fluoroquinolones.

In addition, there have been multiple reports of an increase in the incidence of *S.* Paratyphi as a relative cause of enteric fever over the past three decades

(Sahastrabuddhe et al., 2013). In the same hospital in Kathmandu, Nepal, *S.* Paratyphi cases grew by 34% between 1993 and 2009, and now account for nearly as many blood culture isolates as *S.* Typhi (Zellweger et al., 2017). Researchers observed a 44-fold increase in paratyphoid fever in Phnom Penh, Cambodia, between the surveillance periods of 2007-2010 and 2011-2013 (Kheng et al., 2020; Vlieghe et al., 2013). The incidence of paratyphoid fever has also increased among travellers to countries within Asia, who are prone to consume street food, a risk factor for *S.* Paratyphi infection. However, these patterns have not yet been found in surveillance studies conducted in India and Pakistan. It is therefore unclear whether published rates reflect an absolute increase in the frequency of paratyphoid, a decrease in typhoid incidence due to Vi-based immunisations, or a combination of both (Arndt et al., 2014).

Modelling limitations

Due to the restricted number of surveillance research sites, continental estimates of enteric fever incidence and antibiotic resistance are associated with a high degree of uncertainty. Moreover, although global modelling studies focus on endemic rates of enteric fever, parameters exclude the contribution of outbreaks to disease burden (Appiah et al., 2020; Kim et al., 2019). Enteric fever outbreaks are often caused by natural or man-made disasters that limit access to clean water, hence raising the risk of water-borne infections (Abbara et al., 2021; Ekezie et al., 2021; Kerridge et al., 2013; Makoni, 2018; Rabaan, 2019). It was an outbreak of typhoid fever among packed military personnel which led to the release of the first typhoid vaccine during the first World War (Bollag, 1980; Gradmann et al., 2019; Leishman, 1921; Bradarić et al., 1996; Nawfal Dagher et al., 2020). Subsequently, in regions without access tovaccinations, typhoid outbreaks continue to present a threat to human health; as evidenced in the Democratic Republic of the Congo, where a 2011 typhoid outbreak was connected to water contamination in military camps (E. Ali et al., 2017; Ekezie et al., 2021). These situations thus emphasise the necessity for local surveillance,especially after events that can promote the transmission of enteric fever.

Other poorly surveyed regions with suspected high endemicity of enteric fever include the Pacific Islands of Oceania and parts of South America, for which the majority of data has been derived from vaccine studies and restricted death registries (Aneley Getahun et al., 2019; Ferreccio et al., 1989; Marco et al., 2018; Martins et al., 2015; Salve et al., 2006; Scobie et al., 2014; Sikorski et al., 2020). Increased environmental sampling using low-cost techniques such as Moore Swabs could facilitate future surveillance of enteric fever in these understudied regions (Sikorski & Levine, 2020).

1.2.2 Age-specific incidence of enteric fever

Understanding the distribution of enteric fever across different age groups of children and adults carries implications for the design of vaccination campaigns, and future clinical studies. Globally, the 2-14-year-old age group has the highest estimated cases of enteric fever, with lower incidence estimates in children aged 2 years or less (Meiring et al., 2021; Ochiai et al., 2008; Siddiqui et al., 2006). The reduced burden in the under-two age group is likely a result of the presence of maternal antibodies and a decreased exposure to polluted drinking water while breastfeeding (Baliban et al., 2019; Sinha et al., 1999). However, smaller diagnostic blood volumes for infants and a lower likelihood of blood culture tests may underestimate the true disease burden (Voysey et al., 2020).

The STRAATA surveillance study conducted in Pakistan and Bangladesh, reported the highest prevalence of enteric fever among 5-year-old children, after adjusting for health seeking behaviours and blood-culture sensitivity (John et al., 2016; Saha et al., 2019). Furthermore, in Bangladeshi children aged 2 years, enteric fever was the most prevalent cause of hospitalised infections. In Nepal however, the highest burden of disease was reported within older children and adolescents, aged 5-25 years old. These regional differences in the age distribution of enteric fever cases therefore highlights the importance of local epidemiological knowledge for deploying targeted immunisation campaigns. These findings in

conjunction with higher mortality rates reported in young infants support the WHO recommendations to vaccinate children aged six months to fifteen years. with available Vi-conjugates (Bilcke et al., 2019; Birger et al., 2022; Meiring et al., 2017; World Health Organization, 2019).

Cases of paratyphoid fever exhibited a similar age distribution as those of typhoid (Garrett et al., 2022). However, the median age of children with paratyphoid fever in Kathmandu, Bangladesh, and India was reported to be higher than the median age of typhoid fever infections, likely due to variations in transmission routes and exposure to these serovars (Kanungo et al., 2008; Karkey et al., 2010; Saha et al.,2019). For example, the lower prevalence of *S*. Paratyphi in these areas may result in less frequent exposure, increasing the time taken to build acquired immunity,hence postponing the age at which infection reaches its peak.

1.3 Pathogenesis of typhoidal *Salmonella*

The pathogenesis of typhoidal *Salmonella* is typically studied using mouse models that have been engineered to generate invasive *S*. Typhimurium infections, either by deleting genes needed for preventing *Salmonella* dissemination or, by directly infecting the intestinal peritoneum of mice (Palmer & Slauch, 2017). Due to the distinct genetic and phenotypic features of typhoidal *Salmonella* compared with non-invasive *S*. Typhimurium, as well as the atypical infection routes, there is a limit to which we can interpret results gathered from murine studies of typhoid fever (Sabbagh et al., 2010). In the past fifteen years however; human infection studies and improvements in tissue culture models have expanded our understandingof host-pathogen interactions in enteric fever (A. Barton et al., 2021; Feasey & Levine, 2017), and guided clinical research and therapeutics (Abuaita et al., 2021; Bar-Ephraim et al., 2019).

1.3.1 Mechanisms of entry

Salmonella infections occur through the faecal-oral route, following consumption of contaminated food or water. One of the first barriers *Salmonella* must face is the harsh, acidic conditions of the stomach. Bacteria survival is mediated by the induction of the acid tolerance response in *S*. Typhi, (Foster & Hall, 1990; Parkash Tiwari et al., 2004) whereby hyperosmolar and acidic conditions, switch- on transcriptional factors including *RpoS*, which promotes bacterial survival and replication (Fig 1.3) (Ahirwar et al., 2014). *RpoS* expression has been demonstrated to upregulate bacterial metabolism genes, and *RpoS* knock-out (KO) mutants produced significantly slower growth of *S*. Typhi *in vitro* (X. Zhang et al., 2017).

After passing through the stomach, *Salmonella* reaches the small intestine and may exploit a number of invasion routes (Fig 1.2). Data from murine *S*. Typhimurium models, initially suggested that invasion preferentially takes place using microfold (M-cells) (Clark et al. 1994), which comprise approximately 10% of the intestinal epithelium and surround gut-immune sites known as Peyer's patches (Dillon & Lo, 2019; Lelouard et al., 2012). Initial attachment by *S*. Typhimurium bacteria to the epithelium requires fimbriae, extracellular thread like structures which bind host receptor molecules (C. Forest et al., 2007). However, in *S*. Typhi, many fimbriae genes are pseudogenised, calling into question their importance during typhoidal invasion (Dufresne et al., 2018; Townsend et al., 2001). Following attachment to the cell surface, the *Salmonella* SPI-1 encoded T3SS, injects effector proteins such as SopE, inducing membrane ruffling and cytoskeleton rearrangement. Flagellar expression is also increased under high osmolarity conditions, enabling *Salmonella* to traverse through the epithelial barrier (Fig 1.2). Once through the intestinal barrier, *Salmonella* bacteria can exploit CD18+ macrophages for transport to the nearby Peyer's patch, promoting further replication and eventual dissemination of *Salmonella* into the reticular endothelial system (Vazquez-Terres et al., 1999; Watson & Holden, 2010).

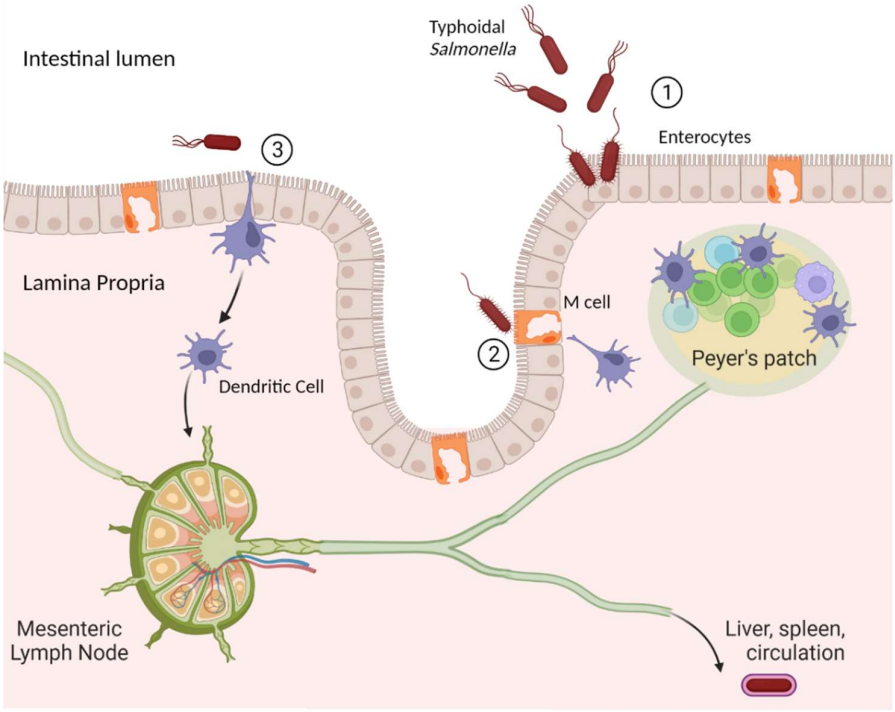


Figure 1.2: Invasion routes of *Salmonella* *S.* Typhi may invade through multiple sites in the small intestine, including by direct invasion of the epithelium (1), through specialised M-cells (2), or by uptake from phagocytic cells including intestinal dendriticand macrophage cell subsets (3). After invasion dissemination to secondary sites including the liver and spleen occurs following access to mesenteric lymph nodes and Peyer's patches. Created using Biorender.com.

However, recent microscopy studies performed using intestinal organoid models, did not observe frequent M-cell based invasion. Instead, *S.* Typhi preferentially invaded the gut via enterocytes lining the intestinal epithelium (Fig 1.2) (Nickerson et al., 2018; Nickerson et al., 2021). Earlier studies comparing invasion routes between *Salmonella* serovars in epithelial cell lines also reported more frequent enterocyte invasion by *S.* Typhi compared with non-typhoidal serovars (Weinstein et al., 1998). One alternate invasion mechanism utilises the STIV protein binding to the Met-receptor kinase, in addition to other potentially uncharacterised proteins that damage microvilli structures (Chowdhury et al., 2018). Cell invasion by the bacterium is potentially further promoted by the secretion of quorum sensing peptides, that were upregulated during models of intestinal invasion; and visualised using electron microscopy data displayed the emergence of 'invasion hotspots'. These hotspots could potentially accelerating barrier damage and bacteria entry

(Nickerson et al., 2018). Following *S.* Typhi invasion, actin is recruited to the enterocyte cell surface, and subsequent remodelling allows the bacteria's transport to the basolateral surface (Nickerson et al., 2018; Vazquez-Terres et al., 1999).

The final proposed invasion mechanism relies upon CD103+ dendritic cells, which sample the intestinal lumen for commensal bacterial antigens, and can take up *Salmonella* bacteria (Aulicino et al., 2018; Schulz et al., 2009). Imaging of intestinal epithelium organoids displayed intestinal DCs extending appendages through tight junctions between enterocytes, and subsequent phagocytosis of *S* Typhimurium (Farache et al., 2013). Furthermore, *S.* Typhi bacteria were also able to infect human derived dendritic cells in vitro, and suppress antigen-presentation, supporting this as a possible invasion route (Aulicino et al., 2022). However, the exact contribution antigen sampling plays in *S.* Typhi invasion remains to be quantified.

1.3.2 *Salmonella* dissemination

Following invasion, *Salmonella* may disseminate to secondary sites including lymph nodes, the liver and spleen (Bravo-Blas et al., 2019) (Fig 1.2). In murine models, large numbers of bacteria were detected in the liver and spleen only after initial replication in the Peyer's Patch and MLN, suggesting this process is critical to establishing infection (Carter & Collins, 1974; Watson & Holden, 2010). Once at the spleen and liver, *Salmonella* replicates within monocytes and macrophages, in a process lasting approximately 7-14 days (Watson & Holden, 2010). Following replication, *Salmonella* can cross over from the lymph via the thoracic duct, resulting in a secondary phase of bacteraemia. Inflammatory mediators such as TNF-α and IL-6 are released, producing clinical symptoms such as fever.

Chronic carriers of typhoid fever

Chronic carriage is estimated to occur within 4% of infected individuals (Chua et al., 2015). The site of chronic infection is established in the gall bladder, which

Salmonella access through the hepatobiliary duct (Gonzalez-Escobedo et al., 2011). In mice, T3SS's encoded on SPI-1 and SPI-2 were necessary for gall bladder epithelium invasion and survival of *S.* Typhimurium, before forming *Salmonella* Containing Vacuoles (SCV's) within epithelial cells, facilitating replication (Menendez et al., 2009). During gallbladder colonisation, pro-inflammatory mediators; TNF-α , IL–6, and monocyte chemoattractant protein (MCP) 1 were released, triggering the influx of neutrophils, and subsequent tissue damage which promotes further invasion of the bacterium (Menendez et al., 2009).

Additional data from studies of *S.* Typhi using epithelial cell-lines, showed that *Salmonella* were resistant to bile degradation, most-likely mediated by the formation of protective biofilms around gallstones (Devaraj et al., 2021; Gonzalez-Escobedo et al., 2011). Within biofilms, expression of the Vi-capsule and the protective extracellular matrix, shields bacteria from immune recognition, reducing the release of Reactive Oxygen Species (ROS) from neutrophils and nitric oxide (NO) from macrophages (Devaraj et al., 2021; Hahn & Gunn, 2020; Sandala et al., 2020). Furthermore, when not replicating, *S.* Typhi bacteria entered a metabolic quiescent state, preventing inflammation and excessive immune activation. Subsequent intermittent shedding of bacteria from the gall bladder then results in the reintroduction of *Salmonella* into the bloodstream, causing secondary episodes of enteric fever (Gal-Mor, 2018; Khanam et al., 2021). However, what makes an individual more susceptible to chronic carriage, and other risk factors are currently not known.

1.3.3 Immune evasion of typhoidal *Salmonella*

In order to survive both extracellularly and intracellularly, typhoidal *Salmonella* employ numerous strategies to evade the immune system, including the regulation of gene expression to minimise immune activation. The expression of virulence genes including the Vi-capsule, SPI-1 T3SS, and flagellar proteins, are tightly controlled

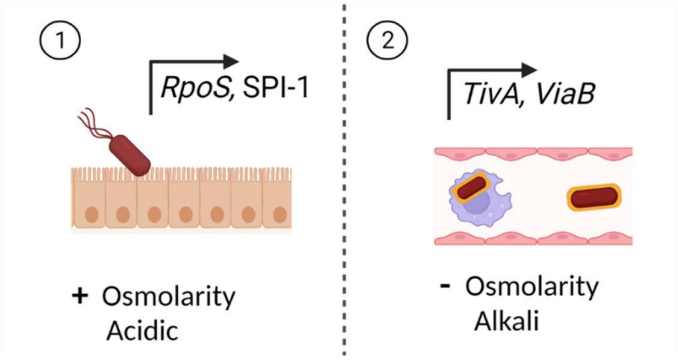


Figure 1.3: Alternate expression of Flagellar and Capsular genes 1) In the acidic hyperosmolar environment of the intestinal lumen *RpoS* and *SPI-1* genes are expressed. 2) Intracellular and bloodstream osmolarity and acidity decreases, enabling the expression of the positive transcription regulator TivA and subsequent *ViaB* locus and capsule expression. *Created using Biorender.com*.

by sophisticated phospho-relay systems that sense changes in osmolarity during pathogenesis, enabling alternate expression of genes at the appropriate anatomical sites (Winter et al., 2010). Transcription of these genes is regulated by the bacterial protein TiVA (Fig 1.3). In the intestinal lumen, *TivA* expression is switched off, reducing transcription of the *ViaB* locus encoding the Vi capsule, enabling the SPI-1 T3SS to make contact with host cells (Pickard et al., 2003).

During extracellular and phagocytic transmission of *Salmonella* bacteria are more likely to be detected by the innate immune system. However, the decrease in environmental osmolarity induces expression of *TviA,* thus downregulating flagellin synthesis and upregulating Vi-capsule expression (Fig 1.3). In doing so, bacteria minimise TLR-5 recognising flagellin on the bacteria cell surface, and has been demonstrated to reduce the number of splenic CD4-T cells that target flagellin (Atif et al., 2014; Winter et al., 2008). During *S.* Typhi infection, *TviA* activation also reduces the expression of the SPI-I T3SS, preventing subsequent NF$\kappa\beta$ signalling and inflammation, commonly observed during *S.* Typhimurium infections (Winter et al., 2014, 2008). The expression of the Vi polysaccharide capsule, also contributes to immune evasion by *S.* Typhi. The capsule acts like a shield, blocking recognition of *S.* Typhi from Toll-like receptors (TLR's) and complement, reducing production of inflammatory Tumour Necrosis Factor α (TNF-α), and IL-6 (Raffatellu et al., 2005; Wilson et al., 2008).

Importantly, it has been shown that Vi expression protects *S.* Typhi from IgM and C3b complement deposition, hence averting C5a-induced neutrophil migration (Rossi et al., 2019). Neutrophil-mediated phagocytosis and the oxidative burst have been shown to aid in the confinement of *S.* Typhimurium and mycobacteria during disease (T. Wangdi et al., 2014). In contrast, intestinal biopsies of *S.* Typhi-infected patients rarely reveal neutrophil infiltration (T. Wangdi et al., 2014). Furthermore, localised intestinal inflammation is reduced during typhoid fever, and diarrhoea is less frequent than in NTS, occurring in just about one-third of cases (T. Wangdi et al., 2014). By evading early recognition by the innate immune system, *S.* Typhi is therefore well-equipped to cause illness and onward transmission.

S. Paratyphi serovars A and B, however lack the SPI-7 locus encoding *tviA* and Vi capsule genes, despite its ability to cause similar clinical disease (Mcclelland et al., 2004). Evasion of complement and neutrophil recruitment is instead thought to be mediated by very long O-LPS chains, which similarly to Vi, can block IgM binding and complement deposition (Hiyoshi et al., 2018). Additionally, the downregulation of SPI-1 flagellin and T3SS genes has also been observed in *S.* Paratyphi invasion of intestinal tissue, in comparison to *S.* Typhimurium (Elhadad et al., 2016). However, the exact regulation of SPI-I expression remains to be investigated further. One hypothesis is that alternate expression is driven by changes in oxygen saturation of the intestinal lumen. Indeed, oxygen dependent regulation of the T3SS and cellular invasion has been observed for *Shigella flexineri* infections (Marteyn et al., 2010).

1.3.4 Intracellular survival

Once inside phagocytes, typhoidal *Salmonella* are adaped to survive the harsh intracellular environment. *S.* Typhimurium survival is mediated by SPI-2 which encodes a specialised intracellular T3SS. Effector proteins secreted by the T3SS induce formation of the *Salmonella* containing vacuole (SCV) which prevents oxidation (Vazquez-Torres et al., 2000). For typhoidal serovars however, many of the SPI-2 T3SS effector proteins are pseudogenised, and a study by C. G. Forest

et al. (2010) suggested SPI-2 was redundant for *S.* Typhi survival in human macrophages *in vitro*. Therefore, whilst SPI-2 genes have been demonstrated to be upregulated by *S.* Typhi during infection of macrophages, their relevance remains unclear (H. Zhang et al., 2016). It's possible that SPI-2 proteins may play a role in invasion and survival of other cell types such as within dendritic cells, where in vitro upregulation of the SPI-1 and 2 loci was also observed (Gogoi et al., 2018). Consequently, more studies are needed to elucidate the mechanisms of *S.* Typhi and *S.* Paratyphi intracellular survival.

Typhoidal *Salmonella*, have also adapted to acquire iron intracellularly, which is necessary for bacterial metabolism and replication. In patients infected with *S.* Typhi, levels of serum hepcidin increased during the clinical symptoms of infection compared with baseline and those who were not diagnosed with typhoid fever (Darton et al., 2015). As hepcidin increases the uptake of iron into cells, it was hypothesized that this is a consequence of *S.* Typhi redistributing iron into phagocyte cells, utilising the metal for its own metabolism (Núñez et al., 2018). Dual RNA-seq of monocyte-derived dendritic cells infected with *S.* Typhi, also revealed increased expression of genes involved in iron uptake and storage, including the transferrin receptor (Aulicino et al., 2022). Furthermore, whilst iron can catalyse ROS and NO release through the Fenton reaction, *S.* Typhi resistance mechanisms, including Vi-capsule expression, may protect the bacteria from these harmful by-products (Leon-Sicairos et al., 2015; G. T. Nguyen et al., 2017).

Non-invasive *S.* Typhimurium bacteria had reduced iron uptake gene expression compared with *S.* Typhi, suggesting the potential importance of this mechanism in causing systemic disease. Typhoidal *Salmonella* have therefore evolved several sophisticated mechanisms of pathogenesis, and overcoming these immune-evasion mechanisms with robust vaccinations will be required for developing sufficient protection.

1.4 The Immune Response during Enteric Fever

1.4.1 Cellular immune response to typhoidal Salmonella infection

Several cell subsets including; macrophages, neutrophils, and dendritic cells (DC), play a crucial part in the activation of the immune system, with dendritic cells triggering the adaptive arm of the immune system to fight infection. Early data from *S.* Typhimurium models illustrated the recruitment of dendritic cells following invasion of Peyer's patches and the lamina propria by *Salmonella* bacteria (Bravo-Blas et al., 2019; Erazo et al., 2021). Dendritic cells can take up antigens secreted from apoptotic infected cells or through direct phagocytosis, and subsequently drain to lymph nodes where they activate T-cells by presenting *Salmonella* antigens on MHC molecules (Farache et al., 2013). In participants who were orally challenged with *S.* Typhi, a peak in *S.* Typhi bound DCs was present in those who were not diagnosed with typhoid fever. Whereas in individuals who did develop typhoid fever, *S.* Typhi binding was delayed, suggesting that early recognition and phagocytosis of *S.* Typhi may be important in preventing infection (Toapanta et al., 2015).

Both CD4 and CD8 T-cells have been implicated in immunity against typhoidal *Salmonella*. During infection, cross presentation of antigens coupled to MHC-class I molecules on DCs may trigger CD8 T-cell activation (Swart & Hensel, 2012). Upon activation with *S.* Typhi, CD8 T-cells release cytotoxic granules which may contribute to pathogen clearance (Wahid et al., 2015). *S.* Typhi infection in Oxford volunteers, resulted in a peak of IFN-γ responses 28 days after infection, along with CD8 and CD4 T-effector memory (Tem) cells (Blohmke et al., 2016). Interestingly, CD8 Tem cells elicited after *S.* Typhi infection were cross-reactive with iNTS and

S. Paratyphi A and B serovars, and high baseline frequencies of multifunctional CD8+ Tem cells correlated with protection from typhoid fever, suggesting a role in prevention of infection (Rapaka et al., 2020). Given that this occurred in volunteers from non-endemic areas, these data suggest that broad cross-reactivity to other enteric pathogens may be driving the T-cell response.

CD4-T cells may also contribute to *Salmonella* immunity by activating the humoral arm of the immune system, and enhancing cytotoxic responses and immune memorycell development (Booth et al., 2020). In patients who developed typhoid fever, and CD4 T-cells were the predominant cell type to secrete IFN-γ when stimulated with *S.* Typhi antigen (Sheikh et al. 2011); and mice deficient for CD4 T cells, or lacking IFN-γ or the IFN-γ receptor could not resolve *Salmonella* infection (Bao et al., 2000; Bukholm et al., 1984). The association of MHC II alleles with infection susceptibility also implicates a protective role of CD4 T-cells in typhoid immunity, and a surveillance study performed in South Africa found higher rates and mortality associated with HIV patients (Dunstan et al., 2014; Keddy et al., 2016). Therefore, these data indicate that CD4-T lymphocytes could play an important role in protection against typhoid fever.

1.4.2 Humoral responses to enteric fever

B-cell responses to *Salmonella* infection

After recognising *Salmonella* antigens, B-cells migrate from the marginal zone of lymph nodes to form germinal centres, where they encounter CD4 T-follicular helper (T-fh) cells, inducing somatic hypermutation of the B-cell receptor (Diamond et al., 2018). Cells expressing the highest affinity antibodies are positively selected, fine-tuning antibody specificity and avidity against infection. In theory, repeated *Salmonella* infections could re-stimulate germinal centre activation, increasing antibody avidity. Increased avidity has been shown to improve neutralisation of bacteria and Fc-mediated responses following vaccination (Dahora et al., 2021). However,

Cunningham et al. (2007) demonstrated that *S.* Typhi in vitro infection reduced GC formation, potentially hindering the humoral response. These interactions are thought to be mediated by SIIE protein, which blocks B-cell lymphopoiesis during *Salmonella* infection (Männe et al., 2019). Sub-optimal antibody responses could therefore be contributing to *Salmonella* persistence. Consequently, boosting these humoral responses through vaccination is required to overcome the immune evasion of typhoidal *Salmonella*.

Antibody production during enteric fever

Most parenteral typhoid vaccines induce *S.* Typhi specific antibodies to mediate protection from disease (Anwar et al., 2014). However, the role of antibody-mediated protection during the primary immune response to *Salmonella* remains debated. The passive transfer of antibodies during murine *Salmonella* infection does not significantly hinder growth or dissemination of bacteria, and mice deficient for B-cell responses can still control infection (Mastroeni et al., 1993; Mastroeni et al., 2000). On the other hand, monoclonal antibodies have demonstrated protection against lethal *S.* Typhimurium challenge (Richards et al., 2020). Transfer of both *Salmonella* specific T-cells and serum from previously infected mice however, did confer protection against infection, suggesting that both the cellular and humoral arms of the immune system are required to mount a sufficient response (Mastroeni et al., 1993).

In endemic settings, antibody titres increase from childhood with age, in conjunction with decreasing *S.* Typhi incidence, however only partial immunity is ever acquired (Oo et al., 2019; Pulickal et al., 2009). Vi-IgG antibodies have been implicated in chronic carriage and long-term immunity, and interestingly, baseline Vi-IgG titres significantly influenced the levels of protection in both vaccinated and unvaccinated individuals (Darton et al., 2016). Furthermore, anti-Vi IgG, but not LPS with complement, were protective in a murine adapted model of Vi-expressing *Salmonella* (Hart et al., 2016). However, in experimental challenge settings, only anti-LPS IgG antibody titres significantly increased following infection, whilst

anti-Vi and flagellar titres did not, nor did they associate with protection (Gibani

et al., 2020). However, anti-LPS titres were significantly higher in individuals who were diagnosed with typhoid fever versus those undiagnosed, suggesting an exposure driven antibody production. In addition, although the complement-binding SBA activity of antibodies decreased typhoid severity, it was not associated with protection (Cardona et al., 2018; Jones et al., 2021). In areas endemic for enteric fever, repeat infections may contribute to the increase in antibody titres observed, giving rise to only moderate levels of protection (Saul et al., 2013).

IgA responses to enteric infections

Compared with IgG responses to typhoidal *Salmonella*, immunoglobulin A (IgA) may play an even more relevant role, due to its capacity to act at mucosal sites. There are two major forms of IgA; dimeric secretary IgA (sIGA) and monomeric serum IgA. In the intestine, sIgA is secreted by mucosal plasma cells and binds to the polymeric immunoglobulin receptor (pIgR) for transport to the intestinal lumen, aiding in immune homeostasis and tolerance (Bemark & Angeletti, 2021). During *S.* Typhimurium infection, sIgA neutralised bacteria at the intestinal epithelium surface, and mice lacking the pIgR receptor displayed increased susceptibility to infection and mortality, emphasising the role sIgA may have in the innate defence against *Salmonella* infections (Michetti et al., 1992; Turula & Wobus, 2018). In later stages of infection, SIgA may also be induced in an antigen-specific manner within Peyer's (Macpherson & Uhr, 2004). Antigen specific LPS-sIGA was protective against *Shigella flexineri* infections in a rabbit challenge model, and reduced inflammation and damage to the epithelium (Boullier et al., 2009; Mathias et al., 2013), however the role of secretory IgA in protection against typhoidal *Salmonella* is yet to be studied.

IgA1 is the dominant subclass in serum and forms 80-85% of the IgA compartment (Leong & Ding, 2014). IgA2 has a shorter hinge region structure which provides resistance to degradation by bacterial proteases, enabling it to operate mostly at mucosal surfaces (Senior & Woof, 2005). IgA-secreting plasma cells have exhibited antibacterial efficacy against a variety of encapsulated pathogens, including Streptococcus and E. coli (Janoff et al., 1999). Furthermore, in

vaccination studies, serum IgA1 titres were moderately associated with protection against typhoid fever (Dahora et al., 2019). Consequently, it is of interest to investigate the function of several antibody subclasses in protection against enteric fever following both infection and vaccination (Jin et al., 2021).

1.5 Interventions to Control Enteric Fever

To reduce the burden of enteric fever, long-term improvements in water and sanitation infrastructure are undoubtedly required, as demonstrated by its elimination in high-income countries following the construction of aqueducts and extensive water treatment facilities (Phillips et al., 2020). Nevertheless, robust immunisations are one of the most effective immediate public health strategies available. There are a number of licensed vaccines for the prevention of typhoid fever,including the oral Ty21a vaccine and the Vi-polysaccharide (Vi-PS) vaccine, both of which are commonly administered to travellers (Meltzer & Schwartz, 2010). However, these vaccines have several disadvantages, such as short-lived immune memory and low immunogenicity in infants, which has made their deployment in endemic countries unsustainable. *S.* Typhi Vi-conjugate vaccines (Vi-TCV) however have so far demonstrated numerous improvements, and the WHO has recommended the use of Vi-conjugate vaccines in typhoid-endemic areas (World Health Organization, 2019). In addition, several *S.* Paratyphi vaccines are currently in pre-clinical development (Martin et al., 2016). Evaluating the immunogenicityand efficacy of these vaccines is therefore essential for their efficient deployment.

1.5.1 Controlled Human Infection Models (CHIM)

For a novel vaccine candidate to achieve complete regulatory clearance, phase III efficacy studies must show vaccination effectiveness, in addition to sufficient safety data. However, owing to the huge number of participants necessary to identify a meaningful proportion of instances of enteric fever, such studies are expensive and time-consuming. Controlled human infection models (CHIM) offer a framework

through which purposely infecting individuals with a measured dosage of the infectious agent increases the statistical power of a smaller sample of participants (Darton et al., 2015). Participants may be vaccinated before infection so that the immunological responses to immunisation may be compared with those in a control or placebo group, and between those who were diagnosed with illness, and those who were not.

Historically, challenge models have been used to accelerate the licencing of several vaccines (Giersing et al., 2019; Porter et al., 2017). Most recently, high vaccine efficacy observed in a cholera CHIM study were used to grant FDA approval of VaxChora, for preventing travel related infections in adults, without requiring field-efficacy data (W. H. Chen et al., 2016). In Maryland U.S.A, the first typhoid challenge studies were conducted on convicts between 1952 and 1974, during which six different vaccine candidates were evaluated (Waddington, Darton, Jones, et al., 2014). The oral typhoid vaccine Ty21a was subsequently approved based on immunogenicity and effectiveness results derived from these trials (Gilman et al., 1977). However, due to ethical issues, these experiments were terminated. In 2010, the Oxford Vaccine Group endeavoured to re-establish typhoid CHIM experiments with the assistance of public volunteers. Preparation began with a dose-escalation trial that yielded a required attack rate of 55-60%. This rate was deemed high enough to ensure appropriate statistical power, whilst being tolerable and safe for participants (Waddington, Darton, Woodward, et al., 2014).

1.5.2 Vaccines against typhoidal *Salmonella*

The development of typhoid vaccines dates back to the early 20th century, with the first vaccine developed in simultaneously by the British researcher Almroth Wright in 1896, and German bacteriologist, Richard Pfeiffer. The outbreak of World War I in 1914 led to widespread inoculation campaigns in the armed forces later along with the TAB (typhoid, paratyphoid A and B) parenteral vaccine

(Hardy A, 2020). However, while the TAB vaccine was effective against *S.* Typhi,

the efficacy for protection against *S.* Paratyphi serovars was unclear (Wally Valla, 1972). Furthermore, due to a high number of side effects and the lower prevalence of *S.* Paratyphi, the vaccine was discontinued (D'Amelio et al., 1988). Subsequent development of monovalent typhoid vaccines were favoured instead.

Live oral vaccines against typhoidal *Salmonella*

The first oral vaccine developed for typhoid fever was Ty21a, a live attenuated vaccine constructed through chemical mutagenesis of *S.* Typhi Ty2. The strain lacks the enzyme uridine 5'-diphosphate (UDP)-glucose-4-epimerase and so is unable to synthesise LPS correctly (Gilman et al., 1977). Following the development of Ty21a, M01ZH09 oral vaccine was developed, with fewer mutations of the parent strain Ty2 compared with Ty21a, in the hope that this would increase vaccine immunogenicity. A randomised controlled double-blind CHIM trial compared vaccine efficacy of one dose of the M01ZH09 vaccine and a three-dose schedule of Ty21a, and a placebo group. M01ZH09 vaccine median efficacy was reported to be 13% and the efficacy of Ty21a was 35%, respectively (Fig 1.4) (Darton et al., 2016).

However, the use of high doses of *S.* Typhi to produce a replicable attack rate, are most-likely higherthan that which one would be exposed to in field settings, and thus makes it difficultfor a vaccine to produce complete protection. In addition, frequently sampling participants can result in the detection subclinical infections from blood cultureand PCR, which may have been missed in larger field trials. Subsequently, whenadjusting for a more representative field diagnosis of an initial fever (≥ 38 ˚C) with a positive, confirmatory blood culture; vaccine efficacy was adjusted as 52% for a single dose M01ZH09, and 80% for the 3-dose Ty21a regime.

As *S.* Typhi and *S.* Paratyphi share a high degree of structural protein homology (Mcclelland et al., 2004), it was hypothesised that there may be a degree of serovar cross-protection when using a live attenuated vaccine. However, while one field study demonstrated moderate protection against *S.* Paratyphi B, using Ty21a,

there is no evidence for an effect on *S.* Paratyphi serovar A (Levine et al., 2007), and this finding is yet to be replicated. The absence of a protective effect for *S.* Paratyphi A is intriguing, as Ty21a generates multifunctional CD4 T-cells responsive to both *S.* Typhi and *S.* Paratyphi A and B, in addition to bactericidal and opsonophagocytic anti-LPS antibodies against the three serovars (Rudolph et al., 2019; Wahid et al., 2012, 2016). It has therefore been postulated that higher antibody titres are needed to induce a significant functional antibody response, necessary for clearing *S.* Paratyphi infection, or that the recruitment of other innate immune mechanisms such as complement are required.

Live attenuated vaccines specific for *S.* Paratyphi are currently in development and include CVD 1902. CVD 1902 is an attenuated *S.* Paratyphi A strain with *GuaBA* and *ClpX* mutations in the parental strain A 915049 (Wahid et al., 2019).The vaccine was shown to be safe and immunogenic in preclinical studies (Gat et al., 2011). Interestingly, a vaccine against *S.* Paratyphi serovar B, CVD 2005 with the same two gene mutations, was also protective in a mouse challenge model (Higginson et al., 2018). Thus CVD 2005 could be considered as a future component in a multivalent live oral vaccine strategy.

A vaccine that provides cross-serovar protection to both *S.* Typhi and *S.* Paratyphi is needed most amongst countries in Asia where both typhoidal serovars are prevalent. One such vaccine is a recombinant Vi-Paratyphi live attenuated vaccine, currently in pre-clinical development (Xiong et al., 2017), in which *S.* Paratyphi was engineered to express Vi by cloning in the *ViaB* locus. Following nasal administration of the vaccine in mice, high levels of Vi-specific IgG titres were detected and resulted in protection from infection with both *S.* Paratyphi and *S.* Typhi serovars. This recombinant strategy may therefore be useful in enhancing cross-protection of future oral vaccines.

Alternative vaccine approaches

The most extensively explored immunogens for *S.* Typhi and *S.* Paratyphi vaccines are the Vi capsule and LPS-O antigens. However other surface antigens

are now being considered, including *S.* Typhi invasin (STIV) protein, which is conserved across *S.* Typhi and *S.* Paratyphi serovars (Das et al., 2019). Immunisation with STIV in mice, resulted in multifunctional CD8 and CD4 T-cells responses against STIV antigens. High levels of serum IgG were observed, capable of opsonophagocytic and complement lysis activity, and immunisation with the STIV vaccine subsequently protected mice from both *S.* Typhi and *S.* Paratyphi challenge. Other vaccine strategies being explored include exploiting *Salmonella* outer membrane vesicles (OMVs) as immunogens. OMVs are secreted by a range of Gram-negative bacteria and contain LPS and other membrane proteins and lipids. Consequently, adjuvants are not required to induce robust immune responses (Roier et al., 2013). Immunisation of mice with *Salmonella* OMVs protected against infection and induced multifunctional T-cell and antibody responses against both *S.* Typhi and *S.* Paratyphi antigens (Howlader et al., 2018). Whilst both vaccines represent early pre-clinical studies, they show promise for future phase I trials.

Polysaccharide typhoid fever vaccines

Vi-polysaccharide vaccines (Vi-PS) have been well tolerated and tested in at least three phase III field trials, where efficacy has ranged from 64% to 72%, and are therefore widely used for travel vaccinations (H. Yang et al., 2001). Furthermore80% protection has been observed from children aged 2 years to 5 years in a phase III trial of a Vi-PS vaccine in India (Sur et al., 2009). So far, TYPHIM-Vi is the only Vi-PS vaccine to gain WHO pre-qualification. However, Vi-PS vaccines induce T-cell independent immune responses; therefore, fail to elicit sufficient booster responses, and waning antibody levels over time, due to the lack of memory plasma cells induced. Currently it is recommended to repeat Vi-PS vaccinations every 2-3 years to maintain immunity (Klein Klouwenberg & Bont, 2008), a non-economicallyviable strategy for many LMIC's.

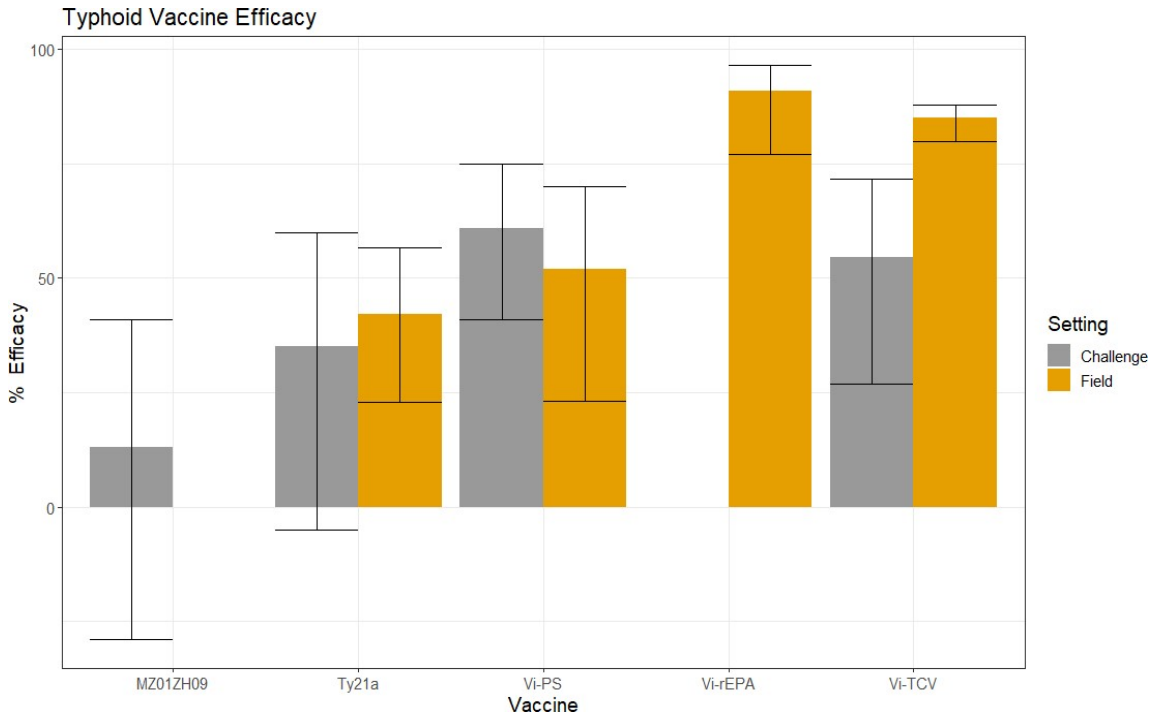


Figure 1.4: Efficacy of typhoid vaccinations measured in CHIM and field trial settings Comparison of typhoid vaccine efficacy measured across both phase IIb human challenge model settings, and phase 3 trials conducted in endemic areas (field). M01ZH09 and Ty21a are live-attenuated oral vaccines, Vi-rEPA and Vi-TCV are glycoconjugate based typhoid vaccinations. The % efficacy is depicted on the y axis. Bar plots show themean vaccine efficacy, while error bars represent the confidence intervals for each trial.

Furthermore, due to a lack of marginal zone B-cells required to mount T-cell independent responses to polysaccharide antigens in children under 2 years, vaccine efficacy is predicted to be limited in this age group (Cunningham et al., 2014; Zhao et al., 2018). Future developments include a new synthetic polysaccharide vaccine which has increased O-acetylation of Vi-LPS, and subsequently demonstrated better immunogenicity in mouse models (Ni et al., 2017). Additionally, the synthetic Vi-PS vaccine was able to generate booster responses and protect from *S.* Typhi infections in susceptible mice. This vaccine could potentially improve on the limitations of current-polysaccharide vaccines, and research into their effectiveness in humans remains ongoing.

Conjugate typhoid fever vaccines

Conjugate vaccines, work by linking the target antigen to a larger carrier protein, so that the polysaccharide(s) are processed in T cell dependent manner, inducing long-lasting memory B cell responses, as demonstrated by the immunogenicity of the successful MenB conjugate vaccine (Schmiel et al., 2011). The first clinical trial of a typhoid conjugate vaccine, conjugated to rEPA protein (Vi-rEPA), was conducted in 2004, Vietnam, and reported a protective efficacy of 91.1% in children aged 2-5 years, however slow licensure of the vaccine and lack of pharmaceutical development stalled vaccine implementation (Canh et al., 2004). A separate Vi-tetanus conjugate vaccine however, was later licensced for use in India and Nepal (Howlader et al., 2017; Mitra et al., 2016).

The efficacy of a Vi-tetanus conjugate vaccine (Vi-TCV), developed by Bharat Biotech, was examined using the typhoid CHIM in Oxford (Waddington, Darton, Woodward, et al., 2014). The phase IIb trial known as, Vaccines Against *Salmonella* Typhi (VAST), compared the efficacy of a Vi-tetanus conjugate vaccine (Vi-TCV) vaccine in healthy adult volunteers to volunteers immunised with the Vi-PS vaccine or a control meningococcal vaccine. Participants were followed over a 2-week period with daily blood culture, and typhoid fever diagnosis was determined by the presence of a continuous temperature of (≥ 38 ˚C) or higher for 12+ hours, or by detection of *S.* Typhi bacteraemia. Using these diagnostic criteria, vaccination resulted in efficacy of 54.6% against disease (Fig 1.4). Subsequently, in a phase III field study conducted in Nepal between 2017 and 2019, Vi-TCV vaccine efficacy of 81% was observed amongst children aged 9-months to 16 years of age, supporting thedeployment and testing of Vi-TCV vaccines in other endemic areas.

For paratyphoid fever, a number of conjugate vaccines are in clinical development, however none have reached licensure (Martin et al., 2016). Several of these vaccines focus on using *Salmonella* LPS O antigen as the vaccine immunogen. The vaccine at the most advanced stage of clinical testing, has been developed by the National Institute of Health, and consists of *S.* Paratyphi O2 polysaccharide conjugated to the tetanus toxoid carrier protein (O2,12-TT) (Konadu et al., 1996), and was safe and immunogenic during phase II clinical trials (Konadu et al., 2000). Following the success of the typhoid conjugate vaccine, it is hoped that paratyphoid conjugate vaccines could be used in a bivalent formula with Vi-TT to give protectionagainst all enteric fever agents. Another *S.* Paratyphi vaccine candidate, includes CRM197, consisting of LPS O2 and 12, conjugated to non-toxic diphtheria toxin,currently in phase I clinical trials (Micoli et al., 2012). As LPS O12 antigen is shared across typhoidal serovars, it is possible that the vaccine might be able to provide protection against both pathogens, however this remains to be tested.

1.6 The Immune Response Following Vi-TCV and Vi-PS Vaccination

Following both Vi-TCV and Vi-PS vaccination, Vi-IgG titres significantly increased post-vaccination from baseline (Jin et al., 2017). However, interestingly, the Vi-IgG titre was not significantly different between those who were diagnosed with typhoid and those who were not diagnosed in participants who received the Vi-TCV vaccine (Fig 1.5). These results therefore suggest that Vi IgG antibody quantity is accompanied by other features which contribute to vaccine protection, such as qualitative functional properties of the antibodies induced. In addition to Vi-IgG antibody titre, Vi-IgA was later measured and found to increase post-vaccination. Vi-IgA titre was significantly different between those who were diagnosed with typhoid fever and those who were not, however no threshold level of protection was able to be distinguished (Dahora et al., 2019).

1.6.1 Correlates of protection against typhoid fever

The approval of new vaccines may be aided by identifying 'correlates of protection', defined as an immunological signature that is significantly associated with protection after vaccination. There have been multiple instances of protective correlates used in vaccine development, including the opsonophagocytosis killing assays (OPKA) for pneumococcal vaccines and serum bactericidal assays for Meningococcus vaccines (Daniels et al., 2013). These correlates may be immunological or transcriptional signatures which can be measured during phase II and III trials. If observed, these data may indicate that a vaccine is protective and encourage vaccine licensure and wider testing.

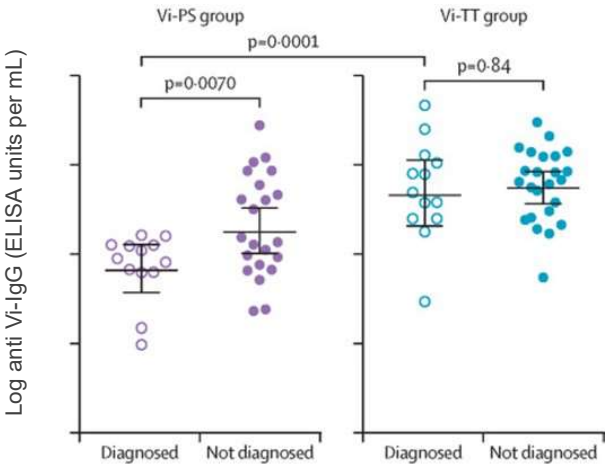


Figure 1.5: Vi-IgG titres 28-days following Vi-TCV or Vi-PS vaccination Vi-IgG titres were measured using ELISA 28 days following either Vi-TCV or Vi-PS vaccination. Groups were further split by typhoid fever diagnosis. *Reproduced from C. Jin et al, NJEM, 2018*.

At the time of starting my PhD there were no correlates of protection defined for typhoid fever, and previous attempts to use Vi-IgG antibody titres had failed to define a threshold of protection (Szu et al., 2014). Given the lack of differentiation between diagnosis groups following challenge by antibody titre alone, high-throughput assays were set up to measure a range of (Fragment-crystallisable) Fc-mediated antibody effector functions, and explore their role in protection against typhoid fever. These assays found that the fold change in antibody dependent neutrophil phagocytosis (ADNP) significantly increased post Vi-TCV and Vi-PS vaccination, and was important factor in distinguishing diagnosed versus non-diagnosed participants within a multivariate model (Jin et al., 2021).

1.6.2 Antibody Fc-mediated effector functions

Binding of the antibody Fc-region to innate immune cells includes downstream effector functions through the induction of ITAM or ITIM mediated cell signalling.

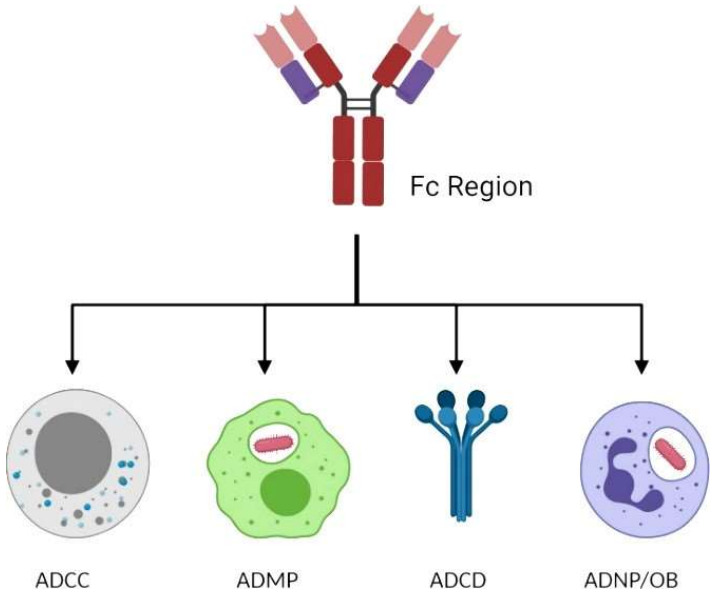


Figure 1.6: Fc mediated antibody effector functions across the immune system These functions include but are not limited to; natural killer cell antibody dependent cellular cytotoxicity (ADCC), antibody dependent monocyte phagocytosis (ADMP), antibody dependent complement deposition (ADCD), antibody dependent neutrophil phagocytosis (ADNP) and antibody dependent neutrophil oxidative burst (ADNOB). *Created using Biorender.com.*

Fc-mediated effector functions include but are not limited to; antibody dependent neutrophil and monocyte phagocytosis (ADNP, ADMP), antibody dependent cellular cytotoxicity (ADCC), and antibody dependent complement deposition (ADCD), and may contribute to control of infections (Fig 1.6). During typhoidal *Salmonella* infection, bacteria have been shown to evade many immune mechanisms such as complement mediated lysis and neutrophil mediated phagocytosis (Wangdi et al., 2012). Therefore, investigating if these responses can be boosted during infection or vaccination through antibody dependent mechanisms, may provide insights into protective mechanisms against infection.

The diversity of Fc-mediated functions if exemplified by the binding of antibodies to several classes of Fc-receptors which are found on distinct cell subsets (Fig 1.7). Belonging to the immunoglobulin protein superfamily, Fc receptors consist of extracellular immunoglobulin domains linked by a hinge region (Woof & Burton, 2004). ITAM or ITIM signalling domains in the cytoplasmic tail of the protein

or as accessory chains mediate downstream signalling and cellular functions upon receptor binding to immunoglobulin (Nimmerjahn & Ravetch, 2007). The receptors are classified with respect to the class of immunoglobulin to which they bind; IgA binds Fcα receptors, IgE binds to Fcϵ receptors, IgG binds Fcγ receptors, and IgM binds Fcμ receptors.

The low affinity receptor FcγRII (CD32) has two forms, the IITAM associated activating receptor FcγRIIA, which is expressed on all myeloid cells (Fig 1.7). In contrast, FcγRIIB is expressed highly on circulating B cells and lowly expressed on monocytes. Its ITIM motif, results in inhibition of phagocytosis and cellular activity (Zhang et al., 2010). Dynamic interplay between expression of the different activating and inhibitory receptors allows for tuning of the immune system in multiple compartments. Lastly, FcγRIIC is thought to be a pseudogene or duplication and its function remains unknown.

FcγRIIIA (CD16a) is most highly expressed on natural killer cells, and activation of Fc-receptor signalling, induces antibody dependent cellular cytotoxicity (ADCC), ensuing targeted killing of infected cells through the release of cytotoxic granules. FcγRIII can also be found on the surface of some monocyte subsets. Antibody binding to Fc receptor has been demonstrated to result in the release of inflammatory cytokines on macrophages.FcγRIIIB (CD16b) is expressed mainly neutrophils and basophils subsets, and acts as an antagonistic, inhibitory receptor, helping to dampen the immune response, preventing overactivation and excessive inflammation (Hargreaves et al., 2015).

The Fcα receptor binds IgA, and is expressed primarily on myeloid cells and. FcαRcontains an inhibitory ITAMi signalling motif, which under normal homestatic conditions results in inhibition of cells upon IgA binding. Due to these interactions several studies have pointed towards an anti-inflammatory role of serum IgA (Breedveld & Van Egmond, 2019). However, under inflammatory conditions FcαRcan bind to IgA complexes surrounding opsonised bacteria, inducing cellular

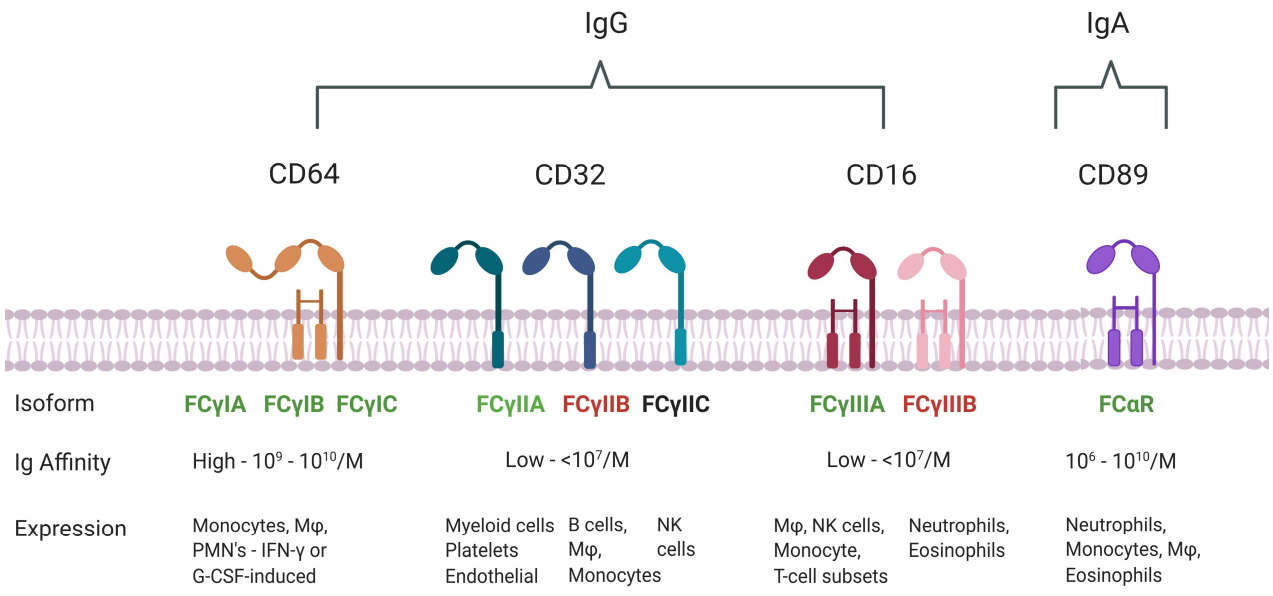


Figure 1.7: Fc-receptor structure, immunoglobulin affinity and cellular expression Illustration of the cellular distribution, immunoglobulin isotype, subclass specificity, binding strength, and downstream consequences for each Fc-receptor. Fcγ receptors bind various isotypes of IgG, and Fc-α receptors bind to IgA. *Created using Biorender.com.*

activation. In *Salmonella* Typhimurium infections, mucosal IgA is thought to play a role in immune defence, as IgA can trap bacterium, contributing to its clearance by binding to the pIgA receptor on the mucosal epithelium (Carlson & Jones, 1998). As serum Vi-IgA was recently found to correlate with vaccine mediated protection in typhoid fever, understanding the role FcαRplays in protection against enteric fever is also of interest.

1.6.3 Antibody Fc-Glycosylation

Fc-mediated antibody effector responses are further altered by changes in Fc glycosylation patterns. Glycans are sugar residues which are added to conserved Fc glycosylation sites by glycosidase and glycosyltransferase enzymes in the endoplasmic reticulum and Golgi apparatus. IgG Fc glycosylation is N-linked, with glycans added to the nitrogen atom of a conserved asparagine residue, in position 297 of

the constant heavy domain (Rudd et al., 1991). IgA contains up to four additional O-linked glycosylation sites, where glycans are added to the oxygen atom of eithera serine or threonine residues within the hinge region (Slauch et al., 1995). The manipulation of Fc glycan profiles to alter antibody effector functions has long been utilised in monoclonal therapeutics (Jefferis, 2009), however a role in immunity against infection is only recently being established.

The core of the IgG glycan structure is a biantennary polysaccharide chain composed of 2 N-acetylglucosamine (GlcNAc) residues connected to a mannose structure with an extra GlcNAc residue on each mannose (Fig 1.8). The variable addition of glycans, such as fucose, mannose, galactose, sialic acid, and a bisecting GlcNac residue, to the core Fc glycan structure, enhances the structural diversity of Fc immunoglobulin, generating over 30 distinct configurations. Furthermore, it has been shown that the modifications of Fc-glycan profiles may result in conformational changes that affect the affinity of antibodies for their respective Fc receptors and the degree of subsequent cellular activation (Ahmed et al., 2014; Rudd et al., 1991).

Glycan structures are often categorised by the amount of varied galactose residues (Fig. 1.8), which impacts downstream functions. In general, the elimination of sialic acid and galactose leads to Fc glycoforms that produce a stronger inflammatory response, linked to the development of a number of autoimmune diseases, such as rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) (Pilkington et al., 1995). Additionally, greater levels of galactosylation were correlated with decreased inflammatory activity in mAb treatments for RA (Alter et al., 2018). Modifying glycosylation has already been achieved in improving monoclonal therapeutics, through increased efficiency and Fc receptor binding (Heemskerk & Egmond, 2018; Jefferis, 2009). Therefore, investigating the relationship between Fc-glycosylation and downstream-effector functions relevant in *S.* Typhi infection, could help elucidate mechanisms of protective immune responses.

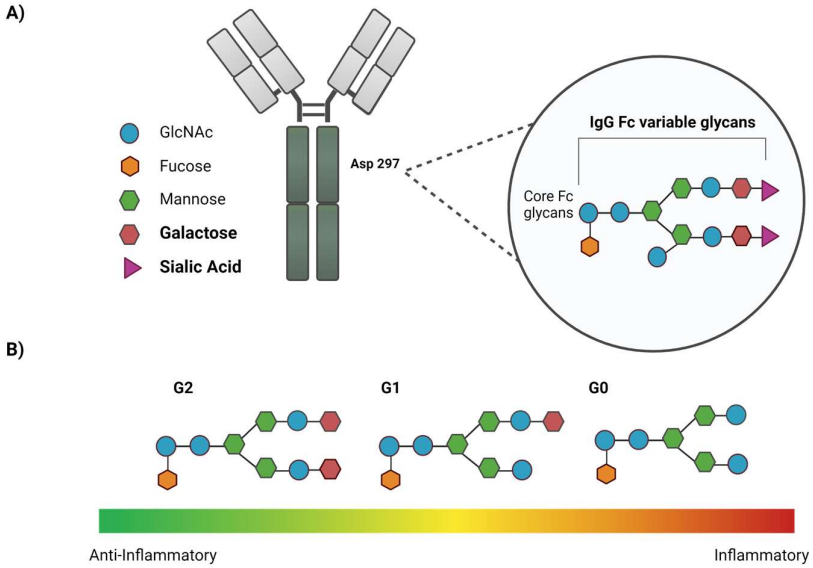


Figure 1.8: Glycosylation features of IgG Variable glycosylation of the fragment- crystalisable (Fc) fragment of immunoglobulin occurs upon an asparagine (Asp) residue, positioned 297. G0, G1, G2 phases depict agalactosylated, monogalactosylated, and di-galactosylated Fc-glycan profiles respectively. *Created using Biorender.com.*

1.7 Human genetic variation and disease

Our response to infection is likely the result of several factors, including environmental exposure, dose, as well as an innate genetic component determined by the inheritance of alleles in genes relevant to the immune response, or host-pathogen interactions. Deciphering the role of genetic variation in these diseases therefore requires a deep understanding of the human genome (Wray et al., 2018). In 2003, the Human Genome Project decoded approximately 93% of the human genome sequence (Abdellah et al., 2004). Since then, vast efforts have been made to reach complete sequence coverage, and in 2022 gapless coverage of all autosomal chromosomes was achieved using advancements in long-read sequencing technology (Nurk et al., 2022). In conjunction with population-based studies such as HapMap and the 1000 Genomes project, these data have provided numerous insights into human genetic variation and function, guiding our depiction of disease inheritance

(Auton et al., 2015; Belmont et al., 2003). GWAS studies of complex diseases such as heart disease, stroke, and alzheimers are currently performed across large consortia, and have revealed several loci implicated in genetic risk of these diseases (Bellenguez et al; 2022; Walsh et al. 2023). Metalanlyses of GWAS studies across populations has also bolstered their impact (Atkinson et al., 2021).

There are several observations which suggest that infectious diseases may have a strong genetic component. Adoption studies observed a marked increase in risk of death from infectious disease in adopted children if one of their biological parents had died prematurely from infectious disease (Sørensen et al., 1988). Twin studies have also demonstrated a heritable component to infection, including; hepatitis B, poliomyelitis and leprosy, whereby identical twins developed infections at more concordant rates than fraternal twins (Herndon & Jennings, 1951; Misch et al., 2010; Nudel et al., 2019; Z. Zhang et al., 2019). Secondly, there is large variability in the response to infection, with often the majority of individuals developing mild symptoms, whilst a proportion may go on to have severe clinical responses, and a proportion of individuals who may not be likely to contract infection in the first place (Mozzi et al., 2018). Recent GWAS studies have implicated genetic factors to an array of infections including *Mycobacteria*, Hepatatis and SARS-COV2 (Thorball et al., 2021).

Controlled human infection models provide well-controlled study designs, which enable the investigation of genetic factors upon disease susceptibility. Participants in these studies are screened for their baseline health and characteristics beforethe administration of the infectious agent. This screening helps to ensure that environmental factors, such as exposure and dose of the pathogen are considered, while also minimising significant variation in lifestyle factors or access to healthcare, both of which can confound association studies that are carried out in field settings (Porter et al., 2017).

In CHIM studies of enteric fever; despite giving participants large oral doses of *Salmonella*, between 30 and 35% of participants do not develop infection (Waddington, Darton, Woodward, et al., 2014). Differences in clinical presentations may therefore be explained by genetic factors, either through modifying the immune response to enteric fever, or by other unknown host factors (Maurya et al., 2022)

Single Nucleotide Polymorphisms (SNP)

Human genetic diversity is primarily driven by single nucleotide polymorphisms (SNPs), which occur as a result of the deletion, insertion or substitution of a single nucleotide base, when compared to the human reference genome, and must occur in more than 1 of individuals (Gagliano et al., 2019; Sachidanandam et al., 2001). Genome-wide association studies (GWAS) utilise whole-genome-sequencing (WGS) and microarray platforms to detect SNPs, and subsequently associate the frequency of SNPs observed in a population, with the frequency of disease, or other measures such as symptom severity and antibody titre.

However, for complex diseases, it is thought that many SNPs with low penetrance (effect size) contribute to disease pathogenesis, rather than a single causal SNP, which would otherwise likely be phased out with evolutionary selection, therefore large sample sizes are typically needed to power detection of statistically significant SNPs (Lynch et al., 2016; Marth et al., 2011). As multiple host-pathways may be involved in pathogenesis, several SNPs significantly associated with disease can also be grouped together to form polygenic risks scores (PRS), which inform individuals of their likelihood of developing disease (Euesden et al., 2015). A PRS was recently tested in conjunction with injury severity scores, as a model for determining risk of sepsis in trauma patients, and could potentially be used as a future genetic screening tool (R. M. Lu et al., 2020).

Insertion-deletion variations

Other forms of genetic variation occur when small lengths of DNA (typically <50bp) are inserted or deleted from the genome, referred to as INDELS. If the INDEL is not a multiple of three, these can result in frameshift mutations that drastically alter the final transcription product (Gagliano et al., 2019; Mullaney et al., 2010). These effects are illustrated by fragile X-syndrome, which is caused by DNA insertions within the promoter region of the *FMR1* gene. The insertions result in increases in DNA methylation which silences *FMR1* gene expression, required for neuronal development (Tassone et al., 2000).

Structural variation of the human genome

Structural variations arise from large alterations of the genetic sequence, and may also contribute to genetic diversity. Discovering these larger variations therefore depends on sufficient genome coverage, acquired through NGS sequencing technologies, or targeted sequencing of suspected risk loci (Handsaker et al., 2011). SV have subsequently been implicated in the development of several conditions; including polycystic kidney disease, ALS, and cardiomyopathy (Abel et al., 2020; Chambers et al., 2010). Structural variation of the genome includes copy number variants (CNV), which affect the number of transcription copies of a DNA segment (Alkan et al., 2011; Sudmant et al., 2015). CNV polymorphisms of the Fc receptor, FCGR3A, were shown to correlate with FCGR3A surface expression CD8 T-cells, and the severity of rheumatoid arthritis (Franke et al., 2015).

1.7.1 From Genotype to Phenotype

SNPs found within coding regions of DNA can be classified into either synonymous SNPs, whereby a nucleotide-base change results in an altered amino acid sequence, or non- synonymous SNPs, in which there is no-effect. Synonymous mutations are therefore analysed for potential changes the SNP may induce in translated protein structure (Dakal et al., 2017). Databases including SIFT and Polyphen have built predictive models of the deleteriousness effects of SNPs, and these results have been found to correlate with disease phenotype (Flanagan et al., 2010).

However, almost 90% of all phenotype-associated SNPs identified by GWAS, are found withinnon-coding regions, including intronic regions, promoters, enhancers, and regions of regulatory non-coding RNA including miRNAs and lncRNA (Edwards et al., 2013; Hrdlickova et al., 2014; Mirza et al., 2014). In a GWAS meta-analysis of participants across 220 sub-populations, lncRNA SNPs in chromosome 5, were associated with increased risk of typhoid fever and dysentery ($P = 1 \times 10^{-8}$, OR : 1.29 [0.84 – 1.73]), and decreases in IFN-γ production, suggesting that it may act on the IFN-γ loci, however this is yet to be determined experimentally. Estimating the effects of these non-coding SNPs on disease phenotype therefore requires an in-depth understanding of regulation and transcription of the genome, and could help characterize protective mechanisms in infection (Shastry, 2009).

1.7.2 Linkage Disequilibrium

Discovering 'causal' SNPs involved in disease phenotypes is further complicated by the phenomenon of linkage disequilibrium (LD), by which blocks of closely linked SNPs on the genome are inherited together, as a result of crossing over events during meiosis. Consequently, SNPs significantly associated with disease, will typically occur in blocks known as genetic risk loci. However, the most-significant SNP within the risk-loci, is not always likely to be the causal variant.

Part of this is explained due to the design of genotyping arrays which utilise the phenomena of LD to impute missing SNPs that are not physically read on the array, allowing researchers to analyse millions of genetic variants at a time (Coleman et al., 2016). SNPs on arrays are therefore often selected based off of high LD rates and population frequency, which increases the downstream imputation power (Das et al., 2016; McCarthy et al., 2016). Yet, a more frequently occurring SNP, may have stronger statistical significance with disease than a less-frequently observed causal variant.

Therefore, GWAS studies with lower statistical power either due to small sample size, allele frequency, or odds ratios (OR), may be limited in the ability to correctly annotate the causal SNP, if based solely on statistical significance (Mitt et al., 2017). Zaykin & Kozbur (2010) demonstrated this phenomenon using GWAS simulations with an equal number of 1,000 cases and controls. In a relatively strongly powered study, with a disease allele frequency of 50% and OR of 1.5, there was a 79% chance that the lead SNP was found to be causal. However, there was only a 2.4% chance of accurate detection when the OR was 1.1 and the risk allele frequency was 5%.

1.7.3 Fine-mapping of genetic risk-loci

Given these challenges, the more knowledge one has about suggestive genetic variants, the more likely one may be to discovering causal variants and mechanisms of disease pathogenesis (Matzaraki et al., 2017). Multiple publicly available databases now exist which can be used to fine-map genetic risk loci. These hold data including linkage disequilibrium (LD) patterns, and the functional consequences of SNPs; including the deleteriousness of variants, their effects on gene expression, and histone-chromatin interaction sites (Broekema et al., 2020; Schaid et al., 2018). Online tools, such as FUMA, or SNP-Nexus, integrate data from several of these repositories including SIFT, Polyphen, the Genotype-Tissue Expression (GTEx) project, the Encyclopaedia of DNA Elements (ENCODE), the Roadmap Epigenomics Project (Landrum et al., 2018; Lonsdale et al., 2013; Watanabe et al., 2017).

Along with ancestral variation data from sources such as the 1000 genomes project, in addition to public catalogues of previously identified significant SNPs and clinical variants (Altshuler et al., 2012; Fokkema et al., 2021; Kichaev et al., 2019; Myers et al., 2011). If these sources are integrated with experimental data such as gene-expression, cytokine, and blood count measures; users can develop powerful context-specific analyses of genetic variation in disease (Cano-Gamez & Trynka, 2020).

eQTL analysis

Expression-quantitative trait loci analysis are particularly useful for uncovering the effects of the abundant non-coding variants observed in GWAS studies (Vosa et al., 2021). As these variants are often enriched in promoter/enhancer regions, it is reasonable to assume that their associations with disease are via the modulation of gene expression profiles (Schadtet al., 2005). eQTLs are classified by the genetic distance between the variant and the gene of interest; SNPs that influence the expression of adjacent genes (<1Mb), are referred to as cis-eQTLs, whilst trans-eQTLs act across larger distances, even chromosomes. Trans-eQTL variants may mediate their effects via changes in master regulatory transcription factors, chromatin modifications, or by altering the transcription of other pathway-linked genes (Breitling et al., 2008). However, due tothe many possible target loci (B. Li & Ritchie, 2021); trans-eQTL's require stringent multiple testing corrections. Subsequently large sample sizes are needed to accurately detect effects, therefore these analyses will be beyond the scope of this study.

Studies of eQTL measured in numerous tissues and cell types have discovered thousands of regulatory polymorphisms. Individually, these are estimated to account for approximately 10% of gene expression variation observed within a population, interestingly these eQTL were markedly enriched for disease-associated variants (Croteau-Chonka et al., 2015). Furthermore, using data from healthy tissue donors in the GTEX consortium; Cis-eQTLs were predicted to account for approximately 11 percent of complex-disease heritability (Yao et al., 2020).

Moreover, recent studies have shown that eQTL effects are strongest when correlating SNP frequency with cell-type and context specific gene expression. Previous analyses which evaluated eQTL effects using healthy, bulk tissue gene expression data, therefore may not provide an estimation of the effects of regulatory variants within disease, which may be stronger than previously estimated (Glastonbury et al., 2019). The use of infection and cell-specific eQTLs, was recently demonstrated by utilising single-cell RNA (scRNA) data to characterise SNPs involved in sepsis, which highlighted several inflammatory pathways linked to genetic variation (Oelen et al., 2022)

1.8 Genetic Susceptibility to Infectious Disease

1.8.1 Evolution of infectious disease traits

Genetic variation arises through both stochastic genetic drift, and the natural selection of traits which may confer a selective advantage (Darwin & Wallace,1858; Sabeti et al., 2006). Humans have engaged in an evolutionary race with pathogens for tens of thousands of years, producing selection pressures on potentially protective or detrimental variants. For example, a mutation in the gene encoding the CCR5 receptor used by HIV for viral entry; decreases receptor expression, conferring resistance to HIV infection (Huang et al., 1996), and are hypothesized to have arisen from selection pressures during historical epidemics including the 14-15th century plague or small pox (Biloglav et al., 2009; Bouwman et al., 2017; Galvani & Slatkin, 2003).

This hypothesis is supported an observed increased frequency of CCR5 mutant variants in European ancestry populations, compared with populations in Sub-Saharan Africa, which did not experience such outbreaks (Martinson et al., 1997). Both poxviruses and *Yesinia pestis* have been demonstrated to utilise CCR5-based entry (Lalani et al. 1999), and macrophages deficient for CCR5,

showed a 30–fold reduction in *Yesinia pestis* uptake, suggesting the variant may

have conferred resistance to infection during these outbreaks (Elvin et al., 2004). Sickle cell anaemia is an Mendelian-inherited disease, whereby an allele conferring a single point-mutation in the *β-globin* gene, results in sickle-like red blood cells which struggle to carry oxygen around the body and cause painful obstruction of capillaries and blood vessels (Lux et al., 1976). However, the mutant most-likely arose as a consequence of human evolution in areas with high malaria incidence, whereby sickle like blood cells result in lower parasitaemia (Williams et al., 2005).

Evolutionary selection pressures placed on the immune system during infection, is further illustrated by the enrichment of immune-related genes in regions of high genetic variability (Jin et al., 2018). These regions typically include genes involved in antigen presentation, innate pattern-recognition receptors, and signalling (Kwok et al., 2020). The genetic adaptations of *Salmonella* Typhi and invasive strains of *Salmonella* Typhimurium to cause human-restricted disease, have been previously been described. However, the role of host-genetics in enteric fever is only recently being elucidated through epidemiological observations and human infection models (Barton et al., 2022; Ma et al., 2021). Understanding the genetic factors involved in responses to infection or vaccination could therefore shed-light on important immune-mediated pathways involved in host-pathogen interactions.

HLA diversity and disease

The impact of genetic diversity on response to infections, is best illustrated by the polymorphic HLA locus, which encodes the major histocompatibility complex, class I and II (MHC). MHC molecules presents antigens to natural killer cells and T-cells, amplifying the immune response. The gene is encoded on chromosome 6 p21.3, and high genetic diversity of the region ensures a broad response and recognition of pathogens. HLA polymorphisms have therefore been implicated in associations with numerous infectious diseases, including, tuberculosis and HIV-1 viral infection, and enteric fever (Barton et al., 2022; Fellay et al., 2007; Leitman et al., 2017; Odera et al., 2022; Toyo-oka et al., 2017).

The importance of HLA alleles in control of HIV-replication was highlighted by a genome-wide association study, in which 50% of the top single nucleotide polymorphisms (SNPs), correlatingwith viral control, were within the MHC locus (Fellay et al., 2007). Polymorphisms in immune-related genes have also been associated with differences in anti-viral treatment responsiveness, as illustrated by polymorphisms in IFNλ3 , which were associated with differences in hepatitis C virus (HCV) anti-viral efficacy (Ge et al., 2009). Screening for the presence of these variants are now routinely performed to inform treatment pathways for these infections (Mosbruger et al., 2010).

1.8.2 Human challenge models for studying disease

One of the benefits of controlled human infection studies, is the ability to closely monitor participants and collect samples throughout the course of infection, allowing the measurement of gene-expression throughout the course of disease, which canin turn be correlated with genetic profiles of participants. In addition, smaller numbers of participants are needed to detect cases of infection, as opposed to field studies, resulting in increased statistical power when comparing differences between the genotype and expression profiles of cases and controls. For example, a human challenge model of norovirus infection detected that the presence of a homozygous recessive allele of the *FUT2* gene, was associated with complete protection from infection (Lindesmith et al., 2003). The *FUT2* gene encodes an alpha-fucosyltransferase protein, responsible for synthesis of the host-H antigen, used for virus receptor-mediated cell entry, and these associations were subsequently observed epidemiological studies (Lopman et al., 2015).

Association studies can therefore highlight important host-pathogen interactions, providing insights for potential targets drug screens targets, or immune responses which could be enhanced by vaccination. In an ETEC human infection model, pathogen-induced transcriptional alterations were compared to those generated by

a database of small molecule medicines. This approach discovered a comparable transcriptomic response during rifampicin treatment, suggesting that in addition to direct-antimicrobial activity, the antibiotic may also stimulate a protective immune response (W. E. Yang et al., 2016). Furthermore, when comparing gene-expression to those who developed acute typhoid fever, to those who did not develop disease, transcriptomic analysis detected significant overexpression of interferon, apoptosis, and neutrophil-related genes (Blohmke et al., 2016).

1.8.3 Analysis of candidate genes and disease susceptibility

There are approximately one million independently assorted genetic loci in the European-American human genome reference which can be tested for associations with disease, requiring stringent multiple testing thresholds typically set at $P = 5 \times 10^{-8}$, to avoid the reporting of spurious findings. (Guinot et al., 2018). Large sample sizes are therefore typically required to detect associations with disease. The study of infectious diseases in LMICs, such as enteric fever, however is generally more limited in resources and scale. Smaller sample sizes reduce the likelihood of observing associations above the gold-standard genome-wide significance level. Instead, associating candidate genes suspected to be mechanistically involved in disease, can provide a realistic means of hypotheses testing within smaller studies. Such candidate gene association studies (CGAS) was the primary association test performed prior to the expansion of next-generation sequencing methods.

Interestingly, a systemic review of polygenic risk score modelling accuracy compared with clinical predictions, found that for most diseases AUC scores based on GWAS-detected SNPs alone, less accurately predicted disease outcome (Yao et al., 2020). Therefore, supporting the case for improving the selection of lead SNPs either through clinically relevant candidate gene association studies, or previously discussed statistical-fine mapping techniques (Kichaev et al., 2019). Furthermore, the complex aetiology of infectious disease which involves a number of host and environmental factors, is most likely responsible for the added difficulty in replicating genetic association studies, opposed to GWAS of metabolic diseases such as diabetes and intestinal bowel disease, which have been well characterised. For example, studies of *m.TB* genetic susceptibility were demonstrated to be confounded by differences in bacterial strains across different endemic regions (Mozzi et al., 2018).

Genes involved in primary immunodeficiencies or autoimmune diseases, may provide a starting point for investigating candidate genes related to either under or over-activation of the immune system (Nielsen et al., 2016). Studies of invasive pneumococcal disease (IBD), found that associated immunodeficiencies involved mutations in genes *NEMO*, *MyD88*, *IRAK4*, and *NFκβIA*; involved in TLR and NF*κβ* signalling pathways, required for controlling infection (Chapman & Hill, 2012; Paciolla et al., 2015; Xu et al., 2017). Variants of these suggested genes were subsequently assessed in candidate gene association studies of *Streptococcus* infection, and correlated with increased susceptibility to IBD, sepsis and disease severity, highlighting potential pathways for treatment targets (Arcaroli et al., 2006; Chapman, 2012). Moreover, blocking NF*κβ* signalling with pharmacological inhibitors during cerebral *Streptococcus* infection provided protective effects against bacterial meningitis in rodents (Koedel et al., 2000).

However, candidate gene analyses whilst more likely to be biologically relevant, also suffer from selection bias, and by design cannot elucidate potentially novel mechanisms in disease pathogenesis, compared with an unsupervised GWAS approach. Furthermore, evaluations of CGAS also struggled with a lack of replication (Lappalainen et al., 2013). Advances in both approaches will likely be achieved through the integration of transcriptomic data, and genetic architecture, to understand the mechanistic roles of genetic variation in disease (Kilpinen et al., 2013; Schaub et al., 2012; Shao et al., 2008). Improvements are also likely to occur through the inclusion of larger, and more diverse study-cohorts, shifting away from the historically Eurocentric approach to the study of human genetics (Popejoy et al., 2018). Several consortia such as TOPMed and PAGE, are seeking to rectify this gap in research, recruiting tens of thousands of participants with diverse ancestral backgrounds to improve on current statistical methods (Roshyara et al., 2016). Recent trans-ethnic analyses have demonstratedthat SNPs shared across ancestral populations, are more likely to be causal SNPs, supporting the use of this approach for discovering causal variants (Atkinson et al., 2021; De Lillo et al., 2021).

1.8.4 Genetic Susceptibility to Enteric Fever

Historically, gene knock-out (KO) mice have been used to model invasive *Salmonella* Typhimurium infection, more representative of *S.* Typhi pathogenesis. Toll-like receptors are innate-recognition pattern receptors that can detect a variety of conserved bacterial antigens; including LPS (TLR4) and flagellin (TLR5). Mice deficient in TLR4 present an invasive *Salmonella* phenotype, unable to control bacterial replication within macrophages or curtail systemic disease (Cervantes-Barragán et al., 2009). TLR5 and mouse-specific TLR11 recognise flagellin, and have also demonstrated increased *S.* Typhimurium invasion and mortality.(Broz et al., 2012; Mathur et al., 2012). Given these associations, defects or varia- tions in TLR-associated genes were investigated in several CGAS, and GWAS of *Salmonella* infection.

In a candidate gene study across Kelentan Malaysia, the presence of non-synonymous SNPS at Thr399-Ile (C/T) and Asp299-Gly (A/G) associated with differences in susceptibility to enteric infection (Table A.1) (Arbour et al., 2000; Bhuvanendran et al., 2011; Rallabhandi et al., 2006). These SNPs alter the extracellular domain of the PAMP binding site, leading to LPS hyporesponsiveness, and susceptibility to gram-negative infections. Additionally, the Asp299-Gly allele was associated with septic shock following bacterial infection (Lorenz et al., 2002). Furthermore, a CGAS of iNTS bacteraemia in Kenyan children, discovered that missense polymorphisms in the TLR4 signalling molecule TIRAP, were associated with disease (Khor et al., 2007), supporting the hypothesis that variation in TLR4-recognition and signalling are significant drivers of *Salmonella* infection susceptibility. Contrastingly, TLR5 mutants whilst resulting in susceptibility to murine *Salmonella* Typhi invasion, did not show any associations with enteric fever when measured in a Vietnamese CGAS (Dunstan et al., 2005).

VAC14 is one of the essential signalling proteins during type I IFNs production. A *VAC14* polymorphism, rs8060947, was significantly associated with susceptibility to typhoid fever in a candidate gene study of a Vietnamese population (Table A.1) (Alvarez et al., 2017). During invasion of HeLa and lymphoblast cell lines *invitro*, mutants of *VAC14* generated through RNAi inhibition displayed increased *S.* Typhi invasion. The mechanism of VAC14/Type I IFN control of enteric disease has been suggested by experimental mouse models in which, treatment with type I interferons, IFN-α β, inhibited cellular invasion by *S.* Typhimurium and *S. flexneri* bacteria, and also enhanced the survival of infected mice (McNab et al., 2015; Niesel et al., 1986). Taken together, these results support the hypothesis that type 1 interferon signalling helps control *S.* Typhi infection, and could be investigated further in susceptibility to disease.

Multiple candidate gene, and genome-wide association studies have implicated alleles of the HLA locus in susceptibility or protection against enteric fever (Table A.1). The strongest association observed so far was detected in a large Vietnamese population, and a replication cohort based in Nepal, in which the HLA-DRB1 *04:05 allele, was strongly protective against enteric fever infection (Dunstan et al., 2014). Additionally, similar associations were seen in a GWAS of enteric fever which took place in Jakarta, Indonesia, suggesting these are true protective effects, and not just artefacts of population ancestry (Ma et al., 2021).

Several other HLA class I and Class II alleles have also been associated with increased severityof enteric disease. The diversity in associations, most likely reflects the diverse polymorphic nature of the locus, and may be contribute to the varying clinical presentations seen during acute episodes of typhoid or paratyphoid fever. The HLA locus, also includes genes encoding the IFN-α receptor. IFN-α has also been implicated in responses to typhoidal *Salmonella* infection, whereby immune evasion mechanisms of *S*. Typhi, dampen the inflammatory response, enabling bacteria dissemination and systemic disease (Keuter et al., 1994). TNF-α alleles were also implicated in host susceptibility to enteric fever, implicating the molecule further in disease pathogenesis (S. Ali et al., 2007).

1.9 Thesis Aims

The overall objective of this thesis is to characterise protective immunological signatures in enteric fever. Broadly, these investigations will be divided into two parts, with the first focusing on the development of methods to measure antibody-dependent phagocytosis in serum from typhoid-vaccinated and unvaccinated individuals, as well as the characterisation of these antibodies by evaluating the effect of different glycosylation profiles on antibody function. The second section will focus on elucidating the genetic basis of disease susceptibility, through the use of genome-wide associations studies of enteric fever, and functional mapping of candidate disease risk loci. Collectively, these objectives will further illustrate the diverse roles of antibody-mediated protection in enteric fever and their associated receptors.

1.1.1 Aims I: Humoral mechanisms of vaccine mediated protection

Chapter II) The development of Fc-antibody functional assays

- To develop methods for measuring antibody-dependent neutrophil phagocytosis (ADNP) and the oxidative burst in serum from Vi-vaccinated adults and children.

Chapter III) Antibody dependent neutrophil functions following typhoid vaccination

- To examine correlations of antibody-dependent neutrophil functions with typhoid fever outcome and symptom severity.
- To assess Vi-TCV immunogenicity in an endemic Nepalese population using antibody-dependent neutrophil functional assays.

- To compare immune responses between those vaccinated in Nepal and Oxford, and assess the impact of age, and baseline titre on antibody-dependent neutrophil phagocytosis.
- To assess the influence of Fc glycosylation profiles upon Fc-mediated effector functions following typhoid vaccination.

1.1.2 Aims II: Genetic Susceptibility to enteric fever

Chapter IV) Genome-wide association study of typhoid fever and vaccination in experimentally infected individuals

- To perform a GWAS of enteric fever, utilising the increased statistical power to detect novel associations in a serovar-specific manner.
- To develop methods for fine-mapping of genetic loci associated with either protection or susceptibility to enteric fever. These analyses will integrate gene expression data, variant annotation, and pathway analyses using both data from typhoid human challenge studies, and public databases.
- To investigate potential associations of genetic variation with vaccine-mediated protection, including Vi-antibody responses.

2

Materials and Methods

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2.1 Fc-mediated neutrophil effector assays

This section details the cohorts and methods used to perform analysis of ADNP and ADNOB following typhoid vaccination in both the UK-based Vaccines against Salmonella Typhi (VAST) study, and Nepal based (TyVAC) study (Table 2.1). Full details of these study protocols can be found at Efficacy and immunogenicity of a Vi-tetanus toxoid conjugate vaccine and Phase 3 Efficacy Analysis of a Typhoid Conjugate Vaccine Trial in Nepal, respectively. The details of the optimisation steps I performed for both ADNP and ADNOB assays are described further in chapter 3. Following optimisation, I then use these assays in chapter 4 to investigate Fc-mediated neutrophil effector functions following vaccination, along with associations of other Fc-features such as glycan profiles, and receptor binding.

2.1.1 Vi-TCV and Vi-PS vaccination in UK adults: VAST

Healthy UK-based adults aged 18 to 60 years without a history of typhoid fever or vaccination received a single dose of either a Vi tetanus conjugate vaccine (Typbar-TCV, Bharat Biotech), a Vi-PS vaccine (Typhim Vi; Sanofi Pasteur), or a control (MenACWY) vaccine (MENVEO; GlaxoSmithKline), randomised 1:1:1 in a double-blind trial. To evaluate the efficacy of the Vi-TCV vaccine, participants were challenged 28 days after immunisation with a dose of $1 – 5 \times 10^4$ CFU of *S.* Typhi in bicarbonate solution and observed for the development of typhoid fever. Participants were diagnosed with typhoid fever if they presented with a continuous temperature of 38°C or higher for 12 hours or longer, or if a positive *S.* Typhi blood culture was obtained within a 14-day period following challenge.

Serum and plasma were collected on day 0 (pre-vaccination) and 28 days after immunisation (D28) prior to *S.* Typhi challenge. 37 subjects were administered the Vi-conjugate vaccine, and 35 participants received the Vi-polysaccharide vaccine. Serum from all typhoid vaccinated participants was run in the initial ADNOB experiments. Follow up experiments comparing responses with the Nepalese cohort utilised plasma from 16 participants who were not diagnosed with typhoid fever in the CHIM study.

2.1.2 Experiment cohorts

Assays	Experiment Aim	Chapter	Study	Vaccine (n)
ADNOB/ ADNP	Development of the ADNOB assay	2	VAST	35 Vi-TCV 37 Vi-PS
ADNOB/ ADNP	Investigating correlates of vaccine mediated protection	3	VAST	35 Vi-TCV 37 Vi-PS
ADNOB/ ADNP	Comparison of Fc-mediated neutrophil responses in UK and Nepal based studies	3	VAST TyVAC	16 Vi-TCV 17 Vi-TCV
ADNP ELISA	Associations of ADNP with Vi-isotype titres and age	3	TyVAC	77 Vi-TCV
ADNOB/ ADNP Luminex	Associations of ADNP/OB with antibody subclass and FcR binding	3	VAST	35 Vi-TCV 37 Vi-PS
ADNP and Fc-glycan profiling	Associations of ADNP with Fc- glycosylation	3	TyVAC	26 Vi-TCV
ELISA Genotyping	GWAS of Vi-antibody titres following typhoid vaccination	4	VAST	34 Vi-TCV 33 Vi-PS

Table 2. 1 Breakdown of the analyses undertaken upon participants vaccinated in both the VAST and TyVAC studies.

2.1.3 Vi-TCV vaccination in Nepalese children: TyVAC

Vi-TCV vaccine efficacy was assessed in children aged 9- to 16-year-old in Nepal in a Phase 3, double-blind, randomised controlled study. See Shakya et al. (ISRCTN 43385161) for complete study information (Shakya et al., 2019). Vaccinations were administered in Lalitpur Metropolitan City, Kathmandu Valley, Nepal between November 20, 2017 and April 9, 2018. Children enrolled in the trial were randomly assigned to receive either the Vi-TCV vaccine (Typbar-TCV, Bharat Biotech), a meningitis vaccine (MenAfriVac, Serum Institute of India PVT Ltd), or a placebo The participants in the immunogenicity cohort underwent blood sampling on day 0 (pre-vaccination) and 28 days post-vaccination. For our investigation, we selected plasma samples from 77 Vi-TCV vaccinees from the immunogenicity cohort, representative of the range of Vi-IgG titres. These participants also included 25 children whose glycosylation profiles had been assessed, and were previously selected at random for study.

2.1.4 ADNP and ADNOB assays

Plasma or serum samples were first heat-inactivated at 56 ˚C for 30 minutes. For experiments just investigating ADNOB in UK participants in VAST, serum samples were diluted at 1:75. When investigating ADNOB and ADNP in both Nepali and UK based cohorts, plasma samples were diluted at 1:160 (ADNOB) and 1:320 (ADNP) in RPMI. Assay dilutions were determined previously by measuring several dilution curves of these experiments on separate occasions.

Red fluorescent NeutrAvidinTM-Labeled microspheres (Thermofisher) were coupled with biotinylated Vi antigen (NIBSC) to simulate Salmonella bacteria in our assay. 2.5 ml of biotinylated 1 mg/ml Vi-antigen was added to 10 ml of red fluorescent NeutrAvidin-Labeled microspheres per plate, and the mixture was incubated overnight at 4 ˚C or for two hours at 37 ˚C . Before the assay, the beads were twice washed with 0.1% and 5% BSA for blocking and resuspended in 1 ml of

RPMI per plate. In 96-well plates, serum or plasma samples were then incubated for 2 hours with Vi-coupled red fluorescent microspheres.

Using an ammonium-chloride-potassium (ACK) lysis buffer, leukocytes, including neutrophils, were extracted from the sodium-heparin-treated blood of healthy, anonymous UK volunteers. Each dish received 5 × 104 leukocytes to each well of antibody-opsonised beads were added. APC-Cy7 anti-CD14 (Clone MyP9, BD Biosciences, Franklin Lakes, USA), Alexa Fluor 700 anti-CD3 (Clone UCHT1700, BD Biosciences, Franklin Lakes, USA), and Pacific Blue anti-CD66b (Clone G10F5, Biolegend, San Diego, USA) were used to stain pelleted cells . Leukocytes were washed and analysed by flow cytometry using a BD LSRFortessa X-20. The programme FlowJo v10.6 was used to manually gate cell populations.

The phagocytic score was determined by multiplying the mean fluorescence intensity (MFI) of red beads within gated neutrophils (CD14, CD3 negative, CD66b positive) by the proportion of bead-positive neutrophils (Fig A.1). Using the following formula, the fold change in ADNP/OB from baseline to 28 days post-vaccination was calculated.

2.1.5 Vi-IgG and IgA ELISA

The anti-Vi IgG titers were determined using a commercial enzyme-linked immunosorbent assay (ELISA) kit (VaccZyme, Binding Site) in accordance with the manufacturer's instructions. Anti-IgA titres were measured by modifying the same commercially available kit by substituting a Vi-IgA conjugate for the TyphiVi-IgG conjugate. The ELISA titres were assessed in plasma samples collected on day 0 (pre-vaccination) and 28 days post-vaccination, and the data has been previously reported (Jin et al., 2017).

2.1.6 Fc-receptor binding assay

Post-vaccination sera were heat inactivated and diluted 1:500 in 1X PBS + 0.1% BSA + 0.05% Tween20. Biotinylated Vi-antigen that was coupled to streptavidin-coated MagPlex beads (Luminex), before incubation with heat inactivated serum for 2 hours. Vi-coated beads and sera were then washed and incubated for 1 hour at room temperature with recombinant biotinylated *FcγR2α , FcγR2β , FcγR2α*, and the IgA binding Fc*α*Rwith tetramerized via Streptavidin-PE. Beads were again washed and analysed using Luminex, and the median fluorescent intensity (MFI) of 30 beads per region was recorded. The greater the measurable fluorescence, the tighter the Vi-antibody binding to its Fc-receptor.

2.1.7 Vi-specific IgG capture and glycopeptide analysis

NeutrAvidin plates (Thermofisher) were coated for 2 hours at room temperature, with 100 ml biotinylated Vi (commercially biotinylated WHO international Standard Vi polysaccharide of *C. freundii* (NIBSC code: 12/244) at 1.25 g/ml in 0.5X PBS to collect species-specific IgG. After three washes with 200 l 0.5X PBS, 200 ml of 1:20 diluted in 0.5X PBS, plasma was incubated for 1 hour at room temperature before being transferred to another Vi-coated blank well for a further 1 hour of incubation. After each incubation, the wells were rinsed four times with 200 ml of freshly prepared 25 *mM* ammonium bicarbonate (Sigma). After the washings, 50 ml of 100 *mM* formic acid (Sigma) was added and incubated at room temperature for 5 minutes whilst agitated.

The 50 ml eluate was transferred to a clean V-bottom 96-well plate, another 50 ml of 100 *mM* formic acid was added, the mixture was incubated for 5 minutes with agitation, and the 2 eluates from the same sample were combined. Samples were dried in a vacuum centrifuge for 1-2 hours at 50-60 ˚C before being delivered to LUMC for total IgG processing. Briefly, materials were resolubilised in 20 ml of 50 mM ammonium bicarbonate (Sigma) for 5 minutes while being shaken.

Twenty microliters of 0.05 g/l of tosyl phenylalanyl chloromethyl ketone (TPCK)-treated trypsin (Sigma) in ice-cold filtered water were added to each well, and the samples were incubated overnight at 37 °C.

2.1.8 Total IgG glycopeptide analysis

On an Orochem filter plate, 2 ml of Protein G Sepharose 4 Fast Flow beads (GE Healthcare) were added per well and washed three times with PBS for IgG purification. Each well received 20 ml of plasma diluted 1:20 (in PBS). Plates were incubated for one hour with 750 rpm shaking. The samples were washed three times using a vacuum manifold and 400 ml of PBS, followed by three times 400 ml of filtered water. Antibody elution from the beads was facilitated by adding 100 ml of 100 mM formic acid (Sigma), stirring for 5 minutes, and centrifuging at 100 g for 2 minutes. Before resolubilisation, samples were dried for two hours at 60 °C in a vacuum centrifuge. Twenty ml of 0.05 g/l tosyl phenylalanyl chloromethyl ketone (TPCK)-treated trypsin (Sigma) in ice-cold filtered water was added to each well, incubated overnight at 37 °C , and then frozen at -20 °C until MS analysis.

2.1.9 Nano LC-ESI-QTOF-MS Analysis

Prior to mass-spectrometry analysis, the purified plasma samples were defrosted and thoroughly homogenized. Five ml of Vi-specific IgG glycopeptide samples or 200 nl of total IgG were loaded into an Ultimate 3000 RSLCnano system (Dionex/Thermo Scientific, Breda, the Netherlands) linked to a quadrupole-TOF-MS (MaXis HD; Bruker Daltonics, Bremen, Germany). The Acclaim PepMap 100 trap column (particle size 5 mm, pore size 100 mm - 20 mm, Dionex/Thermo Scientific) and the Acclaim PepMap C18 nano analytical column (particle size 2 mm, pore size 100 mm, 75 mm, 150 mm, Dionex/Thermo Scientific) were installed in the LC system. Glycopeptides were identified using their mass-to-charge ratio, retention duration, and published data (Falck et al., 2017).

2.1.10 Statistical Analysis:

Statistical analyses were performed using R version 4.2.1. The data on phagocytosis were initially examined for normalcy using the Shapiro-Wilks test. The Wilcoxon paired test was used to compare non-parametric groups, including comparisons between time points, whereas the Wilcoxon test (unpaired) was employed to compare research cohorts. Data on glycosylation were correlated using Spearman's r correlation with a 95% confidence interval. **** = (P<0.0001), *** = (P<0.001). ** = (P<0.01) * = (P<0.05).

2.2 Genome-wide association studies of typhoid fever and vaccination

In order to investigate genome-wide association studies of typhoid fever and responses to vaccination; data was consolidated from both existing genotyping and transcriptomic data, in addition to genotyping additional DNA from participants of the VAST typhoid vaccine study, and the PATCH 're-challenge' study of enteric fever. Existing data included omics gathered from a dose escalation study (T1), a comparative study of live oral typhoid vaccines (T2), and TyGER, which compared typhoid challenge using a wild type *S.* Typhi strain, and a typhoid toxin knock-out (KO) strain.

All participants were aged 18-45 and had no prior history of enteric fever, nor had recently travelled abroad; the exception being in the re-challenge study. For this cohort, only participants in the heterologous re-challenge group (Paratyphi-Typhi) were included in the GWAS analysis, as analysis suggested there was no protective effect from heterologous challenge. Previous challenge was also accounted for as a covariate within the regression model. Further details of the enteric human challenge study protocols can be found within the main study publications (Table A.6).

2.2.1 GWAS Cohorts

Data from participants across *S.* Typhi challenge studies were utilised for genomic and transcriptomic analysis, where samples were available. Following genotyping, data were merged from across study cohorts, quality controlled and batch corrected before being used in the final GWAS and eQTL analysis of typhoid challenge (n = 248). Regarding the GWAS of vaccine responsiveness, only participants from the VAST study were used (n =96) (Table 2.2).

Parent study	Strain	Conditions	Genotyping platform and batch	nTD/TD	N Typhoid GWAS
VAST (Vi-vaccination study)	*S.* Typhi Quailes	Placebo, Vi-PS,Vi-TCV (1:1:1)	Affymetrix GSA V.3 (3)	51/45	96
PATCH (Re-challenge)	*S.* Typhi Quailes, S. Paratyphi A	Heterologous re-challenge vschallenge	Affymetrix GSA V.3 (3)	9/15	24
TyGER (Typhoid toxinstudy)	*S.* Typhi Quailes, Typhoid-toxin KO	WT v TN-(1:1)	Affymetrix GSA V.1 (2)	9/26	35
T2 (Typhoid oralvaccine trial)	*S.* Typhi Quailes	Placebo, MZH09 Ty21a(1:1:1)	Illumina OmniExpress-24 v1.0 (1)	31/36	67
T1 (Typhoid-Dose Escalation)	*S.* Typhi Quailes	High DoseLow Dose	Illumina OmniExpress-24 v1.0 (1)	8/18	26
Total				108/140	248

Table 2.2: A summary of the participants that were genotyped across enteric fever human challenge studies conducted at the Oxford Vaccine Group, and that were included inthe final GWAS analysis of typhoid fever. Bracketed numbers indicate batch.

2.2.2 Sample preparation

DNA for the VAST and PATCH genotyping analysis (n = 142), was extracted from blood clots previously stored at -80 ˚C. Clots were thawed rapidly at 37 ˚C and placed within 50ml falcon tubes fitted with Clotspin baskets (Qiagen; cat: 158932). Red blood cells were removed by the addition of red-blood cell lysis buffer. The remaining clot was homogenised using two rounds of centrifugation (2000G, 5 mins), vortexing (3 seconds) and shaking using an orbital mixer (200 RPS, 5 mins) at room temperature.

Cell lysis solution was added to the final clot mixure, followed by 10 seconds of a vigorous vortex and then incubated for 2 hours at 56 ˚C to complete cell lysis. Lysed samples were then used for DNA extraction with the Qiagen Qiamp Midi DNA extraction kit (Qiagen, cat: 51185), which was followedas per manufacturers protocol. Extracted DNA was then frozen at -20 ˚C and sample quality and yield was quantified using Qubit. Samples with $< 25\ ng/\mu l$ DNA were excluded for genotyping analysis.

2.2.3 Genotyping arrays

Genotyping for the VAST and PATCH typhoid and paratyphoid challenge studies was performed at the Tayside Centre for Genomic Analysis using the Global Screening Array V.3. 142 samples were genotyped with 729,332 SNPs profiled. Genotyping for T1 and T2 typhoid challenge studies was carried out by the Wellcome Trust Centre for Human Genetics on DNA samples (n = 96) on an Illumina OmniExpress-24 v1.0 array, with 716,503 SNPs genotyped in total. Finally, the TyGER typhoid toxin study, and a paratyphoid dose finding study (n = 80) were also genotyped together on an Illumina OmniExpress-24 v1.3 array at the Wellcome Trust Centre for Human Genetics.

2.2.4 Data Pre-Processing

To ensure accurate associations between genotypes and susceptibility to typhoid fever, our data were subjected to rigorous quality control before and after imputation. These data were first converted to the most recent GRCh38 human genome build using UCSC Liftover (Hinrichs et al., 2006), and SNPs were renamed to CHR:BP coordinates to prevent data loss due to differences in array annotation. Genotyped samples underwent pre-imputation quality control as per their batch using PLINK V1.9-V.2 and R version 4.1 (Chang et al., 2015). Prior to imputation and association analysis, SNPs with high levels of missingness (> 10%) are removed. Similarly, samples with a high proportion of missing data across all genotyped SNPs, which indicate a low quality or contaminated DNA sample were removed. A second round of SNP missingness filtering was then performed (Fig 2.1).

The HWE calculates the expected frequency of an allele pair in a population given the laws of independent assortment, Mendelian inheritance, random mating, and non-natural selection. The deviation of the observed allele frequencies from those expected under HWE is calculated using the Pearson's X^2 test. Deviation from HWE may be indicative of violations of the equilibrium, i.e. selection pressures

or non-random mating. However extreme divergence from HWE in GWAS settings, is likely to reflect genotyping error, and subsequently SNPs exhibiting a large HWE deviation are excluded from further analyses ($P < 1 \times 10^{-5}$).

Individual sex was then evaluated against the sex imputed based on X-chromosome heterozygosity rates, with mismatched samples removed from the study ($n = 7$). The X-chromosome heterozygosity (F-statistic), was calculated in PLINK. As different populations might have different mean heterozygosity rates, thresholds were determined based on data inspection in R. Following heterozygosity check, sex chromsomes were removed, as per common procedure (Balding, 2006). Lastly, SNPs were aligned against the TOPMed Hg38 Freeze 5 reference genome (Mbatchou et al., 2021), and mismatching, AT-CG and (Insertion-deletion) INDEL SNPs were removed (Rayner & McCarthy, n.d.).

2.2.5 Imputation

Imputation infers the genotypes of SNPs that were not explicitly measured by each genotyping arrays, and is based on the inheritance of SNPs in linkage disequilibrium (LD) with one another. By increasing the coverage of genomic regions, the statistical power of the study is increased, and the linkage disequilibrium (LD) between the most strongly associated SNPs in a GWAS analysis and unobserved genetic drivers can be reduced, allowing for more accurate fine-mapping of genomic loci of interest. In addition, as the coverage of SNPs increases, the comparability of studies measured on different arrays improves, minimising batch-related biases.

Prior to imputation, haplotypes were estimated and phased using the reference genome and study data and the the Eagle v2 algorithm (Loh et al., 2016). The Minimac4 utility (Fuchsberger et al., 2015), was then used to impute SNP data from the TOPMed r2 genome ($n = 97,256$, SNPs = 308,107,085) using haplotype blocks (Taliun et al., 2021). The deployed Minimac algorithm relies on state- specific

reduction of hidden Markov models (HMM), which searches for similaritiesbetween haplotypes within small genomic windows in order to group haplotypes and reduce the number of modelling states. The HMM algorithm then iterates over each window and infers missing genotype information based on the reference genome. These operations were all executed remotely on the TOPMed imputation server (Fuchsberger et al., 2015).

Following imputation, variants were filtered for those which had allele frequencies $R < 0.8$, leaving only high quality imputed SNPs. At this stage VCF files from separate genotyping batches were merged using bcftools and converted back to PLINK format, and re-filtered for SNP missingness and HWE ($n\,SNPs = 5,708,36$). In addition, SNPs with a minor allele frequency of <10% were excluded from our analysis as the study sample size meant that we would likely be unable to detect associations between rare SNPs. In total $4,577,376$ SNPs were used in downstream association analyses of typhoid fever and vaccine responses (Fig 2.1).

2.2.6 Sample relatedness and population structure

A assumption of genetic association research is that participants are unrelated. As a result of marker linkage and inheritance between related individuals, their inclusion can confound estimates of allele frequencies, which later might lead to false positive results. The relatedness of our participants was estimated after pruning variants for linkage disequilibrium and computing an identity by descent matrix in PLINK, which can also detect cryptic relatedness between samples with common ancestry (Weir et al., 2006). Samples that inherited alleles at a frequency greater than what would be expected without relatedness ($\pi < 0.185$) were documented. In total, one pair of twins, and two sibling pairs were detected. One sibling from each pair was subsequently removed based on SNP missingness.

Underlying population structure due to ancestral differences also has the potential to confound genetic association studies in specified populations, as it may introduce differences in allele frequency in cases and controls that could be misinterpreted as significant traits. Principal components can be included within association models to account for the genetic structure of samples when modelling data. These are calculated using Principal Components Analysis (PCA) from non-related individuals, and a set of pruned independent SNP markers ($R2 < 0.2$), as PCA assumes independent distribution of data. The PCA may also capture other aspects of data structure including batch effects or sex, therefore, components were carefully inspected both with reported ethnicity data, sex, and batch, and alongside imputing ethnicity from the 1000 Genomes Project in R (Auton et al., 2015). The first 5 principal components were subsequently included in genome regression models. Full details of these scripts and processes can be found at: https://github.com/mari-lynne/Enteric_Fever_GWAS

2.2.7 Statistical Power

For the GWAS of typhoid fever, statistical power calculations were used to describe the probability of rejecting the null hypothesis, at a given significance threshold, after accounting for the study design (total sample size, case-control counts) and parameters describing the disease variant (MAF and effect size). For these estimates I used a sample size of 250 participants, 4 million alleles, an average MAF of 0.5, and an effect size of 0.2, or odds ratio of 1.2. When estimating these traits, we were underpowered to detect significant associations at a genome- wide level $P < 5 \times 10^{-8}$. At $P < 1 \times 10^{-5}$ our statistical power was still limited (~0.05). Therefore, a frequentist approach to investigating the top significant genes associated with enteric fever would likely not be appropriate for this study. Subsequently fine-mapping of genomic regions was also used to determine loci of interest.

2.2.8 Genome-wide association regression models

In order to estimate the effect of genotype upon antibody-responses following typhoid vaccination a generalised linear regression model was fitted using PLINK 2.0, with the basic form described below. Whereby, y represents our response variable, titre; G is the genotype dosage matrix, X is the covariate matrix with an intercept vector, e represents the standard error (or residual) of our model estimate, and β are the coefficients of determination.

$$y = G\beta + X\beta + e$$

If $G\beta = 0$ the allele tested shows no relationship with our phenotype score, if $\beta > 0$ the allele is associated with a more positive score, and if $\beta < 0$ the allele is associated with a more negative score. The model slope is then compared with the null model, and significance can be estimated by using the likelihood ratio test under an X^2 distribution. The significance of covariate parameters in the genotype model can also be determined in this way.

For binary phenotypes, such as the case-control studies of enteric fever, logistic regression was used to model the effect of genotypeson the relative risk of typhoid fever. The generalised linear model is first fitted to the data and parameters, with the log-odds ratio estimated through the use of the logit link function as below, where Y represents a vector of 0's and 1's (cases and controls) and the log-odds ratio of our phenotype is stored in logit (P (Y = 1)). Given the small sample size of our study and the unbalanced case-control ratio, a firth regression penalty option, *e,* was also added to the final model.

2.2.9 Variant annotation

SNPs identified in GWAS that were significant above $P < 1\times10^{-4}$, were implemented in the FUMA v1.4.1 variant annotation pipeline (Watanabe et al., 2017), and subsequent downstream eQTL and GSEA analyses. As the FUMA pipeline requires input data to be in GRCh7 build, data was converted the GRCh37 build using Liftover in command line. For the GWAS of typhoid fever, where we did not meet the standard significance criteria of $P < 5 \times 10^{-8}$, our top SNPs ($P < 1 \times 10^{-4}$) were input as candidate lead SNPs for fine-mapping. Variants in linkage disequilibrium with the lead SNP were then examined in a stepwise manner and were grouped into independent loci if inherited together ($R2 > 0.6$). This approach is thus allows us to examine the effects of SNPs nearby to the most significant variant, which may have more downstream functional annotations that could lead to causal variant detection.

The linkage disequilibrium of each independent-loci was calculated, and variants were annotated for genomic features including, genes, ncRNA, introns and exons (H. Yang & Wang, 2015), predicted deleteriousness (CADD), and variants previously reported in the GWAS catalogue (Rentzsch et al., 2018). Chromatin sites and regulatory features of loci were examined using RegulomeDB and the Roadmap database (Boyle et al., 2012; Kundaje et al., 2015). EQTL of associated variants were queered in the GTEx V8 database for tissue specific-QTLs (Lonsdale et al., 2013), as well as cell-specific response eQTL in *Salmonella* infection, previously reported by Alasoo et al. (2018), and immune-cell specific data generated by (Fairfax et al., 2012), and platelet specific eQTL were measured in the CEDAR data set (Momozawa et al., 2018).

2.3 RNA-Sequencing

Participants across the VAST and TyGER challenge studies were sequenced to measure gene transcription. Following quality control, these data were then used in subsequent DEG, eQTL, and pathway enrichment analyses (Fig 2.1).

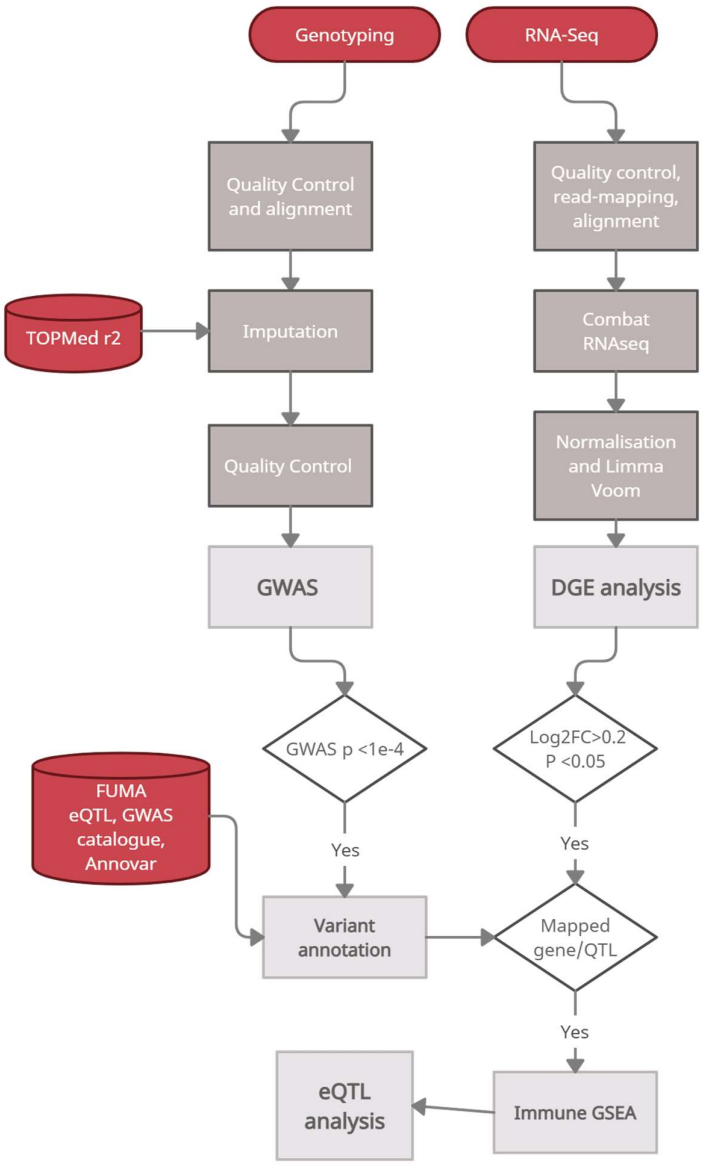


Figure 2.1: Genomic and transcriptomic analysis workflow An overview of the pre-processing quality control steps, dataset integration and downstream analysis undertaken using genotyping and RNA-Seq data from enteric human challenge studies.

2.3.1 RNA-seq cohorts

Study	Strain	Conditions	RNA-seq platform	TD /nTD	GWAS (n)	DGE Baseline	DGE D7-TD
VAST (Vi-vaccination study)	*S.* Typhi Quailes	Placebo, Vi-PS, Vi-TCV (1:1:1)	Illumina HiSeq 200 V4 Wellcome Trust Sanger-Institute	51/45	96	50	24
TyGER (Typhoid toxin study)	*S.* Typhi Quailes, Typhoid-toxin KO	WT v KO - (1:1)	Illumina HiSeq 200 V4 BDI	9/26	35	31	25
Total (n)					101	81	49

Table 2.3: A summary of the participants which were analysed for differential gene expression analysis across typhoid fever human challenge studies in this thesis.

2.3.2 Pre-processing

RNA samples from the typhoid toxin study (n = 40) and the Vi-vaccination study (n = 103) were obtained from whole blood at the following time points (Table 2.3), and extracted using the Tempus Spin RNA Isolation Kit (Life Technologies), with RNA concentration quantified by nanodrop. Libraries were then prepared using a poly-A selection step to exclude ribosomal RNA species (read length: 75 bp paired-end) and samples were subsequently multiplexed for sequencing. Following sequencing, FastQ files from each sample were concatenated, and quality control pre-alignment was performed with FASTQC, before aligning RNA to the human genome (GRCh38 Gencode version 26) using STAR-2.6.1c (Dobin et al. 2013). The number of mapped reads per gene were then tallied using the STAR GeneCounts mode. Probes that did not map to genes or probes that aligned to multiple genes were subsequently excluded from the analysis.

2.3.3 Batch correction

In order to merge data sets, mapped read counts were first batch corrected within their individual study using the Combat RNA-seq package (Zhang et al., 2020). For VAST this included adjusting for the sample sequencing lane which had a significant batch effect, as determined by multiple dimensional scaling (MDS). The TyGER study was batch corrected for the resequenced subset of vaccination samples taken at baseline. The CombatSeq package allows for the inclusion of co-variates during adjustment to ensure biological variation is not removed. For data from the VAST study, I included time-point and vaccine type as covariates, and the strain of typhoid. Data from the TyGER typhoid toxin study included the challenge time point and typhoid strain as covariates in the correction model. Across each batch correction stage, groups were examined to ensure a maximal balanced study-design per batch, so not to introduce confounding effects during correction. Lastly, the corrections were assessed by visualising the PCA of each cohort to ensure that biological effects had not been removed (Fig A.2).

Following individual study correction, data was preprocessed for merging; corrected counts were filtered to include only reads measured in both studies, annotated genes. Lowly expressed genes across both studies were also removed, leaving a final of 12530 gene transcripts, and an average read depth of 12 million reads per sample. These data were then batch corrected again for study, and time-point, vaccination were used as covariates. Merged RNA-seq data (n = 143), then underwent standard RNA-seq processing in limma and edgeR as detailed by (Smyth et al., 2018), and were normalised using the trimmed mean of M values (TMM) method.

2.3.4 Differential gene expression analysis

Limma-voom transformation was applied to count data to correct for sample variance. Next, differential gene expression was estimated by fitting a linear regression model in Limma, with vaccination status, sex, age and challenge strain as covariates and the Participant ID as a blocking variable. The Log_2 fold-change (Log_2FC) was calculated between specified contrasts of interests. Genes that were differentially expressed (DEG) were defined by a $Log_2FC > 0.2$ and a Benjamini-Hochberg adjusted *P*-value of 0.05. For differential expression after vaccination, these thresholds were relaxed to include samples with a $Log_2FC > 0.2$ and a non-adjusted *P*-value of 0.05 when considering genes for downstream analyses.

2.3.5 Expression Quantitative Trait Loci (eQTL)

For eQTL analyses, DEG count data was transformed to transcripts per-million (TPM), to account for biases in gene length that might be introduced when associating genotype data with RNA-seq count data. Analysis of eQTL were performed with the R package MatrixEQTL (Shabalin, 2012). SNPs located within 1 Mb of the gene transcription start-site were classified as cis-eQTL. The R package computes linear models between genotype matrix, expression and covariate matrices. Within covariates, age, sex, BMI, study, vaccination and strain included where appropriate. False discovery rate (FDR), was used to adjust for multiple testing. Variants were then analysed for significant differences in fold-change in expression between baseline, and 7-days post-challenge or time of diagnosis (TD). eQTL of vaccine responses included measurements at baseline and 1-day post-vaccination. Linkage disequilibrium (LD) of eQTL were calculated in PLINK 2.0. LD, gene track information, eQTL and GWAS summary statistics were then integrated in R and visualised using eQTLPlot (Drivas et al., 2021).

2.3.6 Gene set enrichment analysis

Genes that were both differentially expressed (DEG), and mapped to SNP variants either by proximity to gene regions (10 Kb), or as predicted eQTL, and were ranked by fold-change and taken forward for gene-set enrichment analysis (GSEA). GSEA was performed in R using the Cluster-profiler package, against the MSigDB C7 curated immune database (*n genes* = 4782), (T. Wu et al., 2021; Liberzon et al. 2015). Significant enrichment was calculated using the Fisher's exact test, and significance was corrected for multiple testing using FDR.

3

The development of high throughput assays to measure antibody-dependentneutrophil effector functions

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3.1 Introduction

Neutrophils are the most abundant leukocyte in circulation, constituting over 60% of the white blood cell compartment, and are therefore among the first cells to respondto infections (Nauseef, 2007). At the site of infection, neutrophils are capable of a variety of anti-bacterial responses, including phagocytosis, the release of cytolytic granzymes and production reactive oxygen species (ROS) (Hellebrekers et al., 2017; Mantovani et al., 2011). Circulating neutrophils express low affinity Fcγ receptors,

which bind immunoglobulin. During the adaptive phase of immunity, neutrophil effector functions can be enhanced through the increased availability of antibody-Fc receptor interactions (Crockett-Torabi & Fantone, 1990). Antibody-bound neutralised pathogens can engage with multiple Fc-receptors on the neutrophil, leading to receptor cross-linking and phosphorylation of receptor associated Src kinase family members. Scr signalling then induces a signalling cascade which leads to remodelling of the actin cytoskeleton, and the formation of the phagosome (Huizinga et al., 1989; Zhang et al., 2010).

Following pathogen phagocytosis, intracellular signalling results in the formation of the NADPH oxidase enzyme complex. NADPH oxidase can then generates free radicals in a process known as the oxidative, or respiratory burst. Reactive oxygen species (ROS) include the free radicals nitrous oxide (NO-) and hydroxide ions (OH'), which can bind and chelate bacterial DNA, damaging the helical structure, eventually accumulating in damage causing bacterial cell death (Fig 3.1) (G. T. Nguyen et al., 2017). ROS-mediated killing has been implicated in immunity to several bacterial infections including *S*. Typhimurium and *Strep*. *pneumoniae*, following phagocytosis by activated macrophages and neutrophils (Gondwe et al., 2010; Janoff et al., 1999). Furthermore, neutrophil killing of *Strep*. *pneumoniae* isnow assessed as a correlate of protection when evaluating pneumococcal vaccine immunogenicity (Fabbrini et al., 2012). Given the role of neutrophils in preventing dissemination of *S*. Typhimurium, and the importance of the oxidative burst as a bactericidal mechanism, I aimed to investigate the role of these antibody-dependent neutrophil functions in vaccine mediated protection against *S*. Typhi.

To accomplish these aims, I replicated the previously established fluorescent-bead based antibody-dependent neutrophil phagocytosis (ADNP) assay within our laboratory settings, while adapting the method to measure the oxidative burst (ADNOB) in serum from typhoid-vaccinated individuals. Broadly these assays work by linking biotynlated antigens of interest, such as the Vi polysaccharide capsule of *S*. Typhi, to neutravidin-coupled fluorescent beads. Serum is then added to the

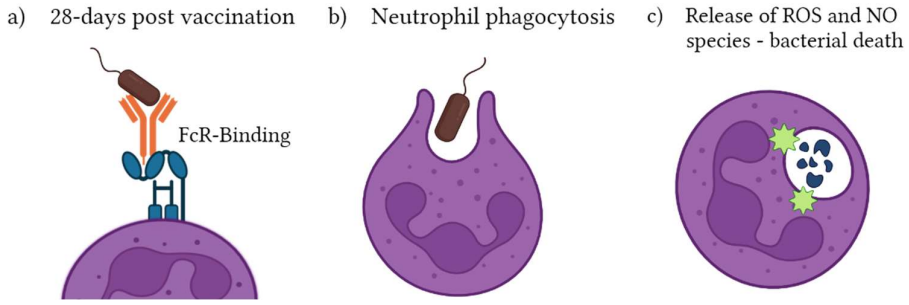


Figure 3.1: Mechanisms of antibody mediated phagocytsosis and oxidiative burst in neutrophils Following vaccination, the Fc-region of antibodies can bind to cognate Fc-receptors expressed on circulating neutrophils. Receptor binding induces phagocytosis of the bacterium, followed by the assembly of the NADPH oxidase complex, and the release of free reactive oxygens species (ROS), which results in bacterial DNA damage and death. *Created using Biorender.com.*

bead-antigen complexes, followed by the addition of the cell type of interest.The source of cellular material can be obtained from either established cell linesof primary cells of the desired lineage. For example, neutrophils from healthy blood donors are added to the ADNP assay, and THP-1 monocyte-like cells, are added to the antibody-dependent monocyte phagocytosis (ADMP) assay. Using flow cytometry, antibody-dependent phagocytosis or other effector functions can then be measured by quantifying the fluorescent signal of beads phagocytosed by cells (Karsten & Köhl, 2012; Ritz et al., 2015).

In order to quantify the oxidative burst induced by sera from typhoid vaccinated individuals; I optimised the ADNP assay by the addition of dihydrohidamine-123 (DHR) to detect the presence of reactive oxygen species released by neutrophils. DHR is a cell permeable probe that becomes oxidized by hydrogen peroxide (H_2O_2), resulting in the formation of its fluorescent form rhodamine, and is commonly used to assess the oxidative burst capacity of patients with suspected chronic granulomatous disease (Y. Chen & Junger, 2012; Roos & Boer, 2014). In this chapter, I describe the optimisation of the DHR-based ADNOB assay, and further refinement of the ADNP assay to measure antibody dependent neutrophil activity following typhoid vaccination.

3.1.1 Chapter Aims:

1. To develop a method to measure the antibody-dependent neutrophil oxidative burst induced by sera from typhoid vaccinated individuals.
2. To evaluate the reproducibility of ADNP and ADNOB assays and their sample capacity.
3. To investigate factors that may influence each assay, and mitigate assay variation where possible.

3.2 Results

3.2.1 Optimisation of high throughput assays to measure ADNP and ADNOB

The addition of DHR in an adapted ADNP assay 50µM detects increasesin ROS production

I introduced Dihdrohadamine-123 (DHR) to the previously established bead-based ADNP assay in order to determine if we could find increases in ADNOB induced by sera collected 28 days after typhoid with either Vi-TCV or Vi-PS immunisation (Jin et al., 2020; Karsten & Köhl, 2012). Briefly, fluorescent beads (Thermofisher) are coated with Vi antigen in a Neutravidin-based reaction, modelling Vi-coated *S.* Typhi. Anti-Vi serum from previously Vi-typhoid-vaccinated volunteers (NIBSC) is then combined with red-fluorescent Vi-conjugated beads prior to the addition of neutrophils from healthy blood donors. Following a one-hour incubation of serum with Vi-beads, neutrophil populations are examined using flow cytometry for both bead uptake, and fluorescence emitted by the oxidation of DHR (Methods: 2.1). A one-hour incubation time was selected based on previous

experimental work within the ADNP assay, which also established that there was no significant observable decrease in neutrophil viability up to a 4 hours incubation time with serum bound beads (Karsten & Köhl, 2012). Likewise, the ADNOB assay did not show significant differences between oxidative burst scores recorded following 45 minutes and 1.5-hour incubation times, or decreases in viability when measured by trypan-blue staining (Fig A.3).

DHR fluorescence was first tested at a concentration of 50 μM, as suggested by the literature (Y. Chen & Junger, 2012; Fuhler et al., 2007). I included DHR at this concentration following incubation with a Vi-positive serum control, and a no-serum negative control, in order to determine if we could observe a discernible shift in the mean fluorescence intensity (MFI). I used a 1:100 serum dilution as per the ADNP assay protocol. Using these conditions, a significant increase in (B530-30) MFI was observed between ADNOB measured in the serum standard andthe negative control, and this concentration was therefore used in future experiments (Fig 3.2). Furthermore, no fluorescence was detected when adding serum without beads, suggesting that the increase in DHR fluorescence was mediated by antibody dependent-phagocytosis of Vi-beads.

Dilution of serum samples for the ADNOB and ADNP assay

To optimise the serum concentration and characterise the linear range of the ADNOB assay, I performed serial dilutions of serum obtained 28 days post-vaccination from typhoid-vaccinated adults. Dilution curves indicated running the ADNOB assay at either a 1:75 or a 1:100 dilution would be appropriate (Fig 3.2 A-B). Given these findings, I chose to test a random subset of pre- and post-vaccination samples within both the ADNOB and ADNP assays at a dilution of 1 in 75, and observed that post-vaccination neutrophil oxidative burst and phagocytic scores increased significantly from baseline scores (Fig 3.3 C-D).

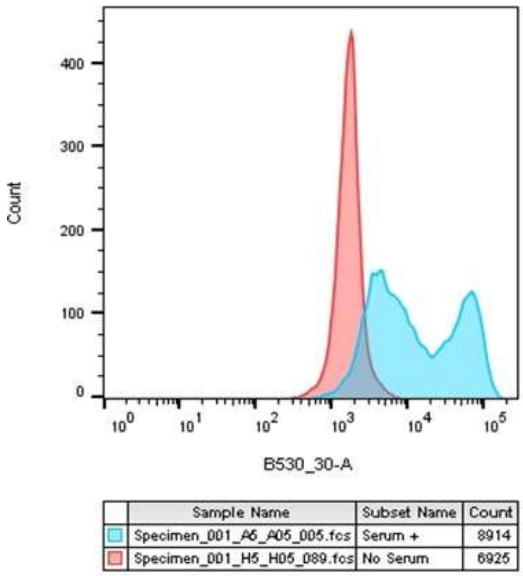


Figure 3.2: Fluorescence intensity of the oxidative burst following serum activation of neutrophils Addition of Vi+ serum standard induces an increase in the oxidative burst within gated neutrophils above baseline that is detectable at a concentration of 50 μM DHR within the assay. The y-axis denotes cell count. The x-axis shows the the fluorescence intensity scale of the DHR channel (B530-30

In addition to measuring antibody-dependent neutrophil function among serum from typhoid-vaccinated adults in the typhoid vaccination CHIM trial (VAST); I aimed to evaluate these assays using sera obtained from a cohort of Vi-TCV-vaccinated children aged six months to sixteen years old in Khatmandu, Nepal, an area endemic for typhoid fever. These samples were collected as part of the phase III Vi-TCV efficacy trial, known as TyVAC (Methods 2.1.2) (Shakya et al., 2019). Consequently, there were several differences between participant cohorts, including age, typhoid exposure, and anti-Vi titres at baseline. These factors could influence the level of antibody induced functional activity, and thus the appropriate serum dilution factor required in each assay (House et al., 2008). Moreover, whereas the VAST study collected serum, the TyVAC study collected plasma-based antibody fractions. Therefore, the dilutions previously selected may not have been comparable for measuring ADNP/OB in plasma from vaccinated children. I therefore ran serial dilutions of TyVAC plasma (n = 23), and a more dilute plasma concentration of 1:160-1:320 was selected for future assay development.

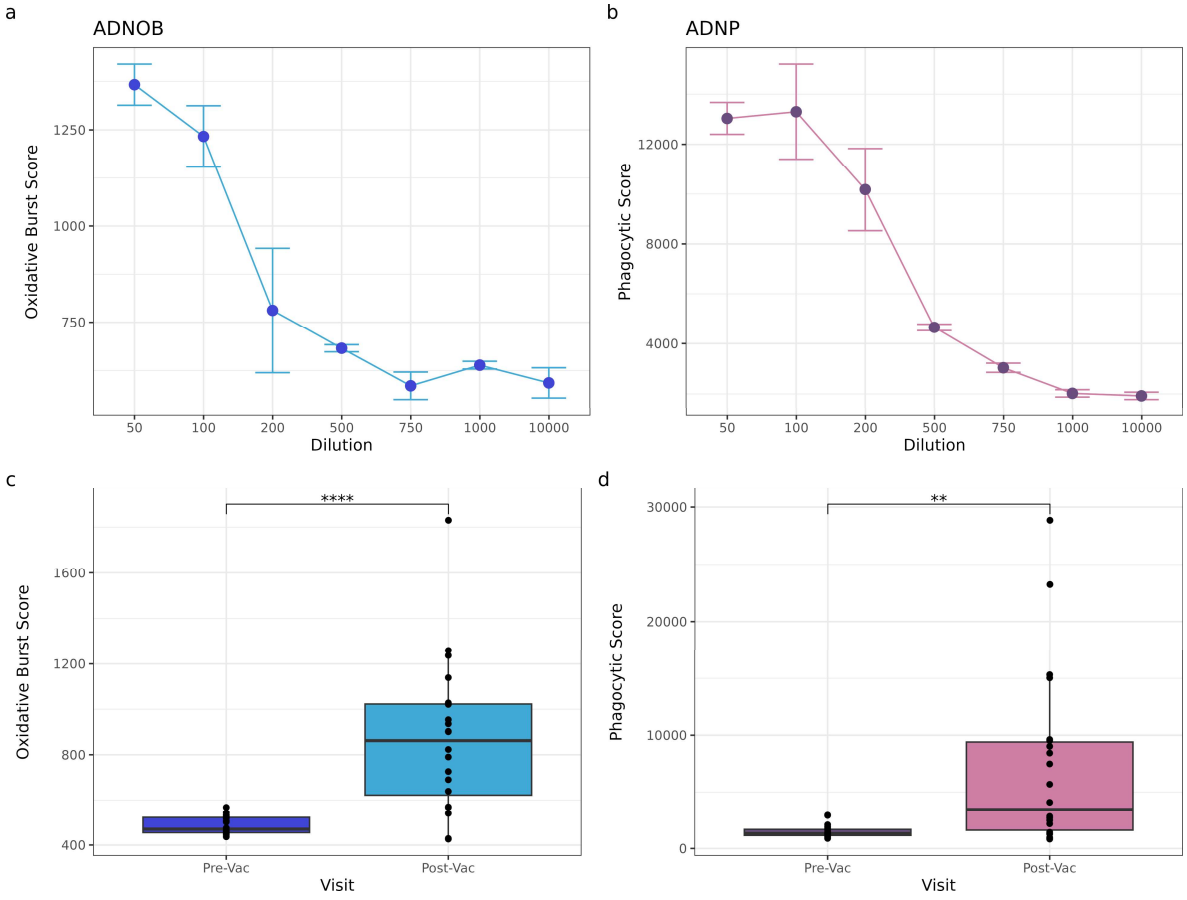


Figure 3.3: Dilution of Serum standard in ADNOB and ADNP assays A- B) ADNP and ADNOB were measured in tandem for the Vi-serum standard in a dilution series. Depicted are the mean oxidative burst and phagocytic scores for technical duplicates, error bars show the standard deviation between duplicates. C-D) ADNOB and ADNP pre and post-vaccination A subset of unpaired serum samples (n = 17) from a mixture ofVi-TCV and Vi-PS recipients, were diluted at 1:75 and measured in technical duplicatesfor ADNOB (A) and ADNP (B) activity pre, and 28 days post-vaccination. Box plots show the median and interquartile range for each group. Statistical comparisons performedusing un-paired Wilcoxon test ($****P < 0.0001, **P < 0.01$).

Technical variation of ANDP and ADNOB assays

To ensure that ADNP and ADNOB scores were reliable, I determined the range of technical variation in phagocytosis and oxidative burst assays was by comparing values between both technical duplicates, and across plates in serially diluted sera (Fig 3.4).

The evaluation of collective experimental data determined that a threshold of 25% CV between technical duplicates was suitable for accepting an ADNP or ADNOB measurement. Furthermore the average variation between duplicates measured on different plates remained below the 25% CV threshold (Fig 3.4). Consequently, multiple 96-well plates may be used within the same experiment to accurately compare antibody-dependent neutrophil functional activity, increasing sample throughput of these assays.

Neutrophil cell concentration for functional antibody assays

To assess suitable leukocyte concentrations in both the oxidative burst and phagocytosis assays, serial dilutions of the Vi-serum standard and positive pool serum were evaluated at multiple cell concentrations, ranging from 3.3×10^4 –20×10^4 cells per well. As per the original protocol, cells were stained with trypan blue before being manually counted using a haemocytometer. These counts were also later verified using a MUSE cell counter. I observed that phagocytosis scores measured between 3.3 – 15×10^4 cells per well were comparable. However, at higher concentrations, there was a considerable decrease in phagocytic scores (Fig A.5). The ADNOB assay was more sensitive to increasing cell densities, with oxidative burst scores dropping at 10×10^4 cells per well. Consequently, the original ADNP-assay cell density of 5×10^4 was utilised for both ADNP and ADNOB experiments.

Consistent oxidative burst scores are measured up to 5 hours post- experiment

Unlike cells which have phagocytosed fluorescent beads, the emitted fluorescence from DHR cannot be reliably preserved with paraformaldehyde (PFA), since it is a transient reaction (Vorobjeva et al., 2017). However, in practice, the user may experience logistical delays when accessing equipment and subsequently the time taken to measuring DHR fluorescence. To confirm that oxidative burst measurements

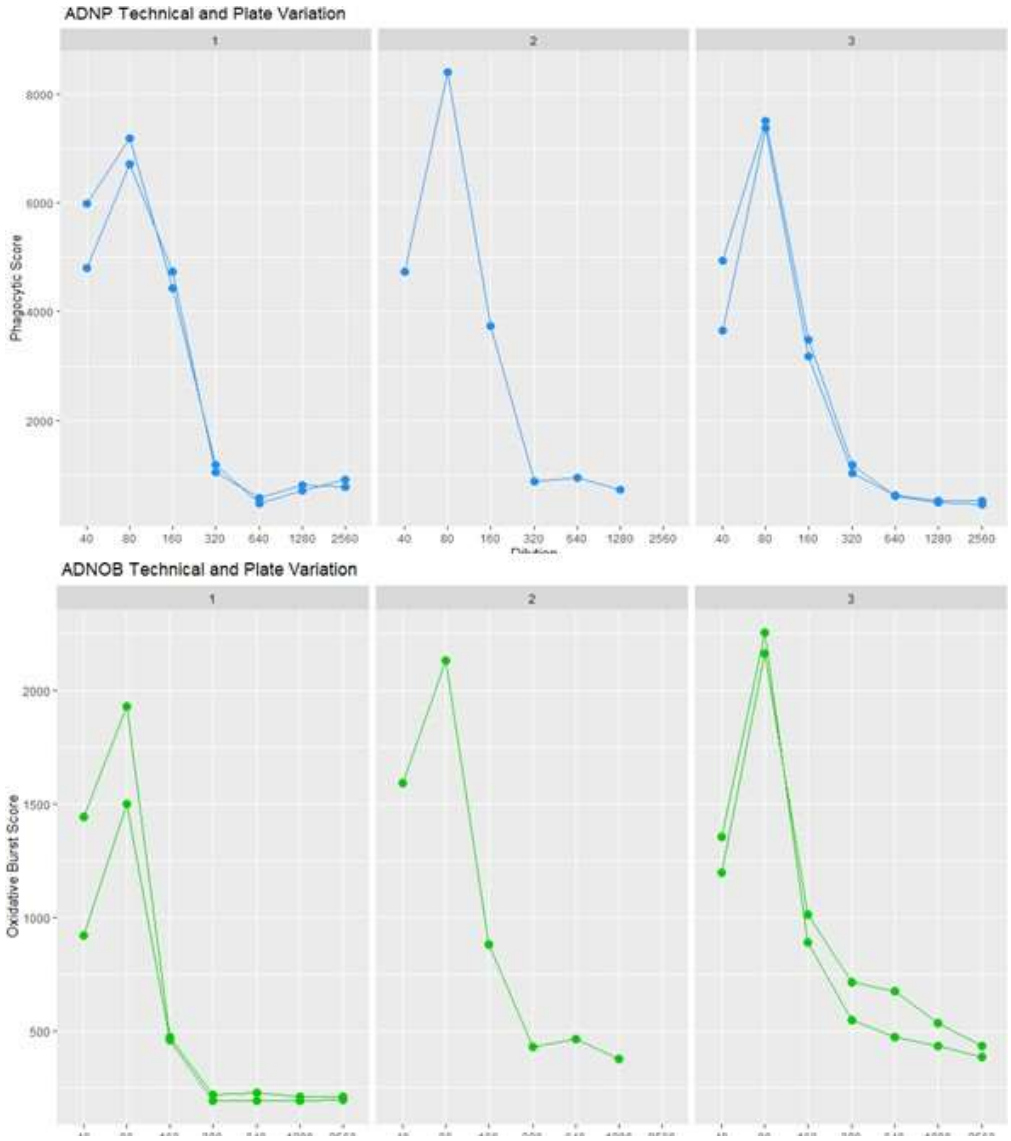


Figure 3.4: Technical Variation of the ADNOB and ADNP assays Technical duplicates of the Vi- serum standard were diluted in a 2-fold dilution series across three separate plates. The phagocytic score (top) or oxidative burst scores (bottom) are shown for each replicate independently.

could be obtained reliably during this period, we assessed the oxidative burst scores measured in plasma 28 days following Vi-TCV vaccination, 1 and 5 hours after the incubation step of the assay (n = 10). Whilst ADNOB scores did decrease 5 hours following the experiment, this was not statistically significant (Fig 3.5).

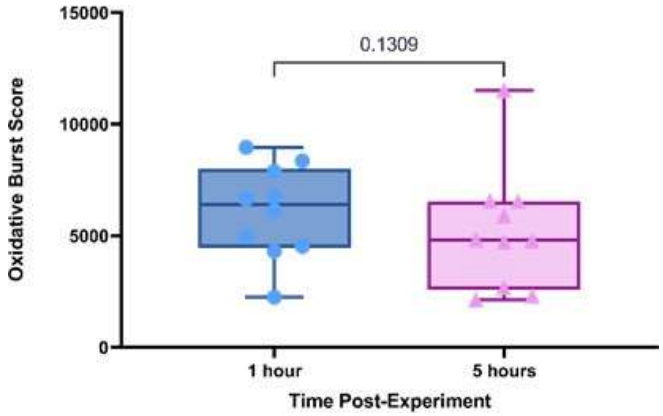


Figure 3.5: ANDOB assay 1 and 4 hours following serum incubation with neutrophils Oxidative burst scores were measured immediately after experiment completion, and 5 hours post-experiment using flow-cytometry (n = 10). ADNOB was assessed at a 1:160 serum dilution and displayed are the means from technical duplicates. Box plots show the median and interquartile range for each group, and whiskers show the minimum and maximum values. Statistical comparisons were performed using the Mann-Whitney U test.

3.2.2 Evaluating inter-assay replicability

ADNP and ADNOB Inter-Assay Replicability

To confirm the reproducibility of the ADNOB assay, a subset of pre-vaccination and post-vaccination sera were measured across 3 separate experiments. Significant correlations were observed between experimental replicates (R = 0.61 – 0.96, P = 0.001), indicating that the assays are reproducible (Fig 3.6 A-B). In addition, the ADNP results generated with sera from typhoid-vaccinated individuals correlated with data previously collected at a separate site (n = 30). (Fig 3.6 C). Inter-operator scores were likewise comparable. Moreover, measurements of the Vi-serum standard and positive pool across multiple experiments demonstrated assay consistency (Fig A.7). A few samples, however, exhibited moderate variation between experimental repeats (CV 25-55%), and mirrored the range of variation measured using the serum standard, indicating that it was not sample-specific. Dilutions of the Vi-serum standard and Vi-/+ pools of sera were also evaluated as a standard curve across experiments; however, the interpolated scores from the standard curves varied by the same magnitude as raw-values (data not shown).

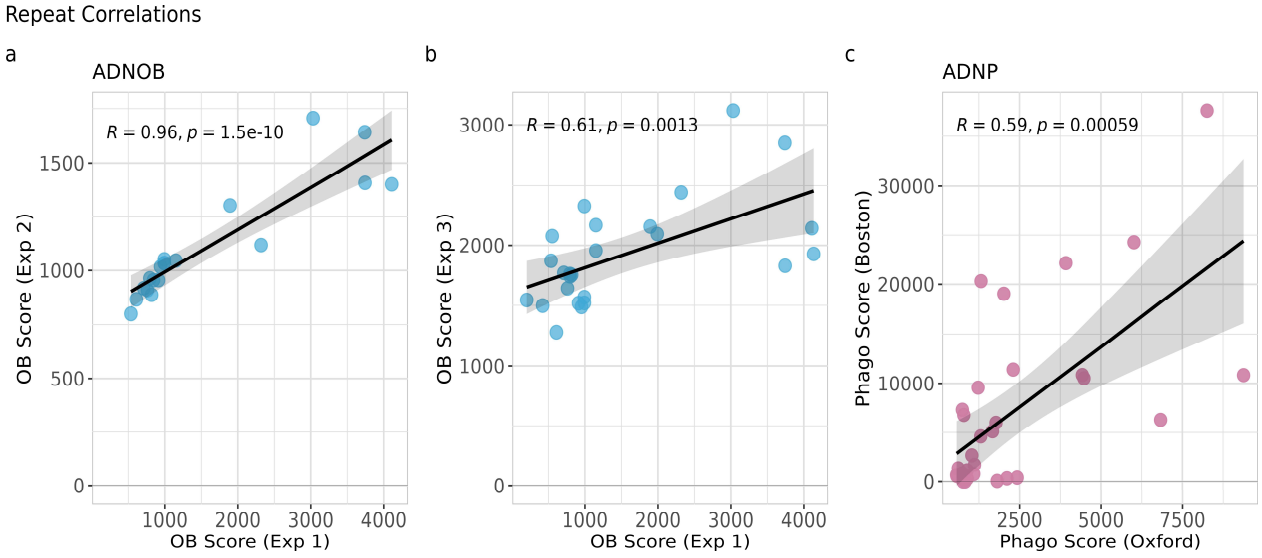


Figure 3.6: ADNOB and ADNP inter-assay replicability Serum samples from the VAST CHIM study were measured on 3 separate occasions, n = 19, and 25 respectively. Presented are the correlations of the oxidative burst scores across the first **(A)** and second repeats. **(B)** R and P values were calculated using spearman's rank correlation coefficient. **(C)** ADNP was measured in serum samples from typhoid-vaccinated adults at the Boston Ragon Institute, and measured again using the same subset in Oxford (n = 30). Depicted are the mean phagocytic scores between technical duplicates. Boston ADNP scores were scaled by 100. Spearmans rank correlation used to calculate the correlation between phagocytic scores measured at separate sites

3.2.3 Exploring sources of inter-assay variation

Neutrophil donor variation across ADNP and ADNOB assays

I hypothesised that the variation in phagocytic and oxidative burst scores measured across experiments could be as a result of differences in neutrophil donors, or their activation during sample preparation (Liles et al., 1995; Zhou et al., 2012). Therefore I measured ADNP and ADNOB across three blood donors as a source of neutrophils for the assay. For each blood donor, I tested the ADNP capacity using a positive pool of sera from Vi-TCV and Vi-PS vaccinated individuals, in addition to a subset of 10 serum samples which reflected a range of Vi-ELISA values. For the ADNOB assay, a subset of 5 serum samples were used to examine the impact of neutrophil donor variation (Fig 3.7 B). Across blood donors, ADNP and ADNOB scores correlated (Fig 3.8, R = 0.78 – 0.91, P < 0.005). In addition, the average

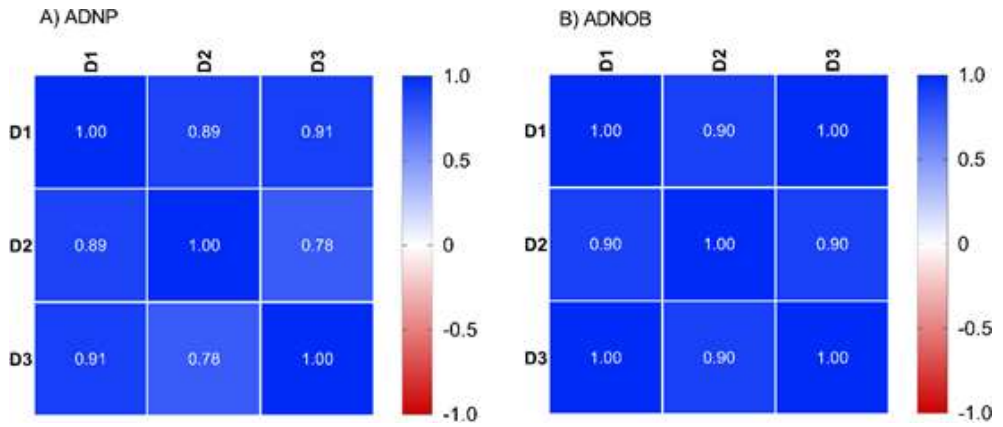


Figure 3.7: Correlation of ADNP and ADNOB scores measured using different blood donors. A subset of 10 plasma samples, and five serum samples from typhoid vaccinated participants were measured for ADNP and ADNOB across three neutrophil donors respectively. Correlation matrix displays Spearman rank scores of samples run using different blood donors.

percentage coefficient of variation (%CV) in sample phagocytic scores measured using different blood sources was not significantly different from the variation observed when measuring samples on different experimental days using the same blood donor (Fig A.7, $R = 0.81$, $P < 0.01$). Therefore, while blood donor variability may influence the level of ADNP, it is not a major contributor to inter-assay variation.

Comparison of RBC lysis and PolymophPrep cell separation

The cell separation procedure chosen for neutrophil functional assays utilises the lysis of red blood cells, after which, leukocytes, including neutrophils, are separated by a centrifugation step. The method whilst being cost-effective and time-efficient, does not produce a pure population of neutrophils. Neutrophil effector functions are instead later measured after manual gating using CD3-, CD14- and CD66+ markers (Methods 2.1.4). Other leukocytes in the assay population may secrete various cytokines, offering an additional possible source of variance. To ensure that the method of red-blood cell lysis was appropriate for functional-antibody assays, we examined two cell separation techniques in the ADNP assay. PolymorphPrep cell separation utilises gradient centrifugation to distinguish neutrophils from other

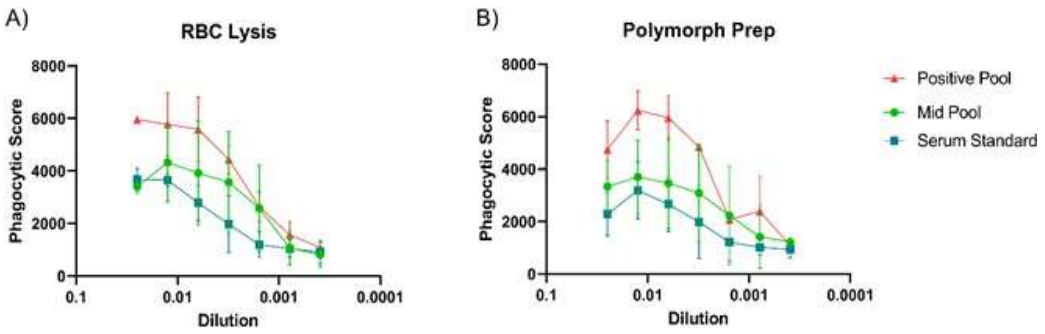


Figure 3.8: Comparison of ADNP scores for the serum standard, positive and mid pools between red blood cell lysis and polymorph prep methods. Pools of sera were serially diluted and measured for ADNP following preparation either with the red blood cell lysis (A) or using polymorph prep gradient cell separation (B). Depicted are mean phagocytic scores from 2 separate experiments across a dilution series of pooled sera, including high and medium Vi titre pools and the Vi-serum standard (NIBSC). Error bars show standard error of the mean across the experiment repeats.

white blood cell types and red blood cells (Zhou et al., 2012). Data showed that the methods were comparable when measuring the phagocytic scores of pooled sera, selected from high, mid, and low Vi-titre samples (Fig 3.8). Furthermore, the use of Polymorph-cell preparation did not significantly minimise variation between experiments.

Neutrophil activation may be sensitive to bystander activation during sample collection or cell preparation, as well as a result of lifestyle factors such as alcohol consumption (Krabbe et al., 2020). It was therefore hypothesised that differences in neutrophil activation both between methods and across different days may contribute to inter-assay variation. Consequently, in order to assess neutrophil activation across different experiments and methods, I measured CD11b expression, a commonly used as a marker for neutrophil activation, before and after stimulation with an agonist, PMA (Diacovo et al., 1996). CD11b expression varied between experiments, but this variation was not statistically significant, nor were differences in CD11b expression between neutrophil isolation techniques (Fig 3.9). In light of these results, and in order to maintain consistency with previously collected data (Jin et al., 2021), I continued to measure neutrophil effector functions using red-blood cell lysis and manual gating of neutrophil populations during flow-cytometry.

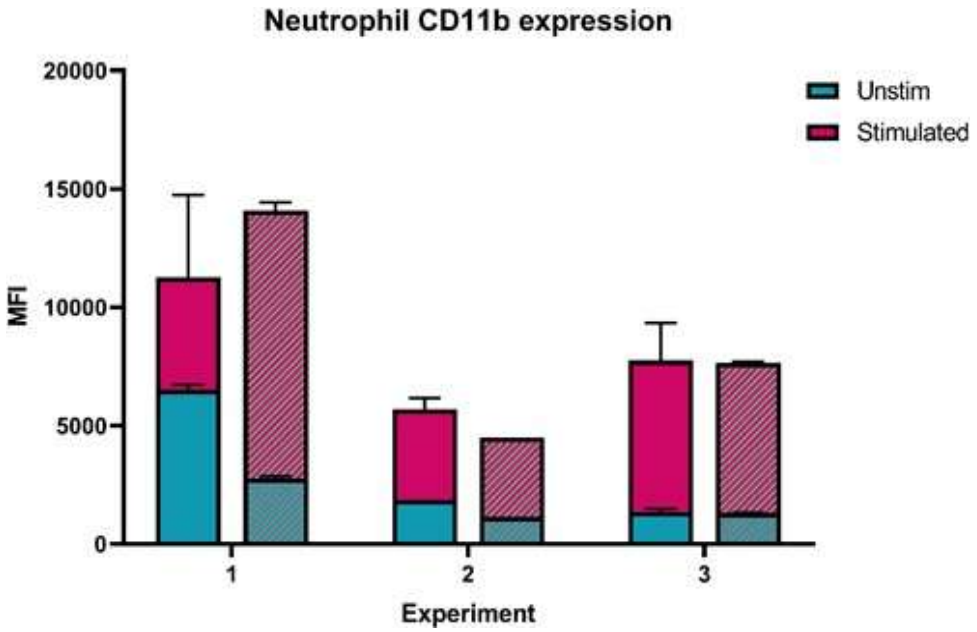


Figure 3.9: Neutrophil CD11b expression across experiments. Neutrophil activation was measured using flow cytometry to measure the relative MFI of fluorescent anti-CD11b of gated neutrophils. Neutrophils were prepared using either red-blood cell lysis (left) or polymorph gradient separation (right), then stimulated with PMA. Error bars show the standard deviation between technical duplicates. An ANOVA model was used to compare methods for significant differences in CD11b expression across experiment repeats and between methods.

3.3 Discussion

The use of high-throughput antigen-bead based methods is becoming a popular technique to assess Fc-mediated effector functions, often across multiple cell types in a 'systems serology' approach. So far, these assays have been used to identify antibody dependent cellular-cytotoxicity as a correlate of vaccine mediated protection for HIV, and more recently investigate humoral responses following SARS-CoV-2 infection and vaccination (Arnold & Chung, 2018; Haynes et al., 2012; Selva et al., 2021). In the case of Vi-typhoid vaccination, Fc-mediated responses were also evaluated in a systems serology approach, and identified antibody-dependant neutrophil phagocytosis as correlating with protection, with the potential for a

mechanistic role in vaccine-induced immunity (Jin et al., 2021). Prior to starting my DPhil, these analyses had been performed at the Ragon Institute, Boston MA, however they were yet to be validated at the Oxford Vaccine Group. Nor had the downstream consequences of antibody-neutrophil phagocytosis been investigated. In this chapter, I have demonstrated the reproducibility of the antibody-dependent neutrophil phagocytosis assay (ADNP) across sites, as well as adapting the assay to measure the release of reactive oxygen species following vaccination.

Several methods have been published regarding the quantification of oxidative burst in neutrophils. The most widely used in clinical settings involves the addition of DHR to neutrophils following stimulation with a PMA agonist, and subsequent quantification of DHR fluorescence using flow cytometry. Regarding antibody stimulated phagocytosis, Llewellyn et al. (2015) evaluated the standardised of an ADNOB assay following *Plasmodium* infection. This method applied comparable antibody concentrations, cell preparation steps, and incubation times as the assay described here; with the primary difference in the fluorescence quantification method. In contrast to measuring fluorescence immediately after phagocytosis using flow-cytometry, their method measures luminosity over an hour incubation using a luminometer. If the sole objective is to measure ROS release, it is possible that a longer read-out time would improve assay consistency and sensitivity, and could serve as a format for future experimental investigations.

However, my goal was to develop a method that could be used for both ADNP and ADNOB measurements in clinical trial settings. By using flow cytometry, there are options to quantify multiple fluorescent markers, as well as other parameterssuch as cytokine release or receptor expression levels. I subsequently evaluated the inter, and intra-assay variation of this method. Despite individual sample assay scores varying across repeats, the overall measurements from each experiment significantly correlated, and replicated previously reported findings (Jin et al., 2020).

Nonetheless, several factors which were hypothesised to influence ADNP and ADNOB measurements were systematically assessed to determine if the inter-assay variation could be reduced. These factors included differences in the time required to perform separate experimental steps, the impact of blood donor heterogeneity, and different cell-preparation methods. Neither of these factors tested in isolation significantly impacted inter-assay variation any more than the threshold for technical variation within an experiment.

There were however, marginal differences in neutrophil CD11b expression measured between experiments. CD11b is a marker of neutrophil activation, therefore differences in the cellular activation may explain a portion of the observed variance. However, this variation could come from a number of sources, not limited to clinical sample collection, or cell-preparation steps (Blanter et al., 2022), and is therefore a current limitation of using primary cells in the assay. Future methods for developing an internationally standardised assay may rely on a cellline such as HL-60 neutrophil like cells (Salehi et al., 2018). However, due to the disruptions caused by the SARS-CoV-2 pandemic, the optimisation of a neutrophil cell-line based assay was beyond the scope of this project.

Nevertheless, given that experimental findings were replicated successfully, and that the inter-assay variation was of a similar magnitude to that obtained in other laboratories, I took forward both the ADNP and modified ADNOB assay to measure Fc-mediated neutrophil responses following typhoid vaccination. Currently, the suggested assay capacity for a single operator is running four, 96-well plates. With the use of plate-reading cytometry equipment, these techniques still ensures that large clinical data sets can be analysed in one experiment and validated. In all subsequent cases, experiments were repeated, and measured alongside the Vi-serum standard, and appropriate controls to ensure the assay was functioning as expected. Future extensions of this work could also include adapted-bacterial based assays which could be used to investigate the relationship between post-vaccination titres with additional *Salmonella* antigens such as LPS that has not been considered here.

4

Fc-mediated neutrophil effector functions following typhoid vaccination

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4.1 Introduction

The Vi-polysaccharide vaccine (Vi-PS) is widely licensed for use in travellers to typhoid endemic countries. Vi-polysaccharide antigens are processed in a T-cell independent manner. Consequently, in the absence of T-cell engagement with antigen-presenting B-cells, neither germinal centres, nor long-lasting memory plasma-cells are formed. As a result, the duration of immunity is typically between 2 and 5 years, requiring booster doses which may not be cost-effective in low and middle income countries (LMIC's) when compared with longer lasting conjugate vaccines. Furthermore, polysaccharide vaccination is generally ineffective in infants younger than 2 years old, who lack of splenic marginal zone b-cells, thought to be required to mount a sufficient T-cell independent response to polysaccharide antigens (Klein Klouwenberg & Bont, 2008; Zhao et al., 2018). Accordingly, Vi-polysaccharide vaccines are unsuitable for the long-term management of typhoid fever which has a high burden in young children residing in LMICs (Howlader et al., 2017). Alternatively, by conjugating the vaccine to a carrier protein, antigens can be processed in a T-cell-dependent manner, and typically induce a more durable immune response.

In 2018, the efficacy of a Vi-tetanus tetanus toxoid conjugate vaccine (Vi-TCV) was compared against a Vi-polysachharide vaccine (Vi-PS), and a placebo vaccine, in a human challenge study: Vaccines Against *Salmonella* Typhi (VAST) (Jin et al., 2017). The trial observed a 54.6% vaccine efficacy in the Vi-TCV vaccine arm, compared with 52% Vi-PS vaccine efficacy, indicating it would be a promising vaccine candidate for further study in endemic settings (Jin et al., 2017). Given the high challenge doses and stringent clinical observations, field efficacy was predicted to be even higher. The Vi-TCV vaccine has since demonstrated above an 80% efficacy in phase III field trials conducted across Kathmandu, Nepal; Dhaka, Bangladesh; and Blantyre, Malawi (Shakya et al., 2019), and is recommended for global use by the World Health Organisation.

Understanding of how Vi-conjugate vaccination results in a robust immune response and protection against *S.* Typhi may therefore provide immunological insights relevant for future vaccine development, as well as evaluating vaccine immunogenicity in smaller cohorts. Initial evaluations of Vi-IgG antibody titres following vaccination in the VAST trial, found that Vi-IgG titres were unable to discriminate between individuals diagnosed with typhoid fever and those who were protected in the model. Furthermore, there was no difference in Vi-PS vaccine recipient serum Vi-IgG titres, and titres measured in serum from those who received the Vi-TCV vaccine (Jin et al., 2017). Subsequently, a systems serology approach was used to evaluate a wide array of immune responses, including Fc-mediated antibody functions and receptor binding. (Jin et al., 2021; Dahora et al., 2019). These results showed that following vaccination, participants who were not diagnosed with typhoid exhibited higher levels of antibody-dependent phagocytosis (ADNP) compared with diagnosed individuals (Jin et al., 2021).

In light of these findings, and the established role for neutrophils in mediating protective immunity against *S.* Typhimurium (Cheminay et al., 2004); it was hypothesised that the recruitment of neutrophils following vaccination may contribute to vaccine-mediated protection. Following engagement of vaccine induced antibodies with neutrophil Fc-receptors, neutrophils may engulf *S.* Typhi bacteria, before killing them via the release of free radicals in the oxidative burst. The antibody-dependent neutrophil oxidative burst (ADNOB) has been demonstrated to result in bactericidal activity against *Strep. penumoniae* in opsonophagocytosis-killing assays using neutrophil like cell lines. (Pérez-Figueroa et al., 2021; Thomas et al., 2018). Therefore, detecting the presence of the oxidative burst in neutrophils following vaccination, may indicate a mechanistic role for Fc-mediated neutrophil effector functions in vaccine-mediated protection.

Furthermore, the identification of these correlates can be used to evaluate vaccine immunogenicity in endemic settings. As the VAST study was conducted on adults in the United Kingdom, it was unknown how the target paediatric cohort would respond to Vi-TCV vaccination. Therefore, I assessed ADNP and ADNOB

responses in plasma from Nepalese children aged 6 months to 16 years who were vaccinated as part of a phase III efficacy study (TyVAC). In addition, using data from both the U.K. and Nepalese cohorts, I aimed to investigate potential factors that could be influencing Fc-mediated neutrophil functions.

These factors included both antibody titre and subclasses, which are determined by class switch recombination in germinal centre B-cells. The glycosylation pathway also provides co and post-translational modifications of the antibody-Fc region, which may result in significant structural and functional antibody diversity. These modifications occur in the Golgi-apparatus as a result of the action of several glycosidase enzymes, and can produce up to 30 different glycoform structures. Through conformational changes, differences in Fc-glycoforms can affect the affinity of antibodies for Fc receptors, and consequently, the magnitude of subsequent cellular activity (Jefferis, 2009). Modifying Fc-glycans has previously been shown to attenuate Fc-antibody responses in immunotherapy, and antibody-dependent cellular cytotoxicity (ADCC), following HIV vaccination (Mahan et al., 2016). However, it is not fully understood how glycosylation might affect antibody-dependent neutrophil functions following vaccination.

4.1.1 Chapter Aims:

1. To assess the antibody-dependent oxidative burst as a potential correlate of protection following Vi-TCV and Vi-PS vaccination.
2. To evaluate the Fc-mediated immunogenicity of the Vi-TCV vaccine in a paediatric population vaccinated in Nepal.
3. To investigate factors that may attenuate Fc-mediated neutrophil functions, including antibody subclass, isotype, Fc-receptor binding, and glycosylation.

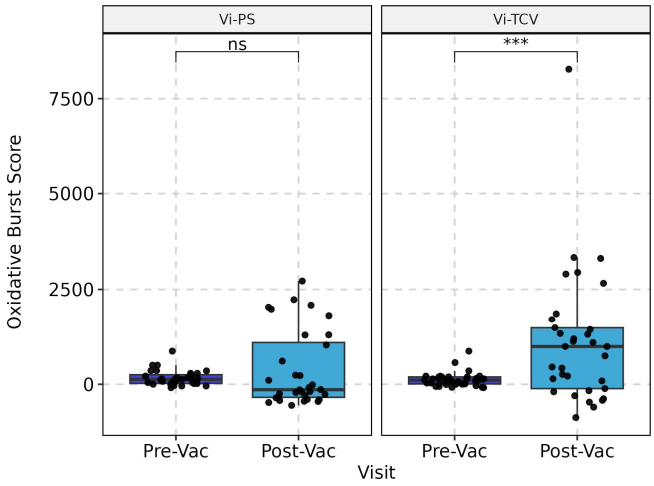


Figure 4.1: ADNOB 28-days following Vi-PS and Vi-TCV vaccination Serum samples were run at baseline and 28 days post-vaccination, the oxidative burst was measured as the MFI of DHR multiplied by the percentage of DHR neutrophils - a no serum control. Samples were run in technical duplicate, and presented are the mean oxidative burst scores for each participant. Each box plot shows the median and interquartile ranges per group, and the Wilcoxon paired test was used to determine statistical significance between time points in each vaccine arm.

4.2 Results

4.2.1 Antibody-dependent neutrophil oxidative burst following typhoid vaccination in UK adults

ADNOB significantly increases from baseline 28-days post Vi-TCV vaccination

Following the optimisation of the antibody-dependent neutrophil oxidative burst (ADNOB) assay (Chapter III); I evaluated levels of ADNOB induced by serum from individuals vaccinated against typhoid in the UK, with either the Vi-tetanus toxoid conjugate vaccine (Vi-TCV), or the plain Vi-polysaccharide vaccine (Vi-PS) vaccine (n = 35, n = 37). I observed a significant increase in ADNOB activity 28 days post- Vi-TCV vaccination from baseline (P = 0.00147), whereas the increase in ADNOB following Vi-PS vaccination was not statistically significant (Fig. 4.1 A). Moreover, sera from Vi-TCV recipients induced higher levels of oxidative burst activity in neutrophils, when compared with sera from Vi-PS recipients (P = 0.0426).

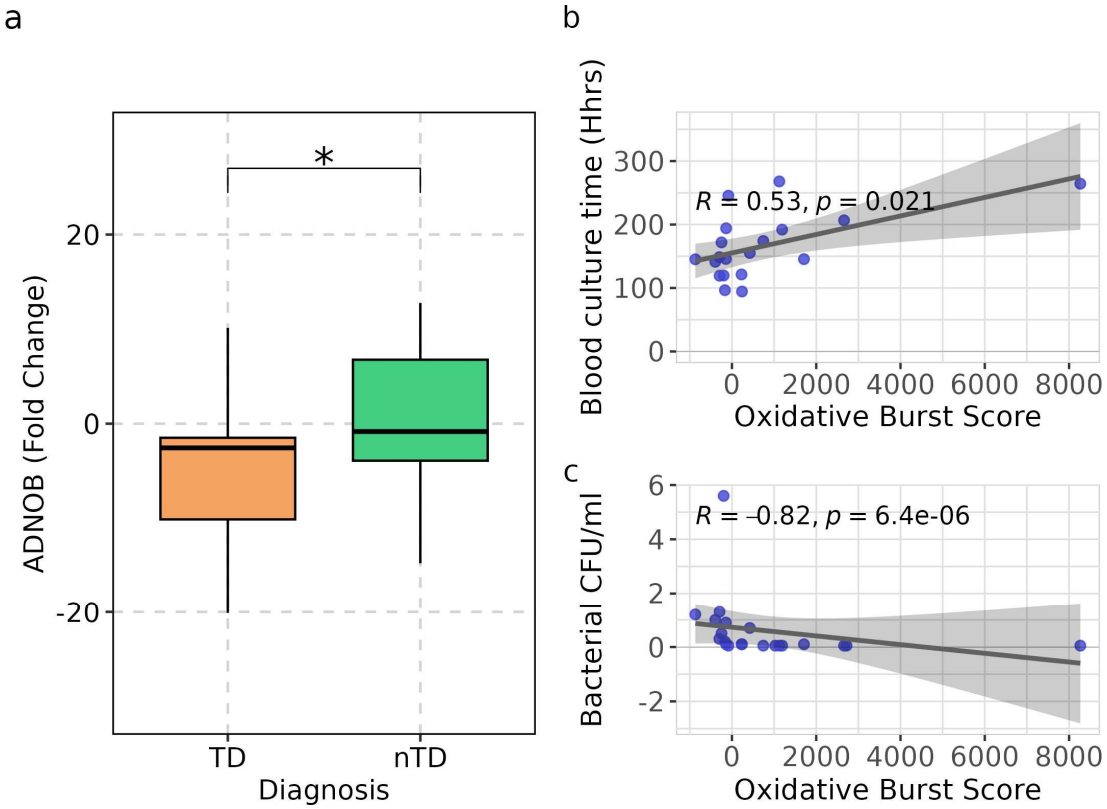


Figure 4.2: Associations of ADNOB with diagnosis of typhoid fever 28 days following vaccinaton Serum samples were run at baseline and 28 days post-vaccination,the oxidative burst was measured as the MFI of DHR multiplied by the percentage of DHR neutrophils minus a no serum control, before calcualting the fold change between timepoints. Samples were run in technical duplicate, and presented are the mean oxidative burst scores. A) Box plots indicate the median and interquartile ranges per group, and the Wilcoxon paired test was used to determine statistical significance between diagnosis outcomes. B) Correlation of oxidative burst scores measured 28 days post-vaccination with the time to detecting a positive blood cultutre. C) Correlations of oxidative burst scorespost-vaccination with the CFU per ml of S. Typhi bacteria measured in liquid culture. Spearmans rank was used to compute r coefficient and p-values for each correlation.

ADNOB associates with protection against *Salmonella* Typhi in a controlled human infection model

In order to determine whether ADNOB may contribute to protection against typhoid fever, I evaluated ADNOB scores between participants who were diagnosed with typhoid fever following infection in the human challenge model (TD), versus

those who did not develop clinically detectable infection (nTD). Encouragingly, I observed a greater fold change in ADNOB 28-days after immunisation from baseline amongst participants who were not diagnosed with typhoid fever, when compared with individuals who did receive a diagnosis (Fig 4.2A), and these findings were replicated upon a subsequent experiment repeat. Moreover, in participants diagnosed with typhoid; higher oxidative burst scores measured from sera taken 28 days after vaccination associated with a longer time to receiving a diagnosis (R = 0.53, P = 0.021), and lower titres of bacteria in blood culture (R = −0.82, $P = 6.4 \times 10^{-6}$) (Fig 4.2 B-C). These results thus further suggest a protective role for neutrophil mediated activity against *Salmonella* infection.

4.2.2 Antibody-dependent neutrophil responses following Vi-TCV vaccination in Nepal

ADNP increases from baseline 28 days after Vi-TCV vaccination in Nepalese children

As Vi-conjugate vaccines will be administered to children in typhoid endemic areas, I assessed ADNP and ADNOB in plasma samples from cohort of children vaccinated in Nepal. Serum samples were not available for this study. Therefore plasma samples which are taken before blood clotting, and so retain clotting factors, were analysed for their capacity to stimulate neutrophil phagocytosis at both baseline and 28 days post-vaccination (D28) in children aged 6 months to 15 years (n = 77). These data showed that ADNP levels increased significantly following vaccination in Nepal (P = 0.0001), with an average 3.6-fold increase inADNP activity measured from baseline (Fig 4.3).

ADNP and ADNOB responses in Nepalese children are comparable withUK adults following Vi-TCV vaccination

As antibody-dependent neutrophil functions associated with protection in the human challenge model of typhoid fever, I then compared the magnitude of ADNP and ADNOB in our target paediatric vaccination cohort with that of UK adult

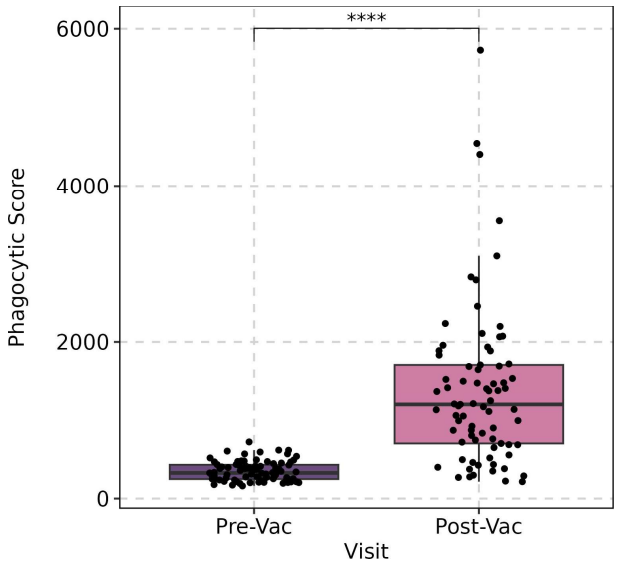


Figure 4.3: ADNP 28 days following Vi-TCV vaccination in Nepal Anti-Vi ADNP was measured for 77 participants prior to and 28 days post-vaccination. The Y axis indicates the phagocytic score, calculated from bead+ neutrophils, multiplied by the MFI of the red fluorescent beads. Samples were run in technical duplicate and presented are the mean values. Each box plot shows the median and interquartile ranges per timepoint, and the Wilcoxon paired test was used to determine statistical significance between time points.

volunteers. Participants included a randomly selected subset of children from the previously measured ADNP-immunogenicity cohort (n = 17). UK participants included those vaccinated with Vi-TCV who were not diagnosed with typhoid fever in order to better reflect 'protective levels' of ADNP activity (n = 16). When measuring ADNP induced by plasma from both cohorts, ADNP levels increased significantly 28 days after Vi-TCV vaccination ($P < 0.0001$, $P < 0.001$) (Fig 4.4A). In addition, the fold change in ADNP between baseline and D28 did not differ significantly between cohorts, despite moderately higher baseline ADNP levels measured in plasma from UK adults compared with Nepalese children (P =0.02). Furthermore, when measuring ADNOB across studies, I also observed a significant increase in oxidative burst scores after vaccination, and the magnitude of this increase was comparable to that observed in our UK participants (Fig 4.4B).

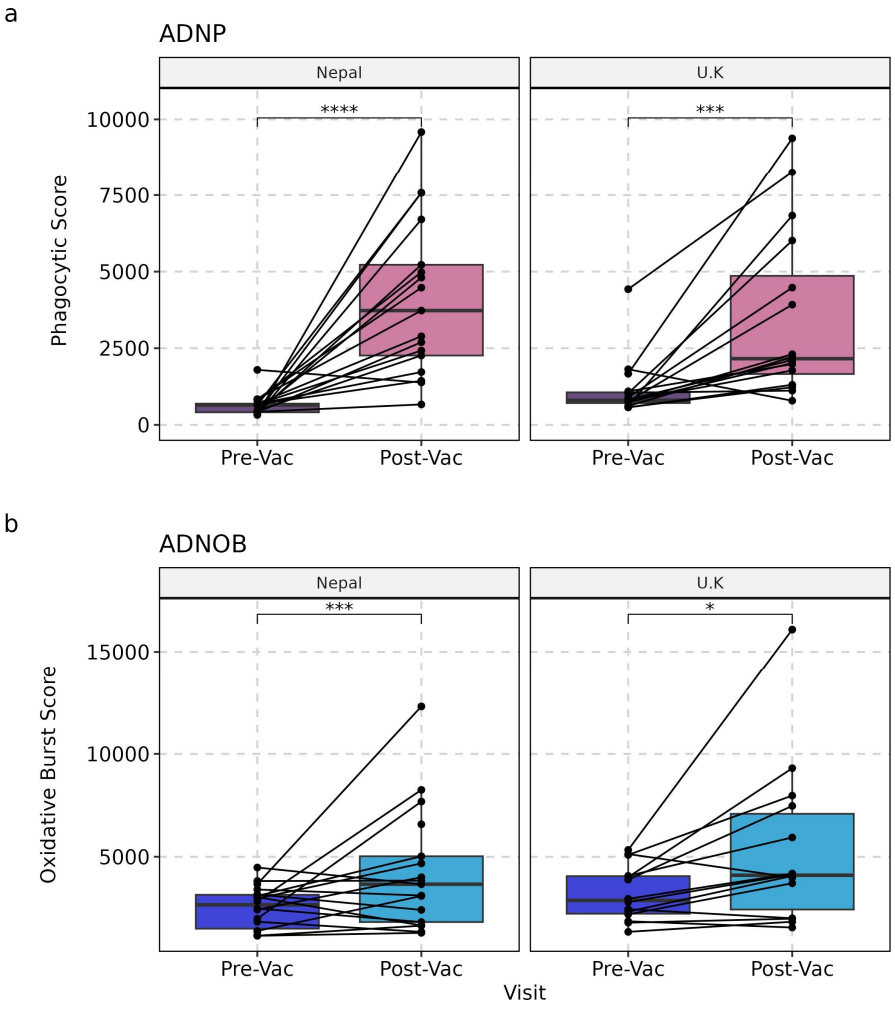


Figure 4.4: ADNP and ADNOB compared between Vi-TCV vaccinated adults and children in Nepal Anti-Vi ADNP was measured pre, and 28 days post Vi-TCV vaccination in participants from the TyVAC Nepal study (n = 17) and adults vaccinated in the UK (n = 16). The phagocytic scores were calculated from bead+ neutrophils, multiplied by the bead MFI. Samples were run on the same day and in technical duplicate. Presented are the mean phagocytic score per participant, pre and 28-days post vaccination. Box plots shows the median and interquartile ranges for each time point, and the Wilcoxon paired test was used to determine statistical significance between time points.

4.2.3 Factors influencing antibody-dependent mediated neutrophil effector functions in Nepalese children

Examining the relationship between ADNP, age and titre in Vi-TCV vaccinated children

Upon starting my investigation, it was not known if there would be differences in antibody-mediated neutrophil phagocytosis across children as they aged, due to possible differences in immune compartments and *S.* Typhi exposure. Therefore, to better understand ADNP responses in Nepalese children, I evaluated ADNP scores using plasma from immunised children aged between 0-4, 5-9 and 10-15 years. Interestingly, at baseline, prior to vaccination, ADNP activity increased along with the age of the children vaccinated (Fig 4.5 A). The oldest children, 10-15 years of age, had significantly higher levels of ADNP when compared with children aged 5-9 and 0-4 years of age ($P < 0.05$, $P < 0.0001$). We were unable to investigate the possible impact of maternal antibody levels due to the small sample size of children aged less than 1 year old.

Larger titres of antibodies with strong Fc-receptor affinity have been previously shown to correlate with Fc-mediated effector functions. Therefore, given that older children have higher *S.* Typhi exposure, which may also result in higher Vi-antibody titres, I hypothesised that this may be driving the higher ADNP levels measured in this group at baseline. Indeed, at baseline Vi-IgG antibody titres were highest in older children (10-15 years) compared with younger children (5-9 years) (Fig A.9). Although, when measuring Vi-IgA titres, there was no significant difference between age groups in our subset of children (Fig A.9). Despite differences in baseline Vi-IgG titres, the correlation between pre-vaccination Vi-IgG titres and ADNP responses was minimal ($R = 0.24$, $P = 0.044$) (Fig 4.6 A).

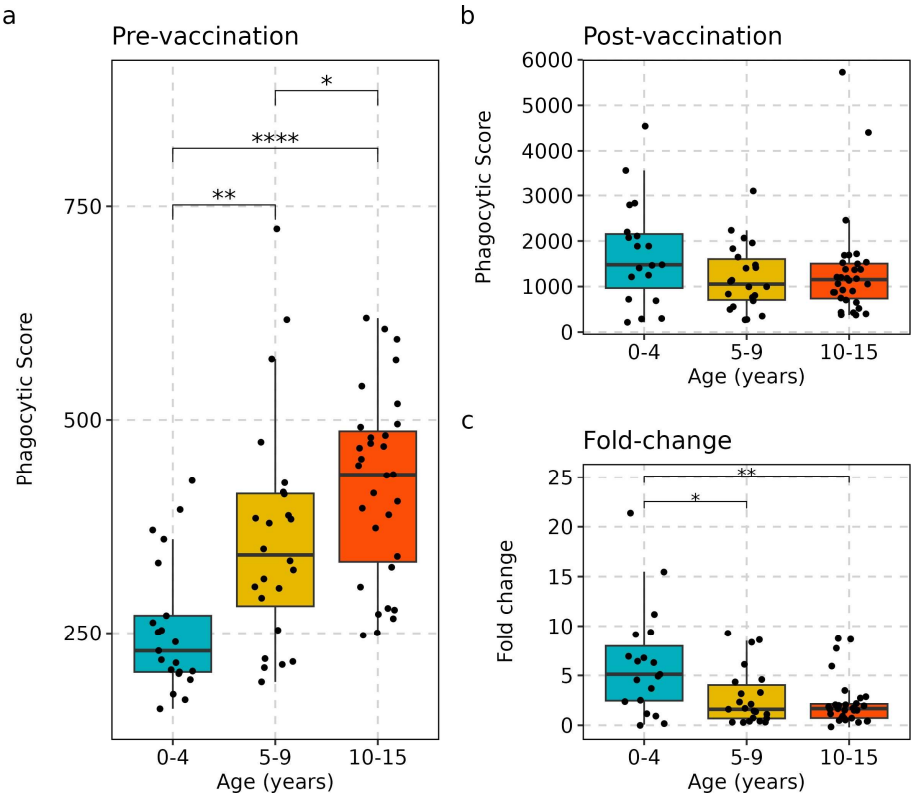


Figure 4.5: Differences in ADNP by age of vaccinated children in Nepal A-B) Participants were separated into groups aged 0-4 (n = 21), 5-9 (n = 24) and 10-15 years of age (n = 32). The MFI of the red fluorescent beads was then multiplied by the number of bead+ neutrophils at the pre- and post-immunization time points, respectively, to determine the phagocytic scores. The Wilcoxon test was used to compare differences in anti-Vi ADNP responses between age groups, and box plots show the median and interquartile range for each age group. Samples were run in technical duplicate and displayed are the mean phagocytic scores. The mean phagocytic scores, which were computed using technical duplicates of the samples. C) Fold change was calculated using the mean phagocytic scores pre and 28-days post Vi-TCV vaccination.

Twenty-eight days following Vi-TCV vaccination, there was no significant difference in ADNP measured between age groups (Fig 4.5 B). Subsequently, due to the lower baseline ADNP levels in the younger children (0-4 years), they exhibited the greatest fold changein ADNP of any age group (Fig 4.5 C). Furthermore, there was an absence of a correlation between ADNP and titres of Vi-IgG and Vi-IgA (R = 0.018, P = 0.89), (R = 0.17, P = 0.16), 28 days following vaccination (Fig 4.6 C-D).

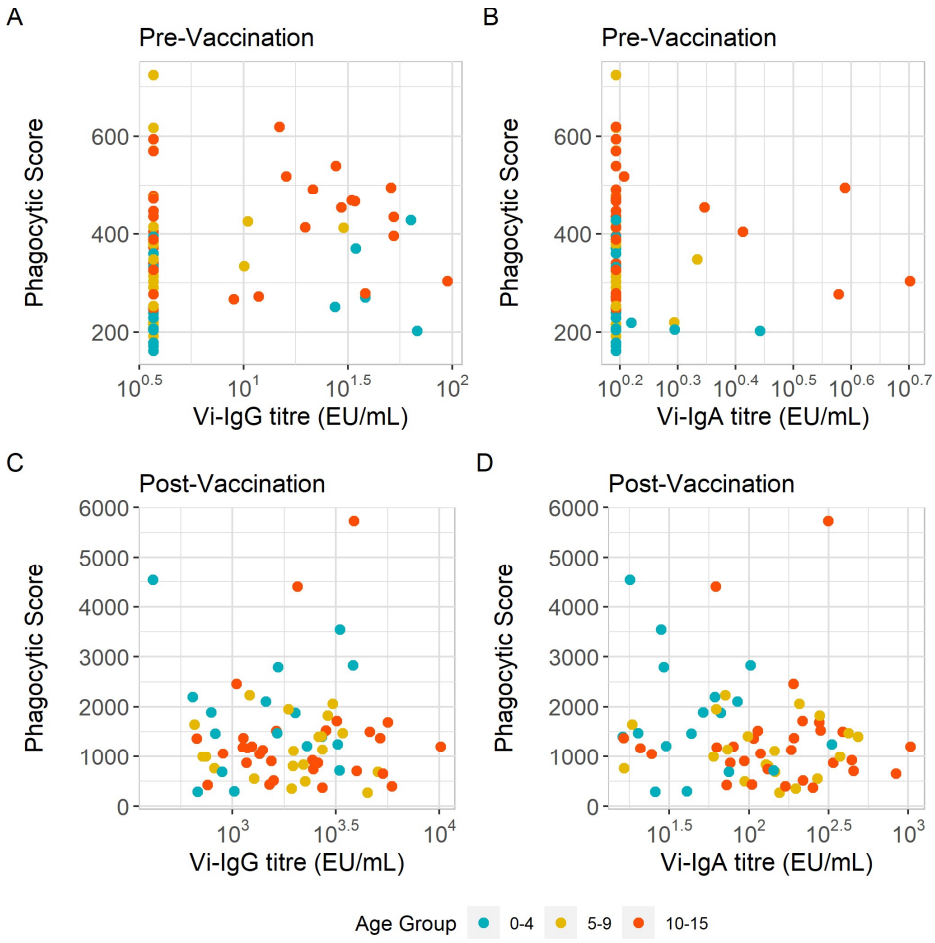


Figure 4.6: Correlations of ADNP with Vi-antibody titres (A, B) Scatterplot displaying anti-Vi phagocytic scores with Vi-IgG, and Vi-IgA titres at baseline (pre-vaccination), and 28 days post-vaccination. (C, D) Scatterplot displaying anti-Vi phagocytic scores and Vi-IgG and Vi-IgA and titres 28 days post-vaccination, respectively. The phagocytic scores were calculated from the percentage of bead-positive neutrophils, multiplied by the bead MFI. Samples were run in technical duplicates and presented are the mean phagocytic scores and ELISA units (EU per ml) per participant (n = 77).

In light of these findings, the observed differences in the magnitude of ADNP measured at baseline and after vaccination in Nepalese children cannot be attributed solely to the amountof Vi-antibodies present, and may instead be driven more by other factors not yet investigated, such as Vi-antibody isotypes, Fc-glycosylation or Fc-receptor binding.

4.2.4 Correlation of Fc-glycosylation profiles with ADNP following typhoid vaccination in Nepal

Given the lack of a direct relationship between antibody titre and ADNP, it was posited that post-translational modifications of the Fc-glycome may be attenuating antibody-mediated neutrophil responses. Changes to Fc-glycosylation patterns allows the immune system to fine-tune antibody effector functions through the addition or removal of glycans, including sialic acid, galactose, fucose, or a bisecting GlcNAc residue. I therefore evaluated the abundance of these Fc-glycans on Vi-specific IgG, 28 days following Vi-TCV vaccination of children aged 6 months-15 years in Nepal (n=35), and potential relationships with Fc-mediated neutrophil phagocytosis. Liquid-chromatography and mass-spectrometry (LC-MS) were used to characterise Fc-glycosylation profiles of Vi-specific IgG subclasses (Chandler et al., 2019). Due to a high similarity in the amino acid sequence around the Fc-glycosylation site in both IgG2 and IgG3, these subclasses could not be distinguished from each other using mass-spectrometry, and data were therefore combined.

When evaluating the relationship between Vi-specific IgG1 and Vi-IgG2/3 glycosylation 28-days following vaccination (D28), and the fold change in ADNP from baseline, I observed that lower sialylation on Vi-IgG1, associated with a greater ADNP fold increase following vaccination (R = 0.47, P = 0.021) (Fig 4.7 C). Similarly, lower Vi-IgG1 galactosylation correlated with ADNP fold change 28 days post-vaccination, however this trend was not statistically significant (R = 0.38, P = 0.067) (Fig. 4.7 D). Additionally, when breaking down Vi-IgG galactosylation quantities into the percentage of agalactosylated (G0), monogalactosylated (G1), and digalactosylated (G2) states, I observed that a higher percentage of Vi-IgG1 (G1) correlated with ADNP levels at D28 (R = 0.48, P = 0.028), whereas the percentage of digalactosylated Vi-IgG1 inversely correlated with ADNP (R = −0.047, P = 0.021) (Fig 4.8).

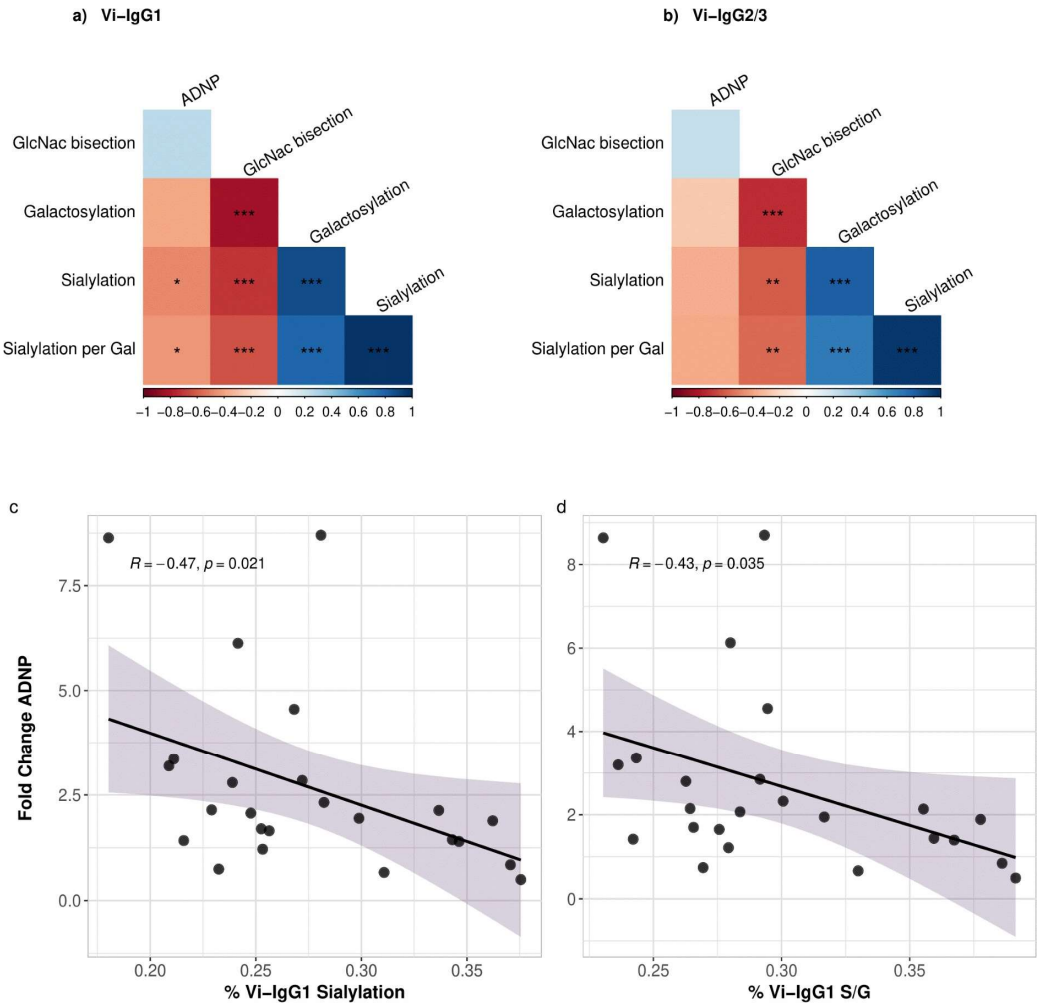


Figure 4.7: Correlations of ADNP fold change with Vi-IgG Fc-glycosylation profiles Spearman's correlation of the percentage of Vi-IgG1 (A), and Vi-IgG23 (B) sialylation, galactosylation, and GlcNAc bisection at day 28, with the fold change in ADNP between baseline and D28, as well as the participant age (n = 24). The colour of the square indicates the strength of the correlation (Rho) and the significance is noted within each box (* $P < 0.05$, ** $P < 0.01$, ***$P < 0.001$). Correlation of the percentage of Vi-IgG1 sialylation (C), and sialic acid per galactose residues (SG) (D) at day 28, respectively, with the fold change in ADNP between baseline and D28 (n =24). Shown for each graph are the Spearman's Rho and unadjusted P-values for significance. The fitted linear regression model is also displayed, and the shaded area indicates the 95% confidence intervals of the model.

When correlating Fc-glycosylation of Vi-IgG2 and3 at D28 with ADNP, I observed similar trends to those described with Vi-IgG1; in which lower percentages of sialylation and galactosylation correlated with a rise in ADNP from baseline, though these were not statistically significant (Fig 4.7 B). However, when examining the relationships between Vi-IgG2/3 galactosylation states and ADNP, monogalactosylation levels did associate with ADNP fold change (R = 0.43, P = 0.038) (Fig A.10 E).

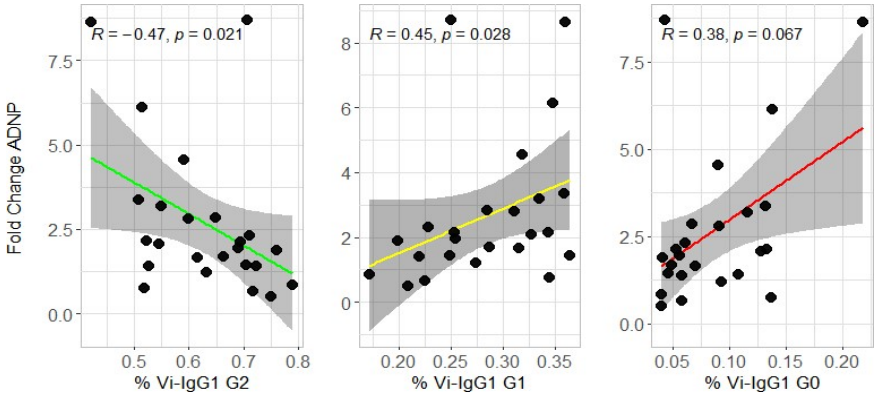


Figure 4.8: Fc galactosylation states of Vi-IgG1 with ADNP fold change 28 days following vaccination Correlation of the percentage of agalactosylated (G0), monogalactosylated (G1), and digalactosylated (G2) Vi-IgG1 antibodies at D28 with Vi-ADNP phagocytic score between baseline and D28, respectively (n 24).The black lineshows the linear regression, and the shaded area shows 95% confidence intervals for the model.

As sialylation immediately follows galactosylation in the glycosylation process, and the two have been demonstrated to correlate in earlier studies (Van de Geijn et al., 2009), I examined the relationship between sialylation and galactosylation of Vi-specific antibodies. I observed a strong correlation (P < 0.0001) between the percentages of Fc-sialylation and galactosylation of Vi-IgG1 with each other (Fig. 4.7 A), indicating that the two processes are linked. Consequently, to determine if sialylation had a specific effect on the magnitude of ADNP following Vi-TCV vaccination, I correlated the amount of sialic acid residues per galactose residue of the IgG Fc-glcyome with the fold-change in ADNP from baseline.

A significant correlation (R = 0.43, P = 0.035) was detected between lower percentages of Vi- IgG1 sialylation per galactose, and ADNP measured 28-days following vaccination (Fig 4.7 D). These findings therefore suggest that sialylation of the Fc region of Vi-IgG1 could specifically alter antibody-dependent neutrophil phagocytosis in a specific manner.

Furthermore, as it is known that sialylation and galactosylation decrease as children age (Gudelj et al., 2018), I investigated whether age might affect the associations we observe with ADNP. Evalauting the interaction-terms in a linear regression model, I found no significant associations between age and Vi-IgG1 glycosylation, when predicting baseline ADNP levels. However, the relationship between the age of the child, Vi-IgG1 galactosylation, and ADNP was close to statistical significance (R = 0.38, P = 0.06).

4.2.5 Associations of antibody titres and Fc-gamma receptor binding in Vi-vaccinated UK adults with ADNP and ADNOB

For adults vaccinated against *S.* Typhi in the UK, multiple measures including Vi-antibody subclass and isotype specific titres were available. These were measured at both baseline, pre-vaccination, and 28 days following vaccination with either Vi-TCV (n=35), or Vi-PS (n=37). In contrast to the lack of correlations observed in children vaccinated with Vi-TCV in Nepal; ADNP significantly correlated with Vi-IgA (R = 0.58, P = 0.00024), and Vi-IgG titres following Vi-TCV vaccination (R = 0.41, P = 0.012). When investigating associations with Vi-PS vaccination, the strongest correlation was observed between Vi-IgG1 subclass titres and ADNP (Fig 4.9). Similar associations between ADNOB responses and Vi-IgA and IgG1 titres following vaccination were also observed, but were only statistically significant when grouping data from both Vi-TCV and Vi-PS vaccinees (Fig A.11).

Interestingly, both Vi-IgA and Vi-IgG1 titres also correlated with protection in the human challenge model, suggesting a protective profile of effective antibody subclasses which may promote Fc-mediated neutrophil functions. In addition, a luminex-based assay was used to determine the Fc–receptor (FcR) binding activity of Vi-immunoglobulin to FcαR, FcγR2α, FcγR2β, FcγR3α, and FcγR3β, 28 days following Vi-vaccination. These analyses found that FcγRand FcαRbinding significantly correlated with ADNP after vaccination, and the strongest association with ADNP was seen with Vi-FcαR binding (R = 0.58, P = 0.00024), (Fig 4.9).

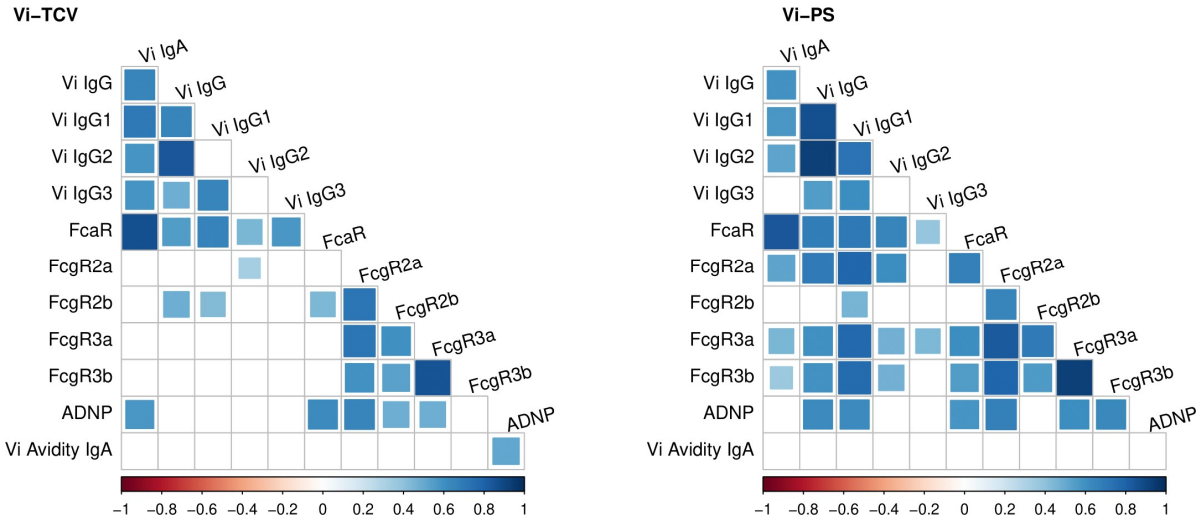


Figure 4.9: Correlation of ADNP with Vi-IgG and Vi-IgA titres, and Fc-receptor binding 28 days following Vi-TCV and Vi-PS vaccination Pairwise Spearmans rho was used to determine the correlation between antibody measures following vaccination (Vi-TCV, n = 35; Vi-PS, n=37). The size of the square is relative to the significance of the correlation. Only significant correlations after adjusted for multiple testing (Bonferroni) are displayed.

4.3 Discussion

Fc-mediated correlates of protection, such as the serum bactericidal assay, have long been used to assess the efficacy of meningitis B vaccines (Balmer et al., 2020; Barrett et al., 2020). For diseases such as HIV, in which developing a robust vaccine has been challenging, Fc-mediated functions have highlighted protective antibody characteristics, helping to focus vaccine design (Arnold & Chung, 2018). In this chapter, I described antibody-dependent neutrophil oxidative burst (ADNOB) as aprotective correlate following Vi-TCV vaccination in the human challenge model, supporting previously identified associations of protection with ADNP. Moreover, I observed comparable magnitude of Vi-ADNP and ADNOB measured in the plasma of our target paediatric cohort in Nepal with that of UK adult vaccinees. In conjunction with vaccine efficacy data from Nepal, Malawi, and Bangladesh, these findings therefore support the expansion of typhoid conjugate vaccines in areas where they are needed (Shakya et al., 2021).

Interestingly, in UK vaccinees, the magnitude of both ADNOB and ADNP was higher in those vaccinated with Vi-TCV, despite similar Vi-IgG and Vi-IgA titres. However, subclass-specific antibody titres and Fc binding differed between vaccine groups (Jin et al., 2021; Dahora et al., 2019). It is possible, therefore, that different antibody subclasses may be more effective at inducing Fc-mediated effector functions, contributing to protection. Vi-conjugate vaccines typically inducestronger immune responses compared with polysachharide vaccines, which reducesthe need for booster campaigns, an important consideration when deploying vaccines in low and middle-income countries. These results, therefore, also suggest that Vi-conjugate vaccination may induce a more robust immunoglobulin Fc-effector profile, which could help contribute to protective responses.

4. ADNP and ADNOB following typhoid vaccination

In order to determine which antibody isotypes may be driving ADNP and ADNOB, I correlated Vi-IgA and Vi-IgG titres with neutrophil effector functions. The correlation between Vi-IgA and ADNP was of particular interest because Vi-IgA titre and avidity following vaccination were also correlated with protection in the human challenge model (Dahora et al., 2021). Neutrophils express the highest levels of the IgA Fc-α receptor compared with other phagocytic cell types (Cheeseman et al., 2016; Morton & Brandtzaeg, 2001), and IgA is the predominant isotype responsible for monoclonal-induced phagocytosis of tumour cells by neutrophils (Heemskerk & Egmond, 2018). In the context of infection, high levels of IgA and ADNP have both been associated with increased inflammation during SARS- CoV-2 infections (Zohar et al., 2020); highlighting the delicate balance between inflammation beneficial to protection, versus inflammation that can cause disease. It is therefore plausible that Vi-IgA titres generated following Vi-vaccination result in higher levels of ADNP, working in tandem to contribute towards protection.

Following Vi-TCV vaccination in the UK, I observed that antibody dependent neutrophil effector functions also correlated with higher titres of Vi-IgA, IgA avidity to Vi-antigen, and Fc-α receptor binding. Implying that efficient binding to both Vi-antigen and the respective Fc-α receptor contribute to antibody mediated neutrophil activity, which may help prevent infection following vaccination. However this relationship was not statistically significant in the Vi-PS cohort. Interestingly, Vi-conjugate vaccination was shown to induce a greater proportion of gut-homing plasma cells than Vi-PS vaccination; therefore, it is possible that the localization of these antibodies to the site of infection also contributes to host protection (Cross et al., 2020; Dahora et al., 2021). For both Vi-PS vaccination and Vi-TCV vaccination, Vi-IgG1 correlated with ADNP and ADNOB magnitude. IgG1 antibodies are the most potent FcγR-binding isotype and bind to FcγR2α (CD32a), which is also highly expressed on granulocytes (Steffen et al., 2020).

4. ADNP and ADNOB following typhoid vaccination

In conjunction with previous associations between Vi-IgG1 avidity and protection in the challenge model; these findings suggest that following vaccination, immunoglobulin isotype switching and affinity maturation result in potent antibody subclasses that help mediate protection against *S.* Typhi infection following challenge. As these assays were conducted with bulk sera, future research evaluating ADNP in the presence of purified antibodysubclasses could confirm these associations.

Interestingly, while Vi-IgA titres and FcαR binding correlated with ADNP following typhoid vaccination in the United Kingdom, I did not observe similar associations in Nepalese children. Despite the fact that higher post-vaccination Vi-IgA titres result in higher levels of ADNP, there seemed to be no strong relationship between the two. However, there are distinct differences in these cohorts, regarding age, ethnicity and exposure which can alter the humoral response, and thus may influence ADNP following vaccination to different degrees. Furthermore we did not have measurements for isotype- or subclass-specific antibody titres, FcR or Vi-avidity for this data set which may have provided better granularity for such an analysis. We can only hypothesise that differences in antibody-functional activities may be attributable to these unexplored characteristics, or to post-translational modifications of the Fc-region that occurs during glycosylation (Johnson et al., 2021).

In addition, I observed unique Fc-glycan features of Vi-specific IgG1 in Nepalese children, which correlated with an increase in ADNP post-vaccination. These included a lower abundance of sialic acid and galactose residues on the Fc domain of Vi-IgG1. Sialylation of IgG Fc has been linked to decreased FcγR3α binding and ADCC activity of IgG (Roberts et al., 2020), and is also responsible for the anti-inflammatory benefits of intravenous immunoglobulin treatments (Ahmed et al., 2014). In addition to IgG sialylation, agalactosylation has been linked to an increase in inflammation in active tuberculosis (L. L. Lu et al., 2020). Removal of sialic acid and galactose on IgG Fc has been shown to induce a conformational change in the IgG Fc hinge region, resulting in antibody structures that are more

accessible to Fc receptors, and thus stronger binding of IgG to Fc receptors (Krapp et al., 2003). Through greater cross-linking of Fc receptors, increased binding of immunoglobulin to Fc receptors has been shown to impact responses downstream from ADNP, including the oxidative burst (Gunn & Alter, 2016; Strohmeier et al., 1995). I also observed a correlation between ADNP and ADNOB and stronger receptor binding in UK vaccinees, suggesting a role for this pathway.

The glycosylation states of Vi-IgG2 and 3 displayed a similar relationship to that of Vi-IgG1 at 28 days after vaccination, although we did not observe any statistically significant associations. Since IgG1 has a higher affinity for Fc receptors than Vi-IgG2 and 3, it's possible that it is a more significant driver of ADNP. I did not examine associations between Fc-glycosylation and ADNOB in this study, however given that ADNP and ADNOB magnitude correlated with each other following vaccination, and are linked processes, it is likely that similar trends would be observed. Future analyses may focus on confirming this relationship.

Of note, these results described are in contrast to the recently reported associations measured in Vi-vaccinated adults, which observed that ADNP correlated with increased Vi-IgG sialic acid levels (Stockdale et al., 2022). This may be attributable to technical differences in vaccinations, or differences in participant age, ethnicity, and typhoid exposure between cohorts (Fig A.12) (Alter et al., 2018). In particular, IgG glycosylation levels have been demonstrated to vary with age, and indeed Vi-specific sialyation and galactosylation was significantly different between the two cohorts at baseline. Therefore it could be that the amount of sialic acid or galactosylation that occurs after vaccination is responsible for driving these associations with ADNP. However due to low sample abundance we were limited in that isotype specific baseline glycosylation data was not available for these samples, due to low abundance. Nevertheless, several improvements in glycosylation capture are under-way, with the ability to better quantify these features in future studies (Luo et al., 2022).

4. ADNP and ADNOB following typhoid vaccination

Fc-mediated antibody functions are playing an increasingly important role in assessing vaccination protection against a variety of diseases, including typhoid fever, HIV, and SARS-CoV2 (Arnold & Chung, 2018; Vicente et al., 2022). Here, I demonstrate how the ADNP assay can be used to assess the immunogenicity of a vaccine in a smaller target population. In the future, these assays could be utilised to assist in the evaluation of typhoid and paratyphoid conjugate vaccine candidates in clinical development (Khan et al., 2017; Steele et al., 2020). Furthermore, I also evaluated factors that could influence Fc-mediated neutrophil functions, highlighting the impact of antigen-specific antibody Fc-glycosylation. Recent research has demonstrated that the type of adjuvant and immunisation site can alter the glycosylation profiles of antibodies and consequently function (Bartsch et al., 2020; Norman et al., 2020). Understanding the factors driving Fc-mediated immune responses could therefore also provide future avenues for targeted vaccine design and implementation.

5

Genome wide association study ofsusceptibility to typhoid fever and vaccination

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5.1 Introduction

The *S.* Typhi human challenge model has shown that, despite comparable baseline characteristics, there is a gap in explaining why one individual can become infected and exhibit little or no symptoms of typhoid fever, while another endures a high temperature and severe abdominal pain. Likewise, within the model, we observe that despite receiving the same vaccination, some participants generate protective immune responses, whereas others do not. Genome-wide association studies (GWAS) aim to detect genetic factors, which can help explain one's risk of disease. Leveraging these association studies alongside genome-wide functional mapping can provide further detail of the human genetic landscape and its influence on infection.

Previous GWAS studies of enteric fever have implicated the highly polymorphic the HLA locus, which encodes the antigen presenting molecules MHC class I and II respectively, with the risk of infection. The strongest protective association was observed in the presence of a HLA-DRB1*04:05 allele, detected amongst both Vietnamese and Nepalese cohorts (Dunstan et al., 2014). In 2021, our group performed a preliminary genome-wide association study of enteric fever amongst participants infected with *S.* Typhi and *S.* Paratyphi in the human challenge model. The study revealed an association between the class I HLA gene HLA*B27 and susceptibility to enteric fever (A. Barton et al., 2022). Although this did not reach genome-wide significance, expression of the HLA*B27 allele significantly increased the colony forming units (CFU) of *S.* Typhi in macrophages, and 9/10 participants with the allele succumbed to infection. Together, these findings imply that MHC alleles with improved antigen recognition are protective against enteric fever and may be responsible for the range of clinical manifestations seen during acute typhoid or paratyphoid fever episodes.

However, approximately 90% of disease-associated genetic variants are typically located in non-coding areas of the genome. Instead of direct deleterious effects to protein structure, their effects are believed to be mediated through epigenetic

regulation, resulting in alterations to promoter, enhancer, or chromatin markers which typically reside up, or downstream of coding regions (Edwards et al., 2013). Expression quantitative trait loci (eQTL) analyses can quantify the magnitude of the effect size of genomic variations on gene transcription. Yet, analyses of the gTEX database of tissue-specific gene expression estimated that just 20% of GWAS variants co-localize with eQTL (Aguet et al., 2017), leaving the majority of variants uncharacterised. Recent research has demonstrated that eQTL effects are most strongly correlated with cell-type and context-specific gene expression (Cano-Gamez & Trynka, 2020), and a similar strategy was used to identify several inflammatory pathways in sepsis that are associated with genetic variation (Vosa et al., 2021).A key advantage of the human challengemodel is the ability to connect biological phenotypes with the progression of disease and outcomes. In this manner, transcriptomic analyses detected significant overex- pression of interferon, apoptosis, and neutrophil-related genes among participants who developed typhoid fever when compared with those who were not diagnosed in the human challenge model, implicating their role in infection (Blohmke et al., 2016).

Since the 2021 GWAS of enteric fever, myself and colleagues genotyped an additional 130 participants from both enteric fever challenge and vaccination studies (Table A.1), increasing the statistical power of the analysis to associate SNPs with susceptibility to enteric fever. Integrating these data sets with measurements of gene-expression has since resulted in a richly phenotyped cohort that can help characterise genetic factors implicated in immunityagainst *Salmonella* Typhi.

In addition to responses to infectious agents, vaccine responsiveness has also been shown to be in part heritable, as demonstrated by a Danish twin study, which found that serum IgG levels following pneumococcal vaccination correlated with genotype (Konradsen et al., 1993). More recently, genome-wide association studies have revealed associations between genetic polymorphisms and vaccine efficacy, whereby HLA genotypes correlated with differences in Hepatitis B-vaccine efficacy

and antibody responses (T. W. Wu et al., 2015). Furthermore, several SNPs were associated with the persistence of antigen-specific serum-bactericidal antibody(SBA) titres following meningitis C vaccination (O'Connor et al., 2019). These variants were located within putative promoter and enhancer regions and expressedin tissues including the thymus, spleen, and red blood cells. However, to date, the role of genetics in typhoid vaccine responsiveness has not been studied.

Chapter Aims:

- To conduct a genome-wide association study (GWAS) investigating the likelihood of typhoid diagnosis within the *S.* Typhi human challenge model.
- To perform a GWAS of vaccine responses following Vi-TCV and Vi-PS vaccination, including Vi-IgA and Vi-IgG titres.
- To functionally map and characterise candidate genetic variants associated with typhoid diagnosis and Vi-based vaccine responses.
- Perform eQTL analyses of relevant SNPs and gene expression following typhoid challenge and vaccination.
- Elucidate pathways relevant in both host and vaccine mediated protection by integrating GWAS, eQTL and genome-annotation data.

5.2 Results

5.2.1 Data quality control and imputation

Genotyping data were consolidated from the typhoid dose escalation study (T1), the typhoid-toxin study (TYGER), Vi-based and live oral vaccination studies (VAST, T2), and the typhoid/paratyphoid re-challenge study (PATCH) (Methods 2.2.1), and samples measured on different genotyping platforms underwent initial quality control (QC) procedures separately. Variants were excluded based on

SNP-missingness (>10%) and significant deviations from the Hardy–Weinberg Equilibrium (1×10^{-7}). Individual samples were also excluded based on missing more than 10% of variants, deviations of imputed sex, and high estimates of genetic relatedness (Methods 2.2.4). One participant was excluded for poor genotyping quality, and an additional 7 participants were excluded based on reported sex-mismatches, 4 of which had been previously reported. Lastly 3 participants were removed due to cryptic relatedness ($\hat{\pi} < 0.185$). In total, 248 participants were included in the final typhoid case-control analysis (Table A.2).

To obtain a wider coverage of the genome, phasing and imputation were performed using the TOPMed imputation server and TOPMed-r2 GRCh38 reference panel (Taliun et al., 2021). More than ten million SNPs were imputed, which were then filtered for high imputation quality scores ($R^2 > 0.8$). These variants were then merged across studies and quality controlled again (Methods 2.2.4). Based on the study sample size and estimated statistical power, variants were filtered for a minor allele frequency of 10% (0.1), leaving 4,577,376 variants in the analysis.

Evaluation of study population ancestry

Samples were evaluated for population ancestry by merging our data set with the richly phenotyped 1000 Genomes (1KG) data (Auton et al. 2015), and performing a principal components analysis (PCA) (Fig 5.1). Across all studies, participants predominantly clustered within the 1KG European super-population. Based on a visual inspection of principal components and the proportion of variance threshold, principal components 1-5 were included in the logistic regression model. The subsequent genomic inflation factor for our regression model λ was 1.0, indicating that our data were not biased by population stratification (Hellwege et al., 2017).

As the human challenge studies included participants infected with a toxin negative mutant *S.* Typhi strain, and those vaccinated with either a parenteral Vi-vaccine or a live-oral vaccine (Table A.2), these factors were included as covariates in our model, in addition to participant age, and sex. Covariates were assessed

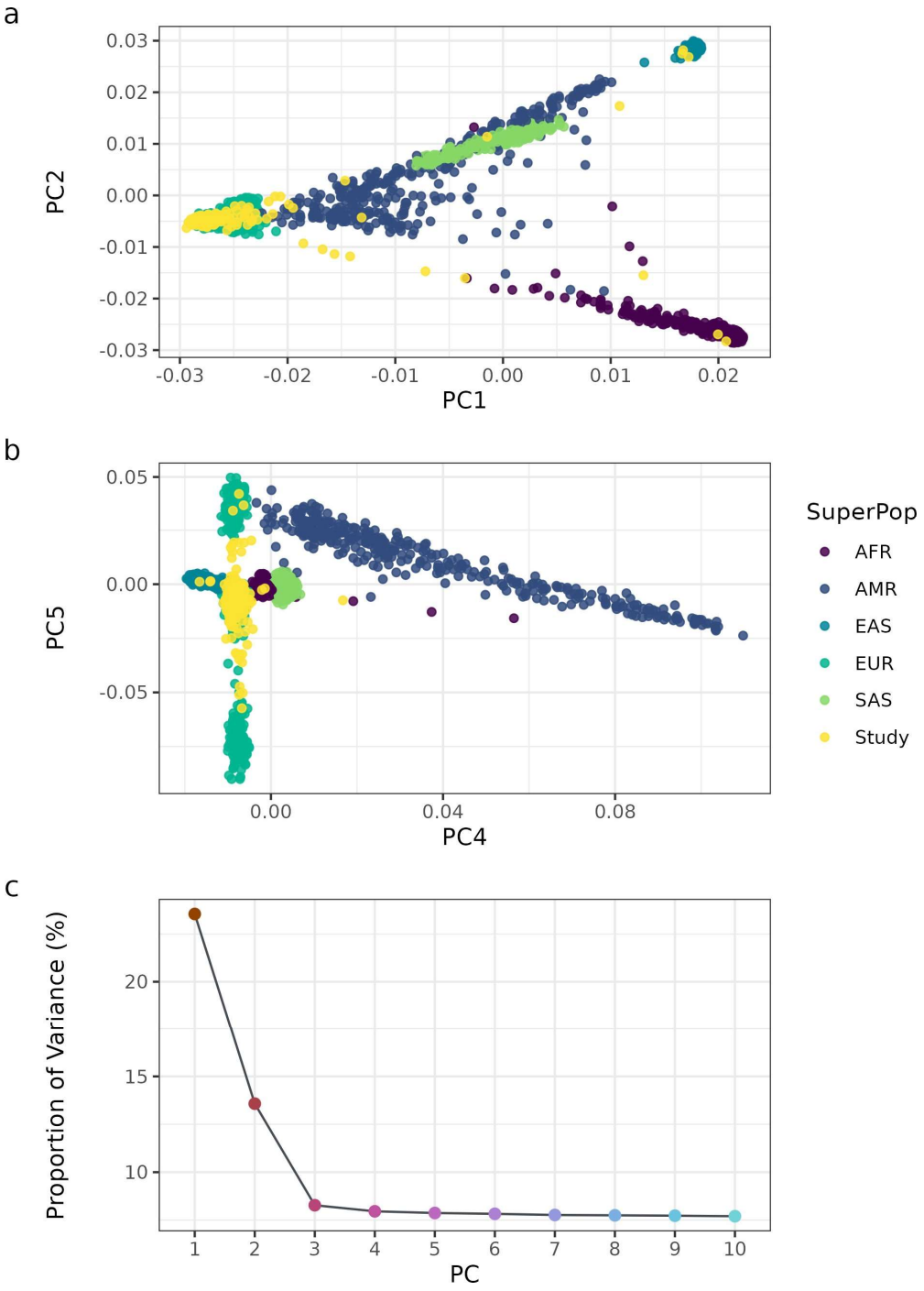


Figure 5.1: Population structure of typhoid fever GWAS subjects GWAS participant data was merged with 1000 Genomes genotyping data and principal components analysis was performed. A-B) show principal components 1-5 within the context of the 1KG super-population data. Individuals from the typhoid GWAS are highlighted in yellow, and 1KG participants are shaded by their recorded super-population phenotype. C) Shows the proportion of variance explained as per each principal component in the final logistic regression model.

through a series of regression model interaction tests, and co-linearity which found none to have a variable inflation factor higher than the threshold of 2.5. I also assessed the inclusion of *S.* Paratyphi challenged participants in study. Many GWAS field studies include *S.* Paratyphi infected participants, however the inclusion of these participants did not improve the significance of the GWAS study, nor did I find sufficient overlap between candidate SNPs across studies (Fig A.13). Given that the two serovars have differences in antigen presentation, invasion proteins and metabolic factors (Howlader et al., 2017), and our sample size for *S.* Paratyphi participants was relatively small (n=77), I excluded *S.* Paratyphi infected participants from the final GWAS model.

5.2.2 Genome-wide association study of typhoid fever following *S.* Typhi infection in a human challenge model

To investigate genetic factors involved in susceptibility to typhoid fever following challenge, I performed a genome-wide association study using a logistic-firth controlled regression (n = 248, cases = 140, controls = 108). Firth regression is a penalised likelihood regression technique that yields unbiased effect estimates and is thus suited for unbalanced or small case-control studies with unequal variance (Mbatchou et al., 2021). Participants were diagnosed on the basis ofa positive *S.* Typhi blood or stool culture, or a persistent fever > $38^{\circ}C$. None of the variants associated with the outcome of *S.* Typhi infection at the genome-wide significance level (5×10^{-8}). However, given the relatively small GWAS sample size, our study was estimated to be underpowered to detect significant associations using the genome-wide threshold (Methods 2.2.7) (Balding, 2006; Barton et al., 2022). Nonetheless, when visually examining the Manhattan plot, loci with greater significance were discernible; and may therefore still be relevant in illustrating mechanisms of protection against enteric fever (Fig. 5.2).

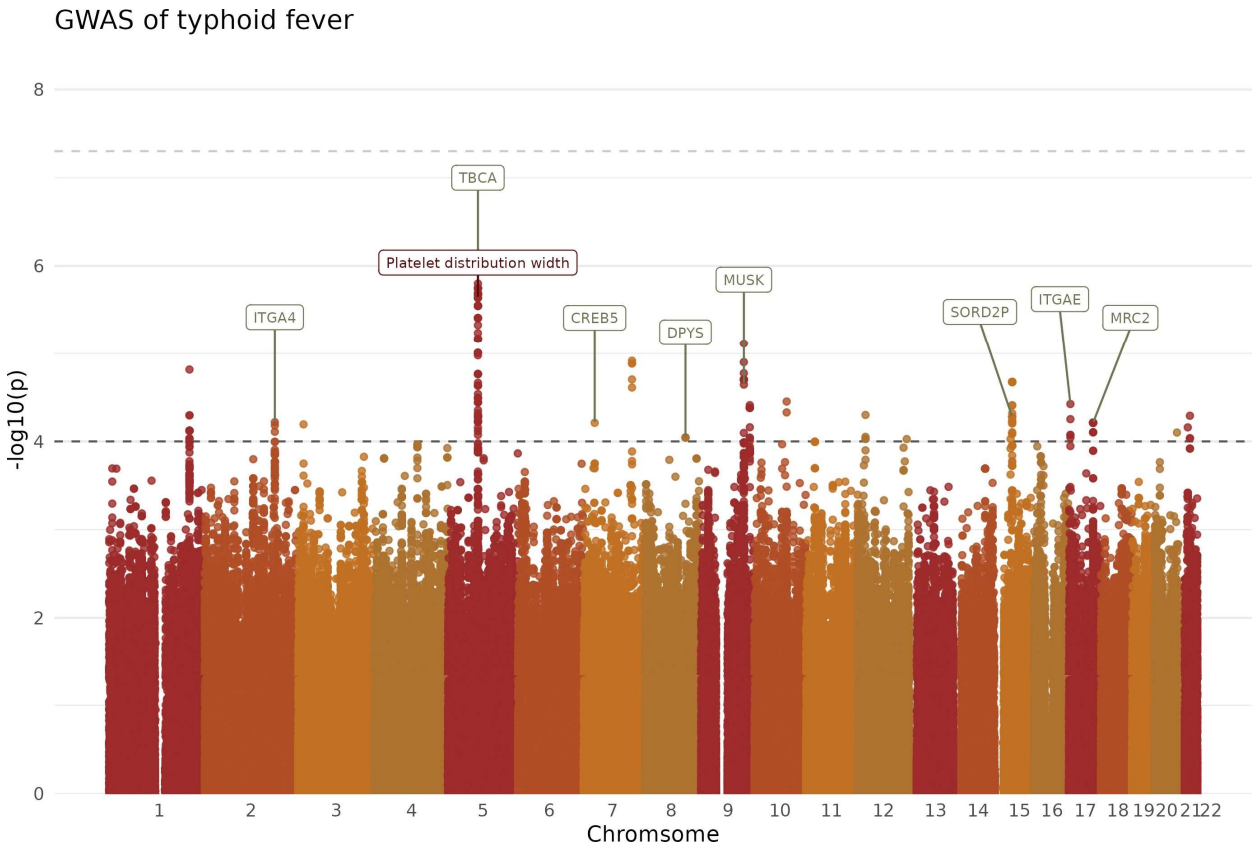


Figure 5.2: GWAS of typhoid diagnosis following S. Typhi challenge Manhattan plot displaying the significance (=log_{10} unadjusted P value) of each variant and the probability of being diagnosed with typhoid fever. SNPs are plotted on the x-axis by chromosome and genomic co-ordinates. The dark dotted line indicates a suggestive p-value of $P < 1_{\times}\ 10^{-4}$ SNPs highlighted in green indicate variants within coding genomic regions. Variants previously associated with GWAS of platelet numbers are also highlighted on chromosome 5.

Fine-mapping of GWAS loci associated with typhoid fever diagnosis

Consequently, in order to prioritise variants that may be functionally relevant in typhoid fever, I focused my analysis using a suggestive significance criterion of $P < 1 \times 10^{-4}$, and performed variant annotation and fine-mapping using the FUMA pipeline (Watanabe et al., 2017). Briefly, the lead SNPs in a possible risk loci were determined based on significance in the logistic regression model. Variants in linkage disequilibrium with the lead SNP were then examined in a stepwise manner and included in the loci if inherited together ($R^2 > 0.6$). This approach

thus allows the examination of nearby SNPs that, while not the most significant in the GWAS model, may have more downstream functional annotations which could lead to the detection of causal variants (Ritchie et al., 2015).

Using this method, I identified 15 independent genomic risk loci, which were subsequently fine-mapped for local genomic structure, and predicted variant deleteriousness. In addition, epigenetic features including, chromatin structure, regulatory regions, as well as prior associations in public eQTL and GWAS databases were evaluated. I further restricted these criteria to features measured in tissues relevant to *Salmonella* infection and dissemination; including whole blood, the spleen and the small intestine, in addition to single-cell RNA-seq eQTL databases for immune cell subsets and infected tissues (Methods 2.2.9). The majority of suggestive variants (95%) were identified in non-coding regions of the genome and were significantly enriched for long non-coding RNA and histone features that can modify gene transcription at both the local (cis) and distal (trans) levels, indicating they may play a regulatory role in gene function (Fig 5.3). Candidate variants in risk-loci were subsequently mapped to their closest overlapping gene if residing within 10 Kb of a transcription start or stop site (n = 184), or to genes previously reported to be in eQTL with the SNP (n = 19).

Pathway analysis

To determine which variants may be most relevant during *S.* Typhi infection and outcome, GWAS-associated genes were cross-referenced with differentially expressed genes (DEGs) from baseline and the day of diagnosis or seven days after challenge for undiagnosed participants (D7-TD). DEGs were further refined to include genes differentially expressed between typhoid-diagnosed and non-diagnosed participants ($adj.P.Val = 0.05$ & $log_2Fc > 0.2$), with the hope that this could reveal key gene pathways involved in protection against typhoid fever. Participants included those challenged with *S.* Typhi in the TyGER and VAST CHIM studies, with vaccinated participants excluded from the analysis (Table A.3) (n=81).

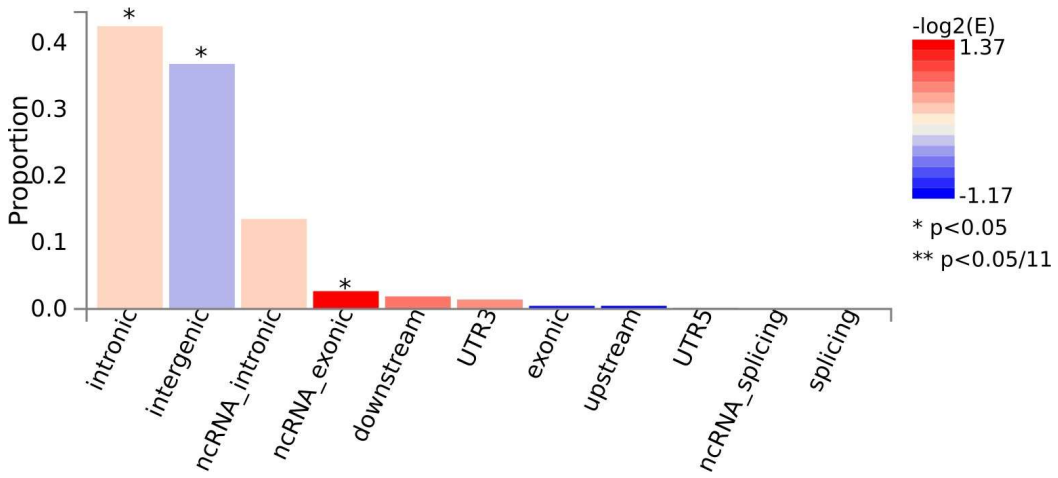


Figure 5.3: Features of top SNPs in GWAS of typhoid fever Top GWAS variants ($P < 1 \times 10^{-4}$) were annotated using the Annovar database. Features labelled with * indicates significant enrichment ($adj.P < 0.05$) in our data compared with random sampling.

Differential expression of 204 GWAS-associated genes was observed between diagnosed and undiagnosed participants at D7-TD (Table 5.1, Fig 5.4a). Since numerous genes may have cumulative effects formulating pathways relevant in protection or susceptibility to disease, I performed gene-set enrichment analysis of overlapping GWAS-DEGs against the annotated MSigDB immune database (n = 4782) (Liberzon et al., 2015). This analysis found that genes associated with protection from typhoid fever were significantly enriched for pathways related to T-cell regulation and activation, neutrophil phagocytosis, and monocyte ratios ($Adj.P < 0.05$) (Fig 5.4 C).

Feature (n)	Typhoid GWAS	IgA GWAS
SNP-tagged genes	184	124
eQTL-tagged genes	19	23
DEG D0-D7	5722	758
Overlapped	204	33

Table 5.1: SNP-tagged genes refers to genes residing within a 1 Kb distance of the tagged variant, or genes reported to be in eQTL with the tagged variant. Differentially expressed genes were determined by those with significant difference in the Log2Fold change gene expression from baseline, to seven days after challenge, between diagnosed and non-diagnosed participant.

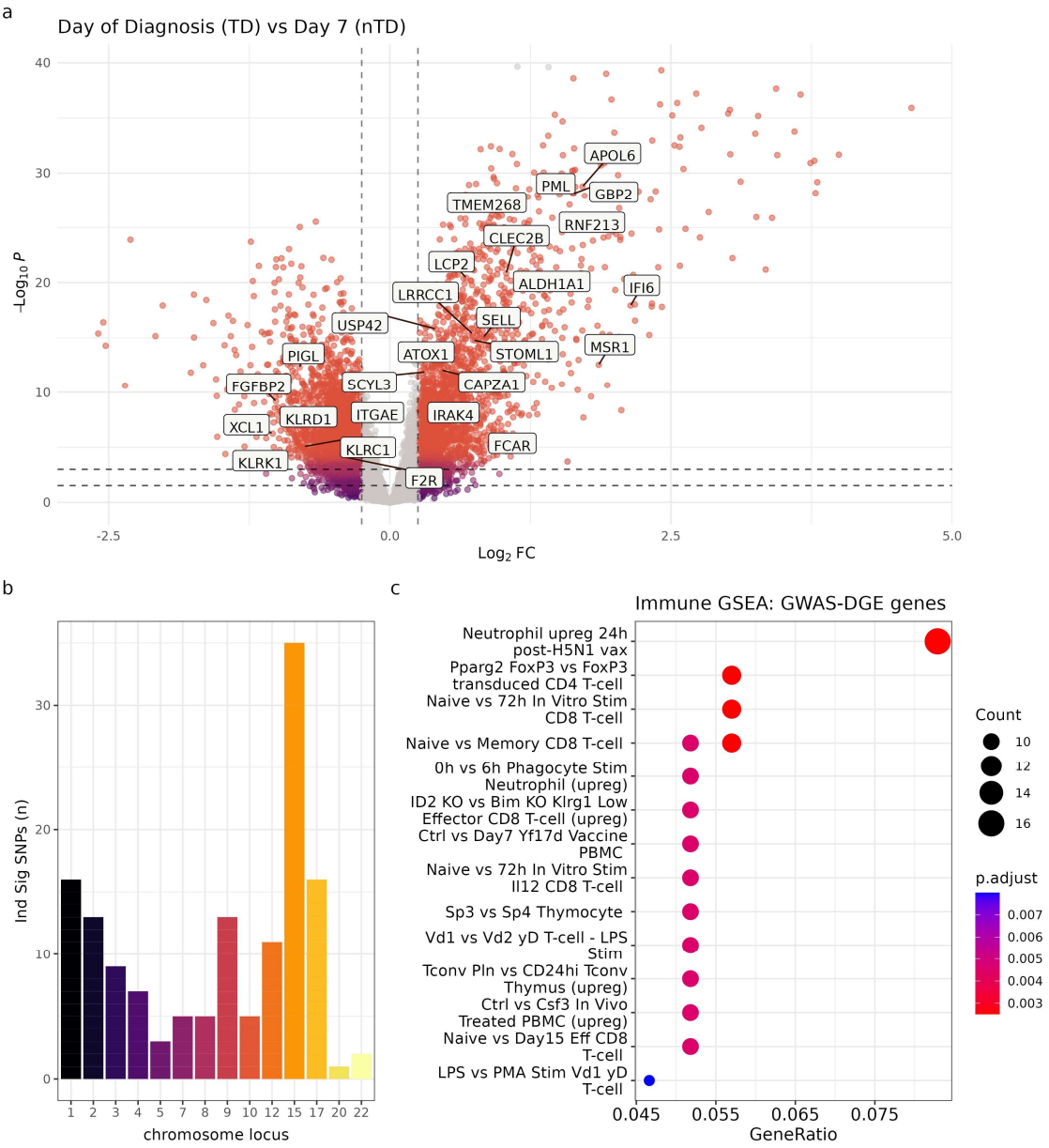


Figure 5.4: GWAS-associated gene expression profiles and pathway enrichment. A) Volcano plot shows the comparison in the Log-FC gene expression between participants diagnosed with typhoid fever (TD) and non-diagnosed participants (nTD). Each point represents an individual gene, and those above the dotted line have a significant fold change between groups ($FDR < 0.05$). Highlighted are a subsample of the top differentially expressed genes linked in the typhoid study. B) Bar plot shows the number of independent significant SNPs located across 15 independent genomic loci. These variants were then annotated for nearest genes and eQTL. C) Genes associated with both outcome of typhoid fever in the GWAS logistic regression model, and those that were differentially expressed between nTD and TD participants were included in MsigDB C7 gene-set enrichment analysis.

Risk-loci eQTL analysis

In order to investigate the influence of genetic variation upon altering expression profiles during infection, I performed eQTL-analysis of GWAS-DGE variants (n SNPs = 204), and gene expression measured at baseline, and either seven-days following *S.* Typhi challenge, or expression measured at the time of diagnosis. Genes located up to 1 Mb either side of the variant were tested for correlations between the variant genotype frequency and gene expression using an additive least-squares regression model. Age, sex, body mass index (BMI), and the study-arm were included as covariates (n=81). Evaluation of these suggestive variants and genes for eQTL did not observe any findings that were significant after correcting multiple testing.

Therefore I prioritised further investigations based upon candidate GWAS variants which had prior associations with either immunological or haematological traits (Table A.5). These included polymorphisms within a 115 Kb locus upstream of the *F2R-F2RLI* genes on chromosome 5 (Chr5:77670380 - Chr5:77786424), which encode protease-activated receptors (PAR 1 and 2), also known as thrombin-coagulation receptor genes. Variants of this loci had been previously linked to increased platelet levels in several genome-wide studies of haematological traits (Astle et al., 2016; Vuckovic et al., 2020) ($P = 5 \times 10^{-12}$, $\beta = 0.02$). In our data set, these polymorphisms were also associated with differential *F2R* gene expression both at baseline and D7-TD, which neared the multiple testing significance threshold in prior eQTL analyses when measured at D7-TD ($adj.P = 0.06$) (Fig 5.5).

Therefore, to investigate the role of the *F2R* gene expression following *S.* Typhi challenge, I modelled the fold-change in gene expression from baseline to seven-days following infection, and observed a significant reduction in the gene expression of *F2R*, along with several other platelet associated genes, possibly in line with thrombocytopenia which has observed during the course of typhoid fever (Fig A.14). Furthermore those who were not diagnosed with typhoid fever had significantly less of a reduction in F2R gene expression following challenge.

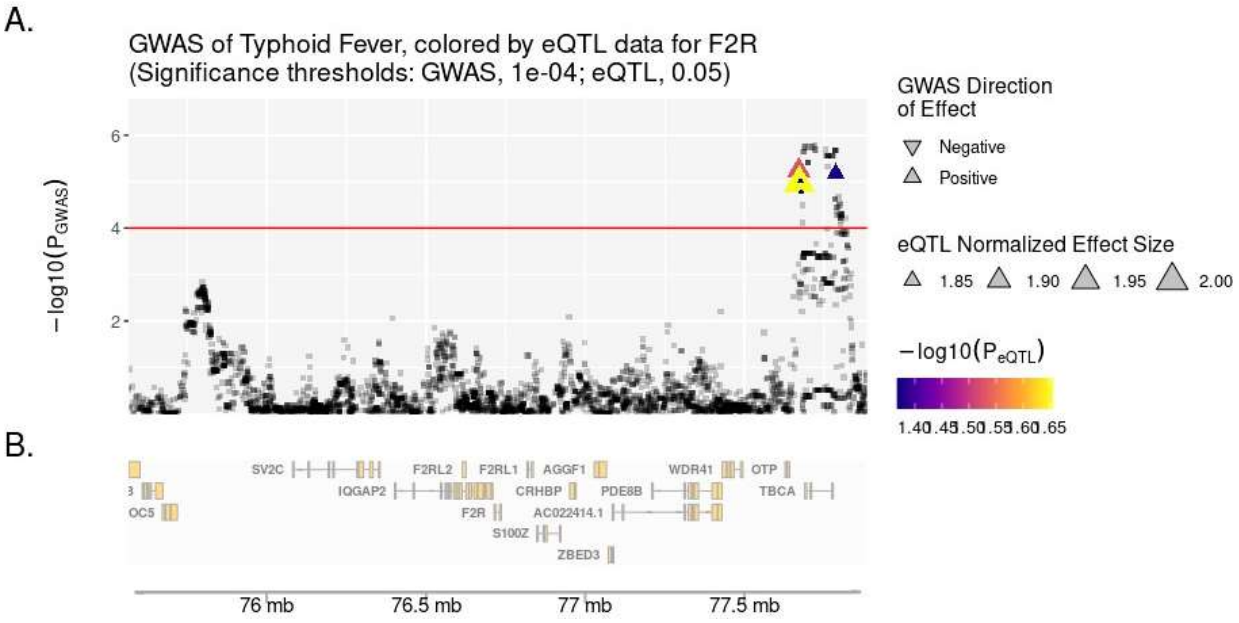


Figure 5.5: cis-eQTL variants associated with F2R gene expression and diagnosis of typhoid fever The x axis denotes genomic co-ordinates of chromosome 5 per Mb, with local genes overlaid on top. SNPs are plotted across the genomic loci, andthe Y axis denotes their genome-wide significance with outcome of S. Typhi challenge. The red line indicates the suggestive GWAS significance threshold of $P = 1 \times 10^{-4}$. SNPs in eQTL with F2R gene expression at D7 are highlighted as according to their significance in the eQTL model.

In order to see if *F2R* genetic variation was also associated with differences in gene expression, I associated the chromosome 5-loci polymorphisms with the fold change in gene expression measured between baseline and seven-days post-challenge, or at the time of diagnosis. Variants that were associated with protection against typhoid fever (*OR* 0.2 – 0.3, $P = 1.4 \times 10^{-5}$), were also associated with a significantly reduced fold-decrease in *F2R* gene expression, seven days following challenge (*adj.P* < 0.05) (Fig 5.6). Conversely, one variant within the loci, (Chr5:77682126:C-T), correlated with an elevated risk of typhoid fever (*OR* 2.4, $P = 7.6 \times 10^{-5}$) and those homozygous for the risk allele had a greater decrease in *F2R* expression. These findings may therefore indicate that maintaining *F2R* gene expression during the course of infection provides some protective benefits.

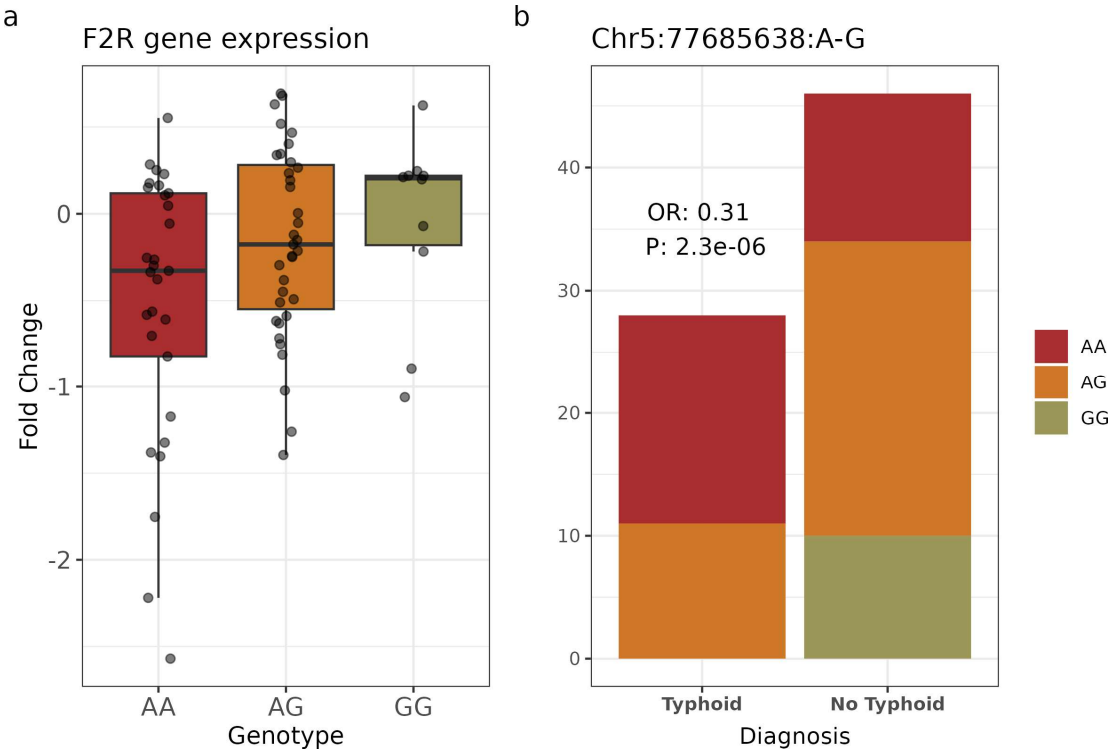


Figure 5.6: Associations of the Chr5:77685638A-G allele with typhoid fever diagnosis and gene expression A) The fold change in F2R gene expression is split across Chr5:77685638A-G genotypes. B) Bar plot shows the proportion of individuals with the either a homozygous AA genotype, heterozygotes (AT), or homozygous (TT) individuals and the proportional outcome of typhoid fever.

5.2.3 Genome wide association study of responses to Vi-vaccination

In addition to investigating SNPs associated with immunity against enteric fever, I also examined associations with vaccine-induced responses and immunity. For this study I included participants from the VAST trial vaccinated with either a Vi-polysaccharide vaccine (Vi-PS) or the Vi-tetanus conjugate (Vi-TCV) (n = 68). Given the associations of Vi-IgA with vaccine mediated protection (Jin et al., 2021), I first focused my analysis on variants that were associated with Vi-IgA levels 28-days following vaccination, to see if these could reveal further insights into vaccine mediated protection.

Despite the relatively small sample size, one SNP located at chromosome 1 reached above the genome-wide significance threshold ($P < 5 \times 10^{-8}$) (Fig 5.7).I consequently investigated genomic features of this variant; however, it was not located near any protein coding regions, nor was it predicted to have an effect on transcription. Subsequently, I annotated variants of suggestive genome-wide significance in the study ($P < 1 \times 10^{-4}$), using publicly available data and feature mapping as described previously, to examine variants that might be casually linked with Vi-IgA production.

Interestingly, the annotation of suggestive GWAS variants included a miss-sense SNP, rs3803800, which had previously been associated with high serum-IgA levels and IgA nephropathy (Jonsson et al., 2017; Kiryluk et al., 2014; M. Li et al., 2020) suggesting that this variant may be located in a genomic region relevant to IgA responses (Osman et al., 2012). Using data fromthe Roadmap Epigenomics Projects which applies DNase peaks and core 15-state chromatin state model, I then evaluated the genomic and epigenetic region of this marker (Kundaje et al., 2015).

eQTL analysis of candidate genes associated with Vi-IgA production

The rs3803800 single nucleotide variant mapped to a genomic loci spanning 100 kb on chromosome 17, (Chr17:7441178 - Chr17:7544686), and in linkage disequilibrium (LD) with several SNPs that were also associated with increased Vi-IgA titres following vaccination (Fig 5.8 A-B). Consequently, I focused my eQTL-analysis to variants in this loci. When examining the epigenetic state of the region, SNPs were co-localised within promoter-enhancer regions upstream of the tumour-necrosis factor 12 and 13 genes (*TNSF12-TNSF13*), which encode TWEAK and APRIL proteins respectively (Fig 5.8). APRIL, along with BAFF, is expressed in monocytes,

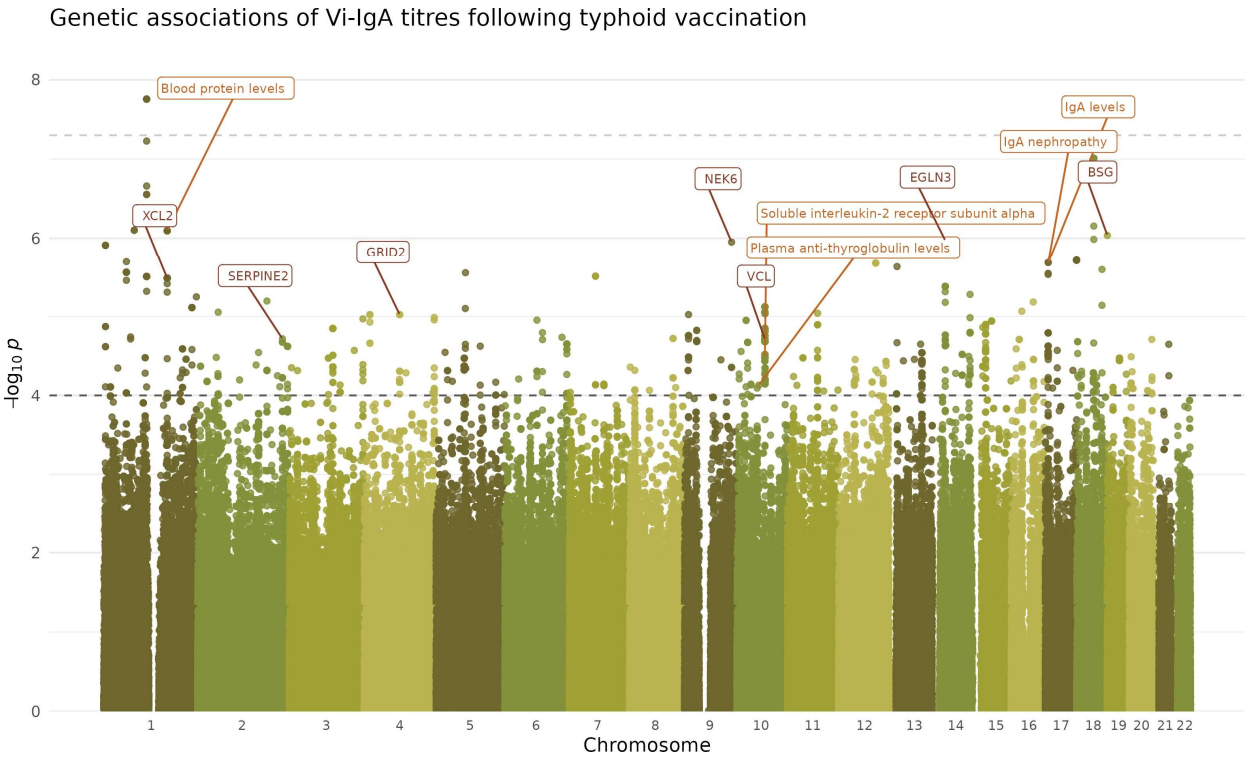


Figure 5.7: GWAS of Vi-IgA titres 28-days following typhoid vaccination

Manhattan plot displaying the significance - log_{10} unadjusted P value of each SNP and Vi-IgA titres 28-days following Vi-vaccination. SNPs are plotted on the x-axis by chromosome and genomic co-ordinates. The dark dotted line indicates a suggestive p-value of $P < 1 \times 10^{-4}$, and the light grey line indicates the genome-wide significance threshold of $P < 5 \times 10^{-8}$. Variants which were previously associated with relevant GWAS are also highlighted.

macrophages, dendritic cells, T cells, and provides survival signals to B-cells undergoing maturation. The TNF-like weak inducer of apoptosis (TWEAK), is a multi-faceted pro-inflammatory cytokine, expressed predominantly in T-lymphocytes, and is also thought to play a role in B-cell survival and maturation (Kumar et al., 2009). Furthermore, data from the Gene-tissue expression (GTEx) database, uncovered that variants in the chromosome-17 loci associated with differential *TNSF12-13* gene expression in whole blood and spleen tissues (Consortium et al., 2015), indicating that these variants might be functionally consequences on gene transcription. I subsequently performed eQTL analysis investigating *TNFSF13* and *TNFSF12* gene expression and associations with genotypes.

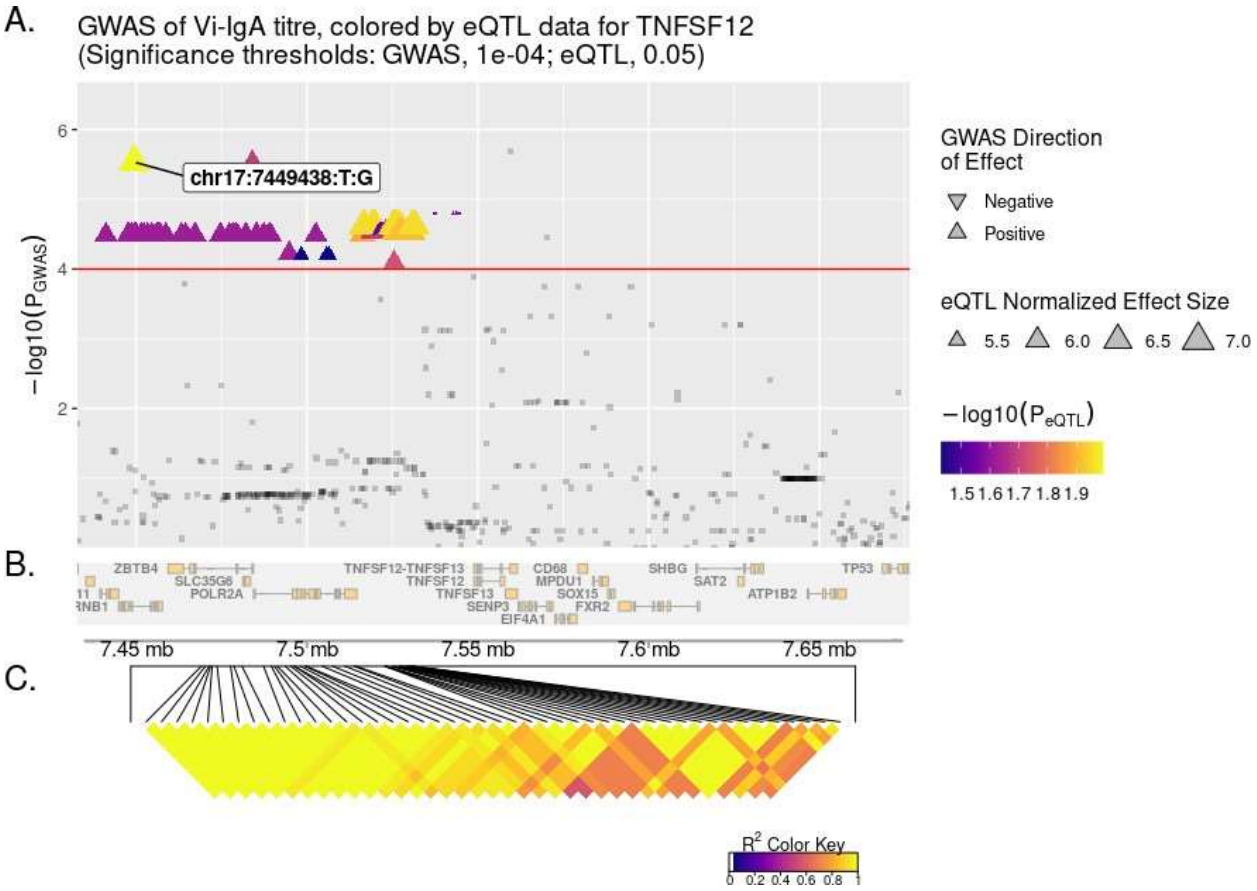


Figure 5.8: eQTL analysis of upstream TNSF12 variants and regional linkage disequilibrium A) The x-axis denotes genomic co-ordinates of chromosome 5 per Mb and local genes are overlaid. SNPs are plotted across the genomic loci (B), and the y-axis denotes the genome-wide significance with outcome of *S*. Typhi challenge. The red line indicates the suggestive GWAS significance threshold of $P < 1 \times 10^{-4}$. SNPs in eQTL are highlighted according to their statistical significance in the eQTL model. C) Linkage disequilibrium of TNSF12-13 variants, shade indicates the strength of the linkage-disequilibrium (R2).

One-day following Vi-vaccination *TNFSF13* gene expression increased significantly from baseline when measured in whole blood. *TNFSF12* expression also increased, however this was not significant (Fig 5.9A). Presence of the rs3803800 G-allele was associated with higher *TNSF13* gene expression 1-day following Vi-vaccination, and subsequent higher vaccine induced Vi-IgA titres (Fig 5.9C). Downstream of *TNSF13*, lies the TWEAK encoding *TNSF12* gene. Variants in the gene region were also associated with increased Vi-IgA production. In contrast to

the effects observed with TNSF13, eQTL were associated with significantly lower *TNFSF12* gene expression following vaccination. Given that TWEAK may play a role in plasma cell apoptosis during B-cell maturation, these results may reflect the alternate roles for the TWEAK-APRIL axis. In addition, genotypes in eQTL with *TNSF12* were more frequently found in participants who were not diagnosed with typhoid, suggesting a protective role for this pathway following vaccination.

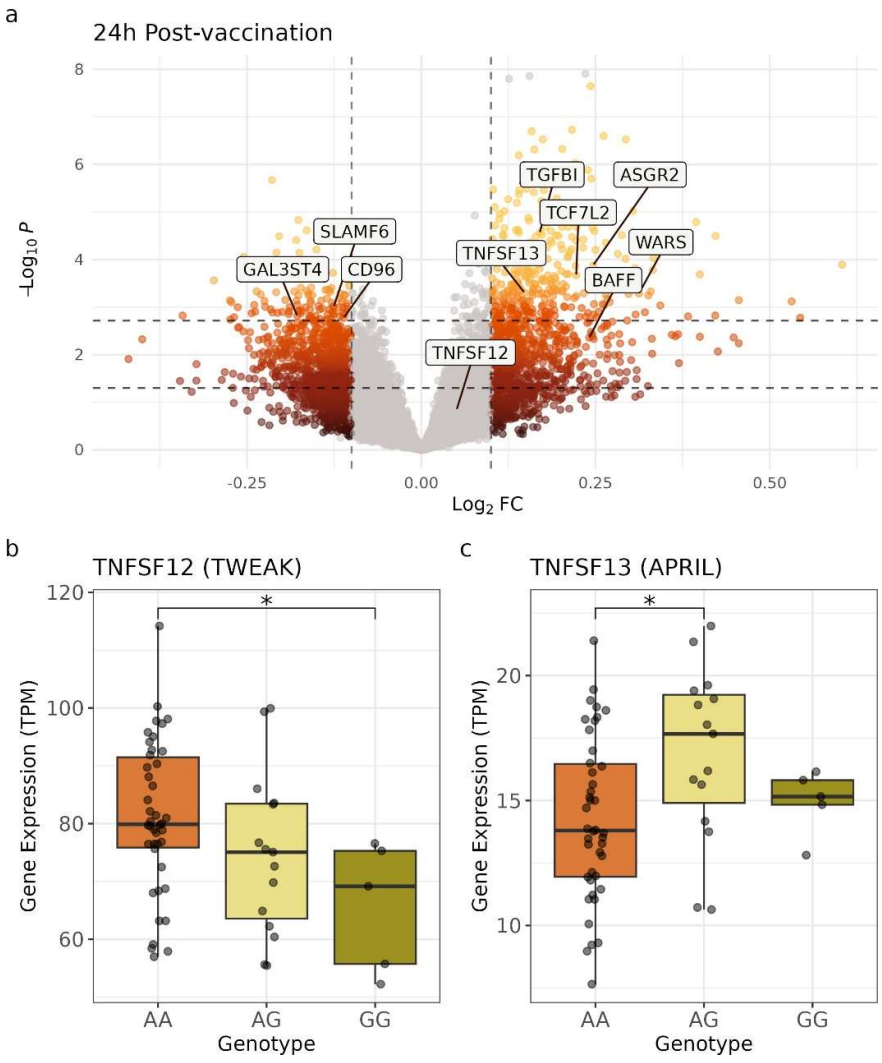


Figure 5.9: Differential gene-expression 24 hours following typhoid vaccination Volcano plot shows Log_2Fc on the X-axis between time points, along with the $-log_{10}$ *P* value of each gene represented on the y-axis. Each point represents an individual gene,and those above the dashed line have significant Fold-change difference in gene expressionbetween groups ($P(unadjusted) < 0.05$). Highlighted are differentially expressed genes that were also associated with Vi-IgA GWAS variants following typhoid vaccination.

Pathway analysis

I next evaluated all GWAS-associated genes ($P < 1 \times 10^{-4}$) for differential gene expression between baseline, and both 1 and 7 days after vaccination. 124 genes were mapped to significant GWAS variants, and 33 of these genes were differentially expressed between baseline and 1 and 7-days following typhoid immunisation (Table A.4). To investigate whether genetic variation and transcriptomic responses might share similar features, I performed gene-set enrichment analysis of GWAS-DEGs using the C7, MsigDB annotated immune database (Liberzon et al., 2015). Pathway analysis revealed that T-cell to monocyte ratios, amongst other vaccine related pathways were significantly enriched in these data (Fig 5.10).

Interestingly, GWAS-DEGs following vaccination were also associated with increased Vi-IgG titres, and included several genes that are differentially expressed during infection (Table A.4). These genes included WARS, which was significantly upregulated 24 hours following Vi-PS and Vi-TCV vaccination, and had previously been identified as a candidate diagnostic biomarker during *S.* Typhi infection using microarray data (Blohmke et al., 2019) (Fig 5.10). Using the Fisher's exact test, I measured significant enrichment of vaccination associated GWAS-DEGs within genes induced following challenge (*adj.P* < 0.05). In addition, these genes were also significantly over expressed in whole blood collected from blood-culture confirmed typhoid fever patients in Vietnam, compared with healthy controls (Thompson et al. 2009). Taken together, these data suggest that variants important in antibody-responses to vaccination, are pertinent amongst pathways also involved in infection induced immune responses to typhoidal *Salmonella*.

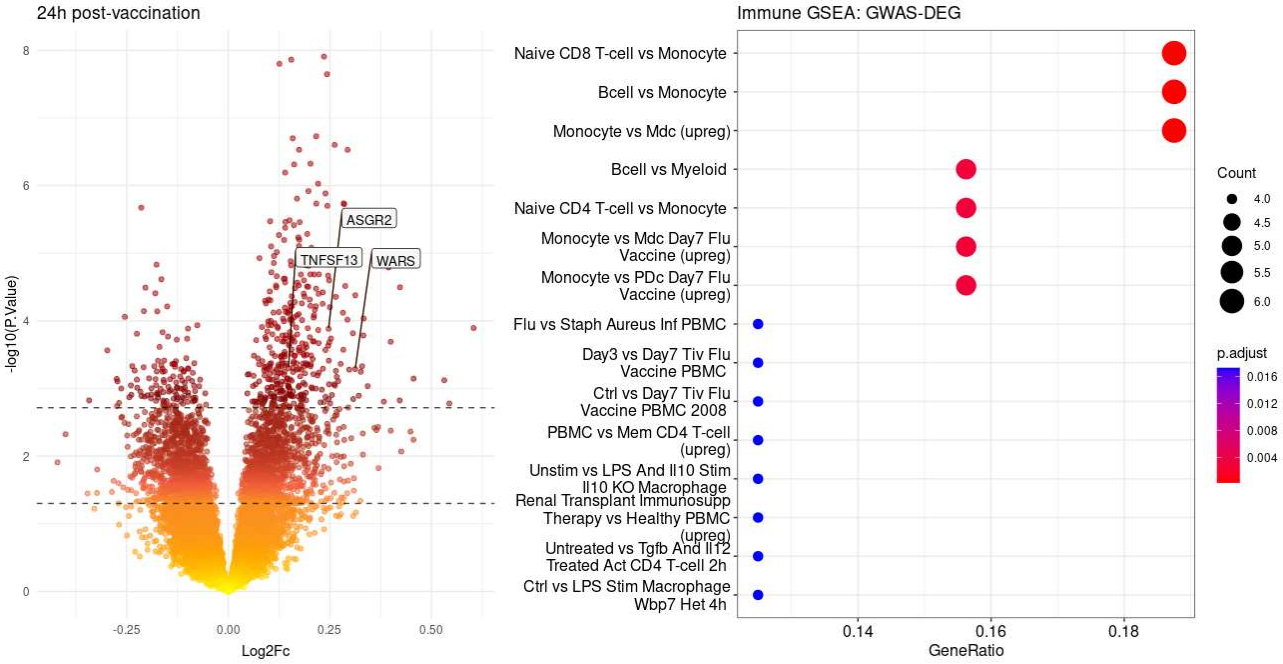


Figure 5.10: Enrichment analysis of SNPs in GWAS of Vi-IgA titres SNPs were annotated if within or 10 Kb upstream or downstream of a gene coding region. The MSigDB-immune gene-ontology database was used to compare the proportion of geneswithin our SNP list and the proportion of genes within a pathway that would be expected without enrichment significance.

5.3 Discussion

Using functional annotation of the genome, in this chapter I describe the characterisation of single nucleotide polymorphisms (SNPs) which are associated with differences in both outcome of *S.* Typhi infection and vaccination. By integrating these data with differential gene expression, I identified potential causal genes associated with outcome that could be influenced by genetic variation. Moreover, gene set enrichment analysis revealed that on a global scale, variants were enriched across multiple pathways involved in both innate and adaptive immune responses to stimuli. Whilst pathway enrichment analysis of suggestive GWAS variants has been employed to study variants in complex diseases such as intestinal bowel disease (J. Li et al., 2016), this is the first application of a pathway-based approach in the context of enteric fever infection and vaccination.

In addition, candidate expression quantitative trait loci (eQTL) were investigated based upon loci containing previously identified GWAS and eQTL traits. For variants associated with increased Vi-IgA titres following vaccination, these included a locus on chromosome 17 encoding the B-cell activating protein ligand, APRIL, and the apoptosis inducer, TWEAK. Regarding variants associated with disease outcome in the typhoid human challenge model, eQTL SNPs were highlighted within a genomic region on chromosome 5 which overlapped *F2R* and *F2RL1* genes involved in platelet adhesion and signalling.

5.3.1 GWAS of typhoid-fever diagnosis

The *F2R*, and *F2RL1* genes encode protease activated receptor 1 and 2 (PAR1/2), respectively. The protease-activated receptor (PAR) family, consists of G-protein coupled receptors, and cleavage of their extracellular N-terminus by serine proteases can initiate downstream signalling and cellular activation; which in platelets may contribute towards eventual haemostasis and thrombosis events (Hollenberg, 2002). While, on the endothelium, PARs have been demonstrated to promote integrin signalling, cell adhesion, and the production of IL-8 by macrophages. (Fender et al., 2017; St-Onge et al., 2010). Research over the past-decade has revealed how platelets can play dual roles in both inflammation and wound repair through cross talk via Toll-like-receptors (TLRs) on the platelet surface. In particular, platelets express TLR4 which can recognise Gram-negative bacterial LPS-A antigen, and subsequent downstream signalling results in the release of inflammatory cytokines and cytolytic granules (Bucci et al., 2012).

However, the exact role platelets and any associated PAR signalling has not been well characterised within the context of *S.* Typhi infection, despite thrombocytopenia being recognised as a known marker for typhoid severity and complications (Malik, 2002). In our cohort of participants infected with *S.* Typhi in a controlled human infection model (CHIM), I observe a decrease in *F2R* gene expression which is significantly higher amongst participants diagnosed with typhoid compared with

those who were not diagnosed. Although I did not have platelet count data for these participants, it is possible that this response is reflective of thrombocytopenia, estimated to occur in 63% of adults with disease. Correspondingly, the down-regulation of platelet-related gene clusters was also observed between culture-confirmed typhoid fever patients and healthy participants studied in Vietnam, a country endemic for typhoid, suggesting that this gene signature is not an artefact of CHIM study conditions (Thompson et al., 2009).

Moreover, variants that were above the suggested GWAS significance in our study of ($P < 1e^{-4}$), were associated with both protection against typhoid disease, and a lower fold-reduction in *F2R* gene expression. Interestingly, polymorphisms in the promoter region of *F2R* have previously been associated with higher gene expression within the context of several diseases including; sarcoidosis, cardiovascular disease and cancer, and subsequent protective outcomes (Gigante et al., 2010; Plate et al., 2015). However, this is the first association of *F2R* variants with protection against infection. Given previous associations with these diseases, eQTL were also modelled with BMI and smoking included as covariates to ensure findings weren't incidental of cohort health outcomes, and these results remained significant.

Interestingly Lippuner et al. (2006) reported that upon treating human mononu-clear phagocytes with either thrombin or a synthetic ligand of PAR2, there was a significant increase in the levels of bacterial killing and reactive oxygen species release (ROS) compared with control cells, and that the bacterial killing induced by PAR2 was comparable with that of IFN-γ -stimulation. Taken together with the 'protective' eQTL associations, these data suggest that alleles linked with higher expression of *F2R* gene may result in increased PAR2 activity which could have a functional role in protection. However, investigating these results in a separate cohort, ideally in an endemic country, would be required to validate these findings. Furthermore, this study is limited in that we did not measure gene expression, nor associate genetic variation with cases of severe typhoid fever, which would likely provide a more relevant phenotype.

In addition to playing a bactericidal role, platelet dysregulation in sepsis cases has been previously associated with poorer patient outcomes, and over-activation of PAR signalling by serine proteases released by enteropathogenic *Escherichia coli* (EPEC) resulted in increased inflammation and haemorrhaging in a mouse model of infection (Greco et al., 2017; Vardon-Bounes et al., 2019). Therefore, the overall down regulation of platelet associated genes and thrombocytopenia following *S.* Typhi infection, is likely a result of the need to maintain homoeostasis. There is thus a delicate balance to be maintained; between inflammation that is protective against infection, and that which may harm the host. Future studies characterising the role of PAR signalling during *S.* Typhi, potentially through either mouse knock-out models, or in vitro studies, may be able to elucidate this relationship further.

Furthermore, when clustering GWAS-associated genes across immune-related pathways, I observed a significant enrichment for genes involved in neutrophil stimulation and viral (H5N1) phagocytosis. Following *S.* Typhi challenge, neutrophil genes are significantly upregulated, and have been investigated as important mediator of the immune response to typhoid infection (S. J. Barton et al., 2019), as well as playing a role in vaccine mediated protection. Interestingly, PAR2 signalling has been demonstrated to stimulate IL-8 release from neutrophils and increase their adhesion and migration (St-Onge et al., 2010), further implicating the role of PARs in inflammation. Moreover, PAR2 neutrophil activation was dependent upon FcγR2a and 3b signalling, also providing a potential link for antibody mediated activation of neutrophils and this pathway.

Interestingly, in a genome wide study of blood-stream candidiasis susceptibility, pathway analysis of genes both differentially expressed and those with suggestive GWAS alleles, amongst uncovered that genetic variation was enriched amongst several immune-activation pathways (Matzaraki et al., 2017). These included Toll-like receptor signalling, and also those involved in platelet signalling and aggregation

24 hours following infection. GWAS-Gene-set enrichment analysis also highlighted clusters of SNPs involved T-cell responses and signalling.

T-cells have a variety of roles during *S.* Typhi infection. CD4 T-cells contribute to *Salmonella* antigen presentation, whereas CD8 T-cells may limit the intracellular spread of *S.* Typhi (Larock et al. 2015). Previous research has linked MHC-class II alleles involved in antigen presentation to CD4 T-cells and MHC-class I alleles involved in antigen presentation to CD8 T-cells with susceptibility to typhoid fever (A. Barton et al., 2022; H. Nguyen et al., 2008). While I did not use these data to fine-map the MHC-locus for GWAS variants, they do support the hypothesis that genetic variation in loci related to T-cell activation are relevant to risk of infection. Future work investigating the role of the genes highlighted in these pathways may therefore uncover novel mechanisms of susceptibility to disease, and remain to be validated in a replication cohort.

5.3.2 GWAS of Vi-IgA titres following Vi-vaccination

In this chapter I also describe the first GWAS study of humoral responses to typhoid vaccination. In particular I characterised variants associated with higher Vi-IgA titres 28-days following vaccination using functional genomics and publicly available data. Vi-IgA titres and avidity for Vi-antigen, have been shown to associate with vaccine mediated protection (Dahora et al., 2019; Jin et al., 2021). Despite promising efficacy results of Vi-conjugate vaccination roll out thus far; understanding why some vaccinees induce higher antibody titres and functional activity, may help to refine future vaccine design and implementation strategies. Here, I identify one variant located on chromosome one, above the GWAS significance threshold of $P < 5 \times 10^{-8}$. In addition, single nucleotide variants within chromosome 17; *TNSF12* and *TNSF13* genes, were associated both with differential gene expression and higher post-vaccination Vi-IgA titres.

Mutations in the tumour necrosis superfamily (TNF) have been previously associated with a number of primary immunodeficiencies, including Common variable immunodeficiency (CVID). CVID is a disease characterised by low levels of immunoglobulin and susceptibility to infection, demonstrating the important role of TNF cytokines within the development of functional humoral responses (Lobito et al., 2011). Compared with wild-type mice, deletion of the *TNSF13* gene significantly reduced B-cell responses to mucosal antigens, and rates of IgAclass-switch recombination. Bucasas et al. (2011) demonstrated 24 hours following trivalent influenza vaccination (TIV), higher expression of *TNFSF13B* correlated with increased vaccine-specific antibody titres and persistence.

Accordingly, I observe that variants which were linked to an increase in *TNFSF13* expression, were also significantly associated with higher Vi-IgA titres following vaccination. Moreover, the *TNFSF13* SNPs identified in this GWAS, have also been linked to higher levels of pathogenic IgA antibodies in IgA nephropathy and systemic lupus erythematosus (SLE) (Ortiz-Aljaro et al., 2022), indicating that these variants are likely playing a causal role in the variability of IgA responses produced following Vi-vaccination. Alternatively, TWEAK is considered to play a regulatory role during B-cell maturation, through NF$\kappa\beta$ signalling and apoptosis induction. Wang et al. (2013) identified that the inheritance of an autosomal dominant mutation of *TNSF12* gene resulted in abhorrent binding of TWEAK to the B-cell activatory BAFF-protein, causing inhibitory effects that were associated with reduced humoral responses and IgA titres. In our study I observe variants associated with reduced *TNFSF12* gene expression, and subsequently higher post-vaccination Vi-IgA titres, thus possibly reflecting alternate roles for TWEAK and APRIL following vaccination.

Whilst two of the variants identified by the association model were located directly within the *TNFSF-12-13* gene locus; the majority of SNPs in linkage

disequilibrium with these variants were located in the downstream *POLR2A* transcription factor gene. Therefore, it's possible that the differences observed in *TNSF12-13* gene expression are rather a result of genetic variation amongst the associated transcription factor. Of interest, a GWAS of immunodeficiencies demonstrated that cis-eQTL variants were also enriched for SNPs in transcription factor genes, which may cause more global effects upon gene transcription (Ishigaki et al., 2022). In this study I did not investigate trans-eQTLs due to the high multiple testing burden and the small sample size of the study; though, one could hypothesise that trans-acting SNPs may also be impacting gene transcription, and subsequently alter infection and vaccination responses. However, in both cases the analysis was underpowered to detect any genome-wide significant associations.

Consequently, future work may involve refining this pipeline to focus on variants located within transcription factors or those residing amongst local promoter or enhancer regions to identify causal eQTL variants which may subsequently alter the response to either *S.* Typhi infection and or vaccination. A similar method prioritising eQTL variants based on the distance to transcription start sites amongst other epigenetic notation, was recently used to improve the detection of eQTL variants involved in immune-related genes using data from the GTEx consortium (Mu et al., 2021). Another study limitation is that in order to maximise functional mapping variants were not pruned for linkage disequilibrium. Therefore, the true number of independent statistical tests is likely lower than the number used for multiple testing corrections. Future statistical approaches could consider either accounting for haplotype structure when making adjustments for multiple testing, or performing grouped associations of SNPs within haplotypes.

In order to identify larger trends amongst genetic variation associated with vaccination titres, I subsequently clustered SNPs and their associated genes based on enriched pathways identified by the immune MSigDB resource. Intriguingly, pathway analysis also highlighted several genes involved in T-lymphocyte activation and regulation, including SNPs in the integrin-B7 (ITG-β_7) subunit gene which is

involved in gut-homing trafficking of lymphocytes (Sun et al., 2020). Interestingly, following Vi-conjugate vaccination, the analysis of cellular subsets through time-of-flight mass-cytometry (CyTOF), revealed that an increase in CD4 T-follicular helper cells was significantly associated with the development of gut homing $\alpha_4\beta_7$ IgA+ plasma cells (R = 0.53, P = 0.017), implicating their involvement in the humoral response (Cross et al., 2020).

Furthermore the observation of genetic variation in T-cell related genes supports findings from previous GWAS studies associating genetic variation in T-cell activatory genes and antigen-presenting molecules with antibody titres (O'Connor et al., 2019; Ovsyannikova et al., 2012; Png et al., 2011). Pathways involved in monocyte ratios were also enriched for GWAS variants in differentially expressed genes, and previous studies of transcriptomic responses to vaccination, have observed how higher baseline monocyte activation may negatively correlate with the outcome of vaccination (O'Connor & Pollard, 2013). While I did not detect genome-wide significant eQTL of variants amongst pathway-linked genes, this study may provide a list of candidate SNPs and associated genes for future investigation.

A final observation to highlight is the enrichment of vaccination-related GWAS target genes, with DEGs following typhoid infection. These genes included *WARS* which is involved in tryptophan metabolism, and had previously been identified as candidate diagnostic marker for typhoid infection (Blohmke et al., 2019). *S.* Typhi, has been shown to alter intracellular tryptophan levels as it acquires the amino-acid for its own metabolic growth requirements (Blohmke et al., 2016). *WARS* also significantly upregulated 24 hours following influenza vaccination, however itsexact role in the response to vaccination remains to be characterised (Bucasas et al.,2011). Differential gene expression following live attenuated rota-virus vaccination was found to mimic that induced by natural infection (Gómez-Carballa et al., 2021), however such an overlap between transcriptional responses following vaccination and infection has not yet been investigated. To investigate this potential association,

future studies could expand the enrichment analysis to investigate all differentially expressed genes following vaccination and infection.

Conclusions

The functional fine-mapping of GWAS data is a relatively novel approach to GWAS analysis. Despite hundreds of GWAS studies, only a small proportion result in functional validation (Gallagher & Chen-Plotkin, 2018). Therefore, I aimed to demonstrate how the integration of data across studies, can lead to the identification of pathways that are important for both disease susceptibility and vaccination responses. In the case of associations with the risk or protection from developing clinically detectable typhoid fever following challenge, these SNPs had previously been investigated in eQTL studies involving *Salmonella* infection, and highlighted genetic variation amongst a relatively understudied pathway of platelet activation. Concerning vaccination, this method also identified an eQTL locus previously associated with IgA titres in disease. Using these methods, we can therefore begin to untangle a wealth of genomics and transcriptomic data, and uncover biologically meaningful phenomena which warrant further investigation

6

Final Discussion

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Human challenge models can provide a plethora of data relevant in furthering our understanding of disease and mechanisms of protection. In this thesis I investigated several aspects of the host determinants and response to vaccination and infection using data from the typhoid human challenge model. In particular, I examined novel aspects of the humoral response following Vi-TCV and Vi-PS vaccination, including antibody dependent neutrophil phagocytosis and the oxidative burst.

I then investigated factors that could be driving differences in humoral responses to Vi-vaccination. These included Fc-glycosylation features, and receptor binding. Finally, at the most fundamental level, relationships between genetic variation and antigen-specific antibody titres following typhoid vaccination was examined. In this chapter I will summarise the key themes of this thesis, as well as their limitations, and potential future work.

6.1 Antibody-dependent neutrophil oxidative burst correlates with protection following vaccination in a human challenge model of *S. Typhi* infection

The release of reactive oxygen species by neutrophils is a potent antimicrobial force and a significant component of the innate immune response against multiple bacterial and fungal infections (G. T. Nguyen et al., 2017). Both *S. Paratyphi* and *S. Typhi* have evolved mechanisms to typically evade oxidative burst mediated killing. For *S. Typhi*, this has been shown to be achieved through the expression of the Vi-capsule which masks LPS from TLR-4 receptors on circulating monocytes (Larock et al., 2015). Additionally, Vi-capsule expression can hinder the binding of IgM to LPS, thereby preventing the activation of the complement cascade and neutrophil recruitment and phagocytosis. In *S. Paratyphi*, extra-long O2-LPS antigen chains also shield the bacterium from detection (Hiyoshi et al., 2018). *S. Typhimurium*, however lacks such features, and is instead thwarted at the intestinal mucosa. The ability for typhoidal *Salmonellae* to spread systemically and cause disease is what makes it so deadly. Therefore, producing vaccines which may limit the spread of bacteria and prevent infection are of particular importance.

In chapter 4, I describe how the antibody dependent neutrophil oxidative burst not only associates with protection in the human challenge model, but also correlates with a lower bacterial load and a longer time to diagnosis in individuals

who developed typhoid fever. These findings therefore indicate that Vi-vaccination generates functional antibodies that can limit the spread of S. Typhi following challenge. Although this study was conducted *in vitro* with donor neutrophils, cellular and gene expression data from the human challenge study suggest that neutrophils are heavily involved in vaccination and challenge responses.

Firstly, 12 to 24 hours following *S.* Typhi challenge, neutrophil numbers increase in whole blood, and neutrophil related genes are overexpressed relative to baseline (A. Barton, 2019). Neutrophils express high levels of Fc-receptors on their cell surface. I also observed that higher levels of Fc-receptor expression 24 hours following *S.* Typhi challenge associated with protection in the human challenge model. Seven days after challenge, or at the time of diagnosis, FcαR and FcγR gene expression was higher still, and significantly increased relative to baseline (Johnson et al., 2022).

Taken together, these data suggest that during *S.* Typhi infection, in vaccinated participants, neutrophils can engage with antibodies via their Fc-receptors, thus contributing to bacterial clearance. Future work characterising the homing markers and specific gene expression profiles of neutrophils, could be used to illustrate the precise localisation of neutrophils during infection, as well as the role of other uncharacterised molecules such as complement. Complement has been shown to enhance both Fc-receptor expression and antibody dependent phagocytosis in neutrophils, and may therefore be relevant for future research (Van Der Maten et al., 2017).

6.1.1 ADNP and ADNOB as a correlate of protection

This study also represents the first assessment of a functional correlate of typhoid vaccine protection measured in endemic settings. Encouragingly, I observed similar ADNP and ADNOB responses using serum from vaccinated Nepalese children, compared with sera from UK adults. These data therefore suggests that the same protection benefited to UK vaccinees, is present in those vaccinated in Nepal.

However, a potential limitation of this interpretation is that neutrophil donors were from healthy adult volunteers. Due to differences in the development of the granulocyte compartment in very young children, nutritional differences, and anaemia (Hellebrekers et al., 2017), the neutrophil effector functions of Nepalese children may behave differently.

Compared with older children and adults, the ability to induce the respiratory burst is typically diminished in children younger than 2 years old (Biasi et al., 1996). In spite of this, a study examining antibody-dependent neutrophil phagocytosis and ROS release using serum and neutrophils from Malawian children infected with iNTS found that neutrophils from these children were able to successfully induce the oxidative burst (Gondwe et al., 2010). Although in infants younger than 6 months, this response was significantly reduced. Nonetheless, ADNP and ADNOB following vaccination are only one component of antibody-induced protection; adequate levels of cellular and humoral immunity are still necessary to mount an effective response.

Following *S.* Typhi challenge, Vi-antibody titres do not significantly increase, unlike LPS antibody titres (Darton et al., 2016). Subsequently, adapting these methods in future using whole *Salmonella* bacteria as a base, could be used to investigate the contribution of other antigen specificities such as LPS (Johnson et al., 2019). Preliminary steps to develop these protocols, have been taken, as well as adapting ADNP and ADNOB assays to investigate Fc-antibody responses against *S.* Typhi bacteria. Given that *S.* Paratyphi also evades the neutrophil respiratory burst during infection (Hiyoshi et al., 2018), it will be interesting to see if *S.* Paratyphi vaccination results in similar responses, if so these may be indicative of a protective immune profile, and support further clinical testing.

6.1.2 The role of IgA in response to typhoid vaccination

Secretory IgA is produced by intestinally derived plasma cells as a dimer and can neutralise enteric pathogens. There is evidence to suggest that both serum and

mucosal IgA may be involved in protective immune responses following typhoid vaccination. Serum IgA responses correlated with protection and Fc-mediated neutrophil activity in participants vaccinated in the UK. Moreover, there is evidence to suggest that serum IgA responses may reflect responses produced at mucosal sites, as circulating IgA+ plasma cells and those obtained in the intestinal mucosal were shown to be clonally related. Indeed, Vi-TCV vaccination reduced *S.* Typhi shedding in stool samples following challenge (Gibani et al., 2019).

Cellular data also suggests a role for antibody localisation at mucosal surfaces. 7 days after Vi-TCV vaccination, gut homing plasma cells expressing CCR10 and $\alpha 4\beta 7$ significantly increase from baseline (Cross et al., 2020). Furthermore, the proportion of $\alpha 4\beta 7$ cells was demonstrated to correlate with ADNP and Vi-IgA levels. Together, these data suggest that following Vi-TCV vaccination, functional antibody responses at the intestinal mucosa are contributing to protection. The association of SNPs within the *integrin* $-\beta 7$ gene and Vi-IgA titres also implicates a mucosal homing pathway following vaccination. Future work investigating the association of Fc-mediated functions induced by mucosal IgA, would be able to elucidate whether parenteral vaccination against an enteric pathogen may be mediating protection at mucosal sites.

6.2 Fc-glycosylation profiles associate with ADNP following challenge

In this chapter, I also presented the first investigation into the relationship between Fc-glycosylation and antibody-dependent neutrophil phagocytosis. Fc-glycosylation is also a relatively new frontier for vaccine development, but it has been shown to influence humoral responses after influenza and HIV vaccination and to be associated with antibody-dependent cellular cytotoxicity (Alter et al., 2018). It was unknown whether Fc-glycan profiles would exhibit the same associations as observed in these studies and monoclonal design. Low levels of sialic acid on

pathogenic IgA antibodies have been associated with inflammatory profiles in rheumatoid arthritis and systemic lupus erythematosus (SLE) (Vattepu et al., 2022). Consequently, I observed similar associations between lower levels of sialic acid and galactose and ADNP, as demonstrated by studies. Future functional studies, such as those that remove sialic acid from post-vaccination serum with enzymes such as neuraminidase, could confirm this association.

6.2.1 Limitations and future work

This study is currently limited in that I did not directly associate ADNOB with Fc-glycosylation. Characterising the impact of Fc-glycan profiles more thoroughly may require additional research into ROS release in neutrophils and other downstream functions, such as NETosis. Lastly, functional antibody assays were conducted with bulk-sera, which may include the contribution of other immunoglobulin subclasses, such as Vi-IgA. By investigating these assays with subclass-specific sera, the antibody subclasses most relevant for mounting ADNP/OB responses could be better profiled.

In addition, I did not evaluate Vi-IgA glycosylation; given the relationship between Vi-IgA and protection, this could be the next direction for this project. In general, antigen-specific IgA glycosylation has not been investigated; therefore, understanding its potential role in antibody-mediated effector functions for a variety of vaccines, such as the glyconjugate *Shigella* LPS vaccine, where serum IgA responses also correlated with protection (Clarkson et al., 2021).

6.3 Genome-wide association study of Vi-Ig titres following typhoid vaccination

6.3.1 Genetic variation in *TNFSF loci* associate with Vi-IgA antibody levels

In the final chapter of this thesis, I analysed the relationship between SNPs with genome-wide significance and Vi-IgA titres. GWAS studies so far have only examined vaccine responses against hepatitis-B, smallpox, *meningococcus*, *haemophilus*, and tetanus-toxin (O'Connor et al., 2019). This study is the first to examine genetic associations with responses to typhoid vaccination, and therefore represents an important contribution to the field. While I did not detect significant overlap between GWAS hits identified in response to typhoid vaccination and those in prior GWAS studies, there are numerous differences between these vaccine cohorts and outcomes. Future work harmonising these studies could be of interest to detect potential pathways that are applicable across vaccine types. Pathway enrichment analyses has recently been used to assess transcriptional responses, both indicative and predictive of responses to 13 types of vaccinations (Fourati et al., 2022). Consequently, integrating typhoid genomic and transcriptomic data with other systems vaccinology data could also uncover broader responses relevant to antibodyoutput following vaccination.

6.3.2 From target gene to function

There are several uses of genome-wide association studies and casual gene detection, the first being the development of genetic risk scores that could be used in the future for genetic screening in vaccination and treatment protocols. However, given the economic limitations to distributing vaccines against enteric fever, it is unlikely that genetic screening will be implemented in the near future, nor were GWAS performed in populations where enteric fever is endemic. Resultantly, this study was not suited

for such an investigation. GWAS in recent years have also be used to uncover novel loci and pathways involved in disease. This approach has been highlighted by the use of GWAS which identified autophagy as an important mediator of Crohn's disease, and neuronal pathways involved in the genetic risk of obesity (Cano-Gamez & Trynka, 2020; Joslin et al., 2021; McGovern et al., 2015).

Using functional genome-mapping I identified loci associated with genetic variation that may be playing a mechanistic role in Vi-IgA production. These loci included variants in the tumour necrosis family genes *TNFSF12* and *13* which encode ARPIL and TWEAK respectively, which are relevant for the regulation in B-cell maturation and antibody production. These SNPs were also associated with differential gene expression 24 hours following vaccination. These SNPs had previously been associated with pathogenic IgA titres, therefore this approach demonstrates the utility of integrating prior GWAS data. A review by Gallagher & Chen-Plotkin (2018), also pointed towards the benefits of using prior GWAS data when informing the search for candidate genes, and such an approach has been taken by GWAS of frontal temporal disorders, which have consistently found associations with *TMEM106B* which has led to the development of novel treatments for these diseases (Nicholson & Rademakers, 2016).

6.3.3 GWAS limitations

The primary limitation of this study was the small sample size, and European-centric demographic. As this study was conducted with individuals of a majority European ancestry, and not our target vaccine cohort, validation of these results in vaccinated individuals within the TyVAC Nepal study could confirm these associations. Recently, trans-ethnic analyses have revealed how variants identified across divergent populations are more likely to be associated with causal genes (Peterson et al., 2019). In this study I did observe that SNP targeted differentially expressed genes, were also differentially expressed in enteric fever patients from

Vietnam. These data therefore indicate that the genes highlighted by this GWAS study are involved in relevant immune pathways are not merely an artefact of the human challenge model, nor restricted to a European-dominated population.

Additionally, I did not specifically investigate genetic variation among participants who were classified as vaccine non-responders, i.e those with very low antibody titres. There is evidence to suggest that the strongest genotype-phenotype associates are found within individuals of an extreme phenotype (Amanat et al., 2020). Identifying individuals at risk of not mounting an effective vaccine response could be used in future vaccine administration programs, and may also highlight relevant pathways involved in poor immune responses to vaccination. Furthermore, I concentrated eQTL and pathway analysis on immune-related genes. However, this approach has the disadvantage of overlooking potential causal loci that may be involved in other pathways such as protein folding, processing or glycosylation. Expanding this analysis with a larger cohort could be used to further characterise relevant genomic and transcriptomic responses in vaccination. Future research could also examine the impact of more complex genotype models, such as those incorporating epistatic interactions.

6.3.4 Potential applications of functional genomics

Lastly, I ensured that all stages of the analysis could be easily applied to other data sets by making the necessary code publicly accessible. Recently, I applied the described pipeline to a genome-wide association study (GWAS) investigating susceptibility to respiratory syncytial virus (RSV) infection (data unpublished). The target genes of SNPs with suggestive significance were also involved in actin remodelling pathways that are known to play a role in viral budding and secretion. Inhibition of a Rho-GTPase identified by the GWAS was shown to reduce viral titres in inhibition assays (Malhi et al., 2021), supporting the utilisation of functional genomics when mapping disease variants. Although online tools such as FUMA

and SNP Nexus provide easy access to numerous databases (Watanabe et al., 2017), they are not integrated with essential pre-processing steps such as quality control and imputation, nor do they provide the option to associate user-entered gene-expression data. By completing this pipeline in an accessible Docker container, those with beginner to intermediate R knowledge could investigate their own GWAS hypotheses using this approach.

Overall, I hope to have highlighted the use of a functional-GWAS approach to investigate potential candidate SNPs, bridging the gap between genotype and phenotype. This is a relatively novel method for analysing GWAS data, but it offers numerous translational avenues to explore. Specifically, this method could be used for other smaller studies with genotyping data, which is especially relevant for studies of infection and vaccination, which compared with GWAS of other polygenic traits tend to be of a smaller magnitude.

6.4 Conclusions

A number of factors, including genetic and environmental, must be considered when examining the immune response to vaccination. What is promising is that typhoid conjugate vaccines tested are showing high efficacy rates across diverse populations, and are being prioritised in areas where incidence and antimicrobial resistance is high (Birger et al., 2022; Shakya et al., 2021). However, estimates of vaccine distribution suggest that it could take up to 50 years for typhoid vaccines to reach every country with endemic disease. Therefore, it is likely that several vaccines will be required if we are to successfully limit the spread of disease. Currently at least 7 typhoid-conjugate vaccines are in clinical or pre-clinical testing (Vashishtha & Kalra, 2020). Furthermore, a vaccine against *S*. Paratyphi, which causes approximately 10,000 deaths per year, is not yet licensced.

Alongside interventions such as novel diagnostics, antimicrobials, and a continued effort to improve access to clean water and sanitation, vaccines are critical for tackling disease. Therefore, I hope that the methods and approaches described in this thesis could be used to evaluate immunogenicity of future vaccines, helping to reduce the toll of enteric fever.

A

Appendix

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A.1 Introduction

Below is a summary of all Genome-wide association studies of susceptibility towards enteric fever (*S.* Typhi and *S.* Paratyphi blood culture confirmed cases). The summary is up to date as of December 2022.

Gene	Variant	Consequence	Significance + 95% CI	Population	Method
TLR4 Chr 9	Missense mutation **Thr399Ile (C/T)** **Asp299Gly (A/G)**	Increased and decreased susceptibility to typhoid fever	P= 0.039; OR 2.309 (1.019-5.23) P = 0.012; OR 0.714; (0.582 - 0.877)	Kelantan, Malaysia (n=304) typhoid fever cases(n=250) healthy controls	Allele-specific PCR
VAC14 Chr 16	Single-nucleotide Polymorphism **rs8060947**	Increased susceptibility to *S.* Typhi invasion of cell lines and typhoid fever	P=0.01; (infection) OR=1.38 (allelic model) OR=3.60 (recessive)	Ho Chi Min, Vietnam (n=496) typhoid cases, (n=500) matched controls	Allele-specific PCR
PARK2 Chr 6	Single-nucleotide polymorphism **PARK2 e01(T-2599)**	Increased susceptibilityto enteric fever	P= 0·02 OR: 1·51 (1·02-2·23)	Jakarta, Indonesia (*n* = 90) typhoid cases (*n* = 26) paratyphoid cases (*n* = 337) fever controls (*n* = 322) healthy controls	Allele-specific PCR
CFTR Chr7	Polymorphic dinucleotide repeats and SNP variants in ***CFTR*** gene **Poly-T *CFTR*** gene variants	Susceptibility to enteric and typhoid fever Protection against enteric fever	P = 0.003 EF OR=2.1 (1.3-3.3) P = 0.0001 TF OR=2.6 (1.6-4.3) P <0.1 OR 0.57 (0.37-0.87;)	Jakarta, Indonesia Enteric fever GWAS study	Allele-specific PCR
***HLA* Class** II TNF*α* loci Chr 6	**HLADRB1*0301/6/8** + **TNFA*2 (-308)** haplotype ***HLADRB1*04:05*** + ***TNFA*1 (-308)*** haplotype	Susceptibility to typhoid fever Resistance to typhoid fever	P = .0066; OR, 6.416 (1.4-29.0) P = .0003 OR, 0.084 (0.02-0.36)	Dong Thap, Vietnam (n=113) typhoid fever cases (n=77) healthy controls	Allele-specific PCR
HLA Class II	SNP - **rs7765379** LD with **HLADRB1*04:05**	Resistance to enteric fever	P = 1.3 × 10-4 OR = 0.18 P = 2.60 × 10-11 (HLADRB1) OR = 0.14 P =0.0007 (HLADRB1) OR = 0.34	Ho Chi Minh City and Dong Thap,Vietnam (n=432) typhoid cases (n=2011) controls Kathmandu, Nepal (n =402) typhoid cases (n= 188) paratyphoid cases (n=386) heathy controls	Genome-wide genotyping followed by Exome-wide genotyping (Illumina) Imputation of East-Asian HLA alleles (SNP2HLA)
***HLA Class II TNF α* loci**	**TNFA*2 (-308)**	Severity of disease	OR 4.42 (1.11-38.41) OR 2.87 (1.00-11.28) P <0.05 (TF, PF) only when compared with fever controls	Jakarta, Indonesia Enteric fever GWAS study	Allele-specific PCR
HLA Class I	**HLA-B*27:05**	Subtle increase in susceptibility to enteric fever Increased CFU/ml	P= 0.011 OR = 1.04 (1.01-1.09) P = 0.02	Oxford, U.K, CHIM S. Typhi and S. Paratyphi (n= 154) enteric fever cases (n=122) no enteric fever diagnosis controls	Genome-wide genotyping (n=173) (Illumina) RNA-sequencing (n=174) HLA imputation (SNP2HLA or HISAT)
CAPN14 *Chr3*	Single-nucleotide polymorphism **rs4952069**	Increase in susceptibility to enteric fever	P = $<10^{-5}$ OR = 5.8	Oxford, U.K, CHIM study	Genome-wide genotyping (n=173) (Illumina)

Table A.1: Summary of previously conducted GWAS studies of enteric fever and associated genes and risk alleles.

A.2 General Methods

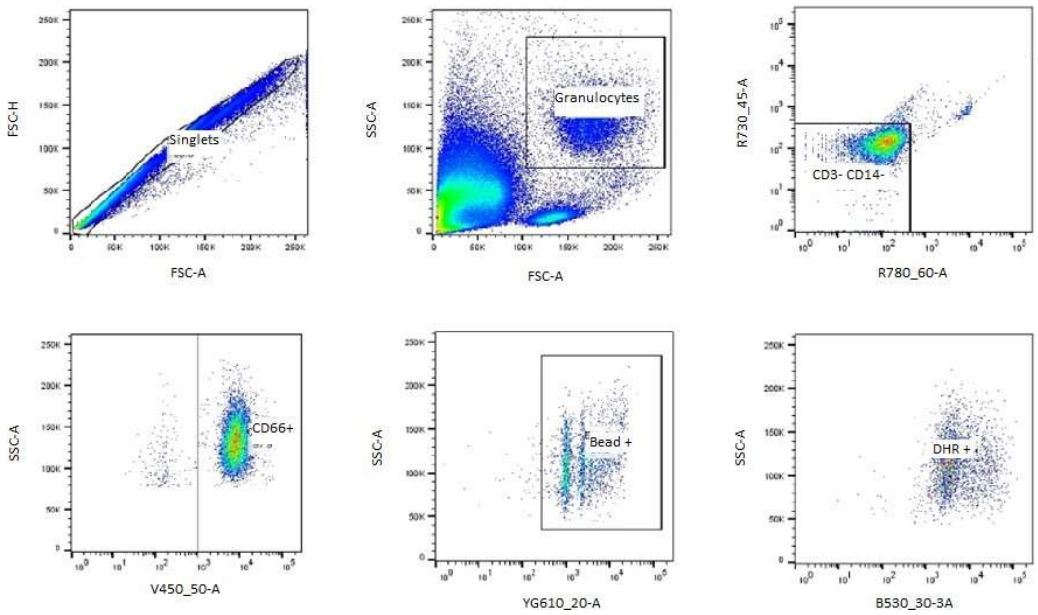


Figure A.1: Gating of neutrophils within ADNP and ADNOB assays Neutrophils following red-blood cell lysis and incubation with Vi-positive serum, were examined for phagocytosis and ROS release. Granulocytes were first gated using forward and side scatter, followed by a neutrophil positive gate CD66 +, and a CD14 negative gate. These cells form the neutrophil population of which uptake of bead + cells is assessed by the MFI in the YG610-20 channel, and DHR fluorescence is measured by the MFI of the B530-30 channel.

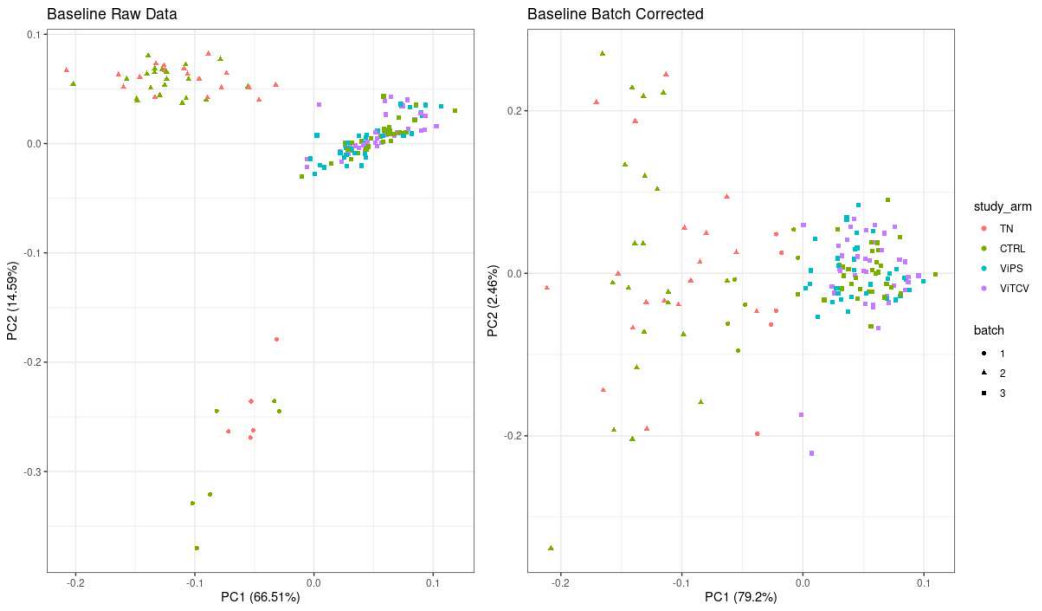


Figure A.2: Combat-seq batch correction RNA-seq count data was merged between sequencing batches using the Combat-Seq package in R. Plots shows the principal components of variance (PCA) before and after batch adjustment. Each point represents an individual sample at baseline (n = 101). Shapes denote the batch whilst colours indicate the study arm.

A.3 Development of ADNP and ADNOB assays

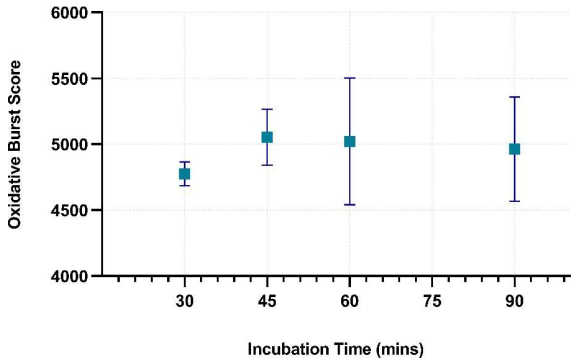


Figure A.3: Time course of DHR and neutrophil incubation times during ADNOB assay Neutrophils from healthy blood donors were isolated using red-blood cell lysisis. Cells were incubated between 30 and 90 minutes with a Vi + serum standard, Vi-antigen coated beads, and DHR, in order to measure ADNOB. DHR fluorescence was measured using flow cytometry and CD66b+ and CD3- gated neutrophils. Depicted are mean oxidative burst scores of the serum standard measured following different incubation times in the ADNOB assay. Error bars depict the standard error of the mean (SEM) between technical duplicates.

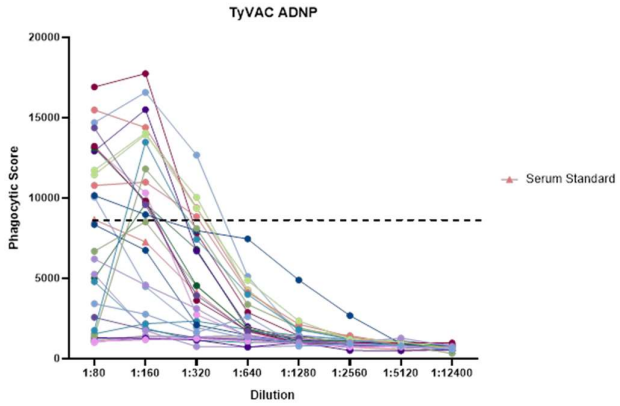


Figure A.4: Serial dilutions of plasma from Vi-vaccinated children in the ADNP assay Plasma samples (n = 23) were serially diluted before being used in the antibody-dependent neutrophil phagocytosis assay (ADNP). Phagocytosis was measured using the MFI of fluorescent beads, multiplied by the number of bead positive cells to give a phagocytic score (y axis). Depicted are the mean phagocytic scores of technical duplicates across serum dilutions (x axis).

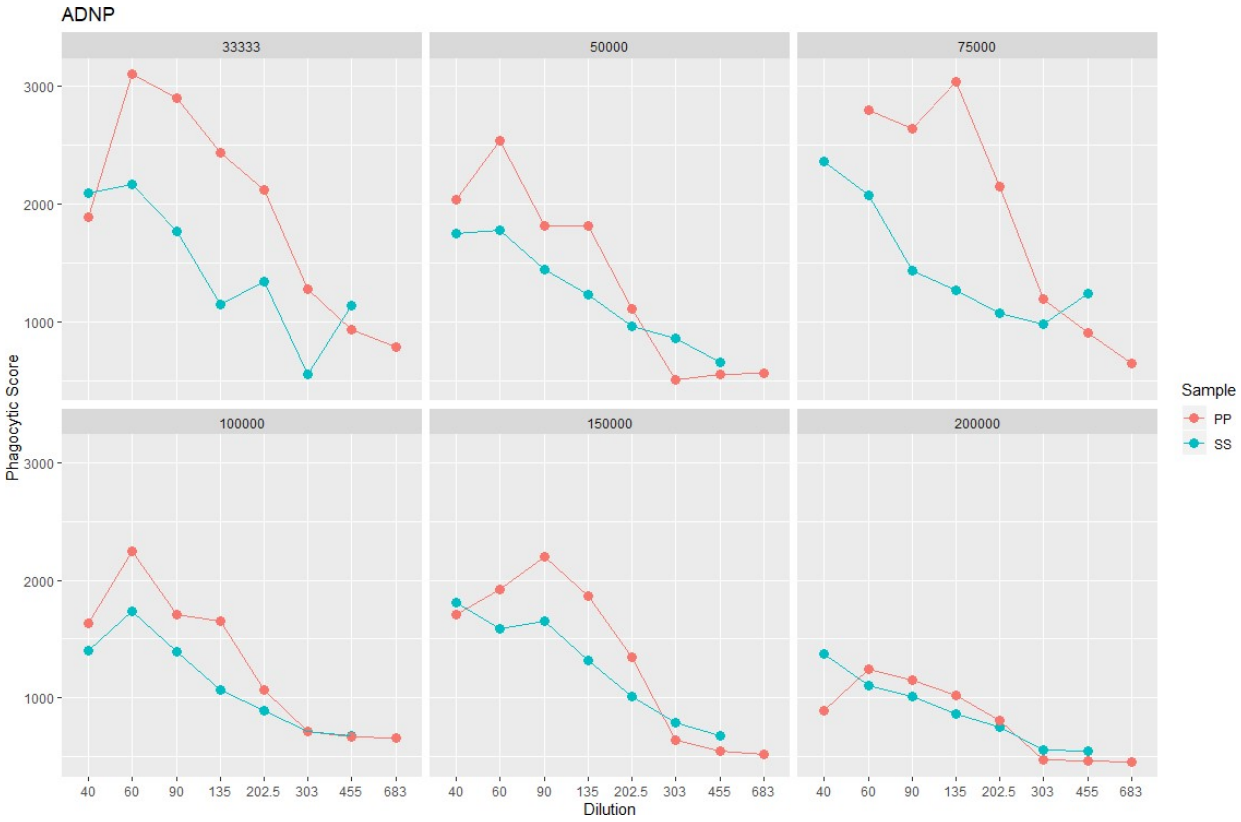


Figure A.5: Phagocytic Scores of the Serum Standard and Positive Pool across different cell concentrations The Vi-serum standard (SS) and Vi-positive pool sera (PP) from Vi-TCV and Vi-PS vaccinated healthy adults in the UK were serially diluted and run in singlicate in the ADNP assay. Y axis denotes the phagocytic score, and serum dilutions are spread across the x-axis. Different cell concentrations of leukocyes following red-blood cell lysis were added to the ADNP assay and are indicated by the plot titles (number of cells per well).

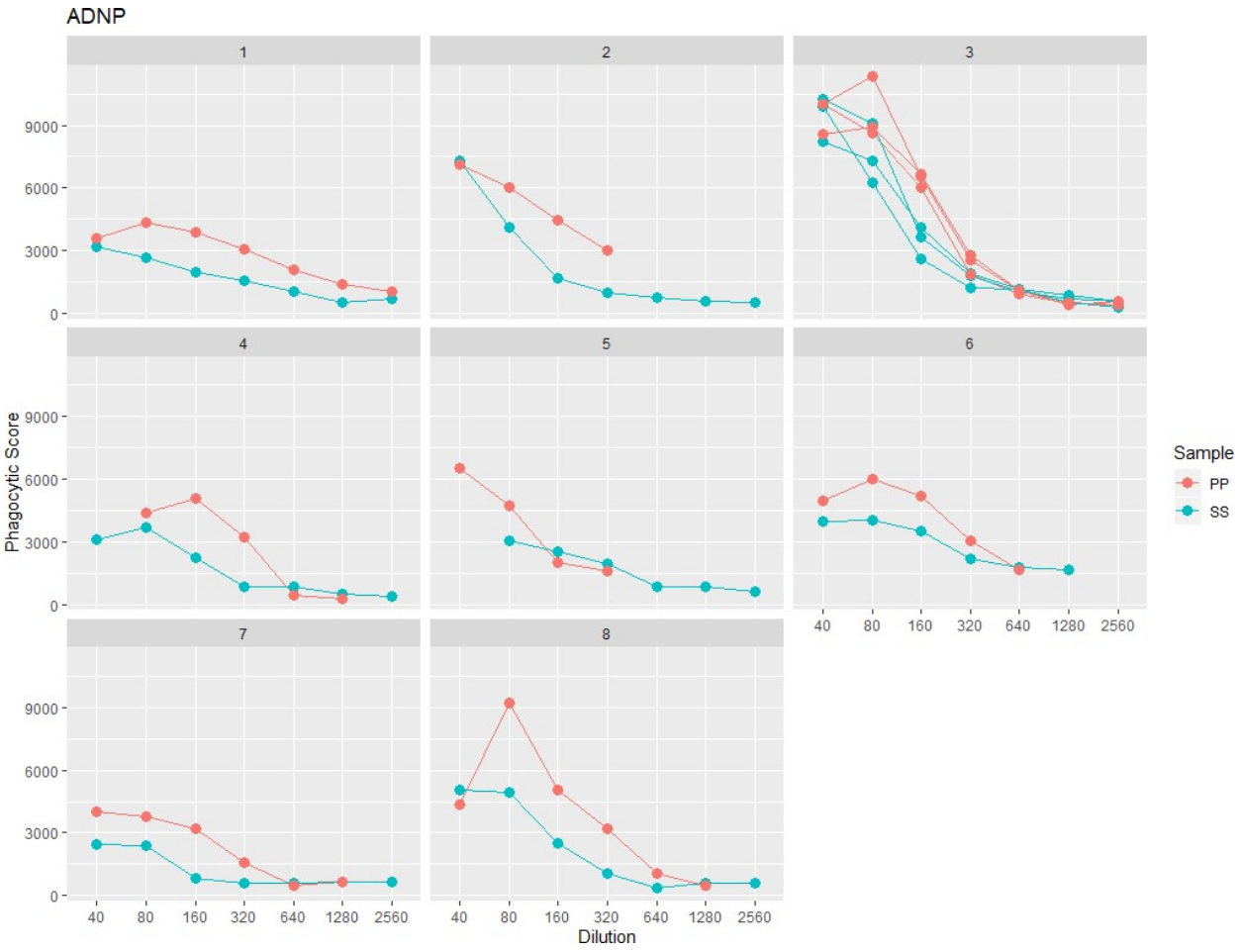


Figure A.7: ADNP assay performance across experiments Serial dilutions of the Vi serum standard (SS) and Vi-positive pool (PP) were used across 8 independent ADNP experiments. Each sample was measured in technical duplicate, and presented are the mean phagocytic scores. Experiment 3 consisted of 3 different neutrophil donors run within the same experiment.

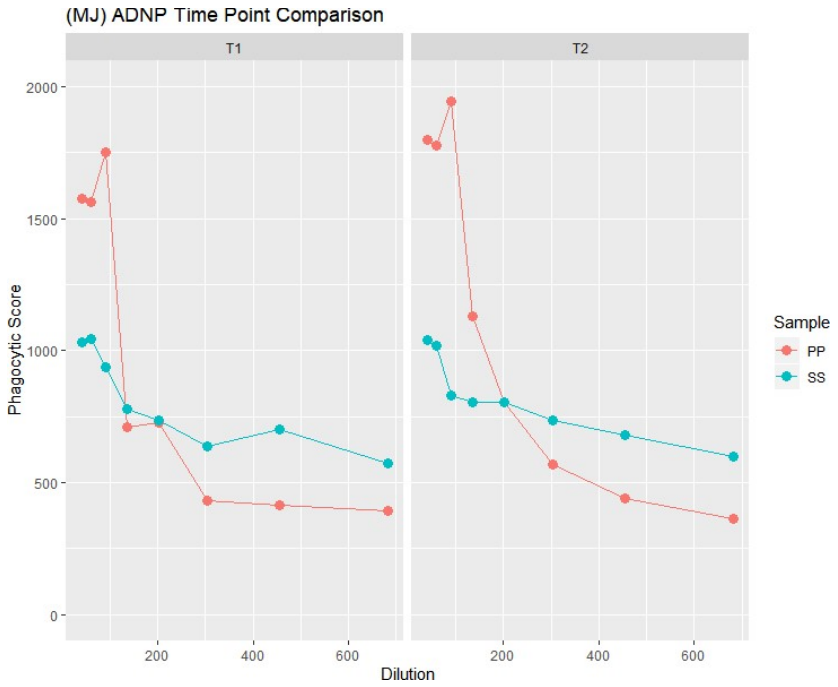


Figure A.8: Comparison of phagocytic scores of the serum standard and positive pool between blood resting time. Phagocytosis was measured in the ADNP assay following either a 1 hour (T1) or 4-hour (T2) incubatory period of whole blood samples prior to red blood cell lysis for both the serum standard (SS) and positive pool (PP). Shown are the mean phagocytic scores for each serum sample, across dilutions.

A.4 Fc-mediated neutrophil effector functions following typhoid vaccination

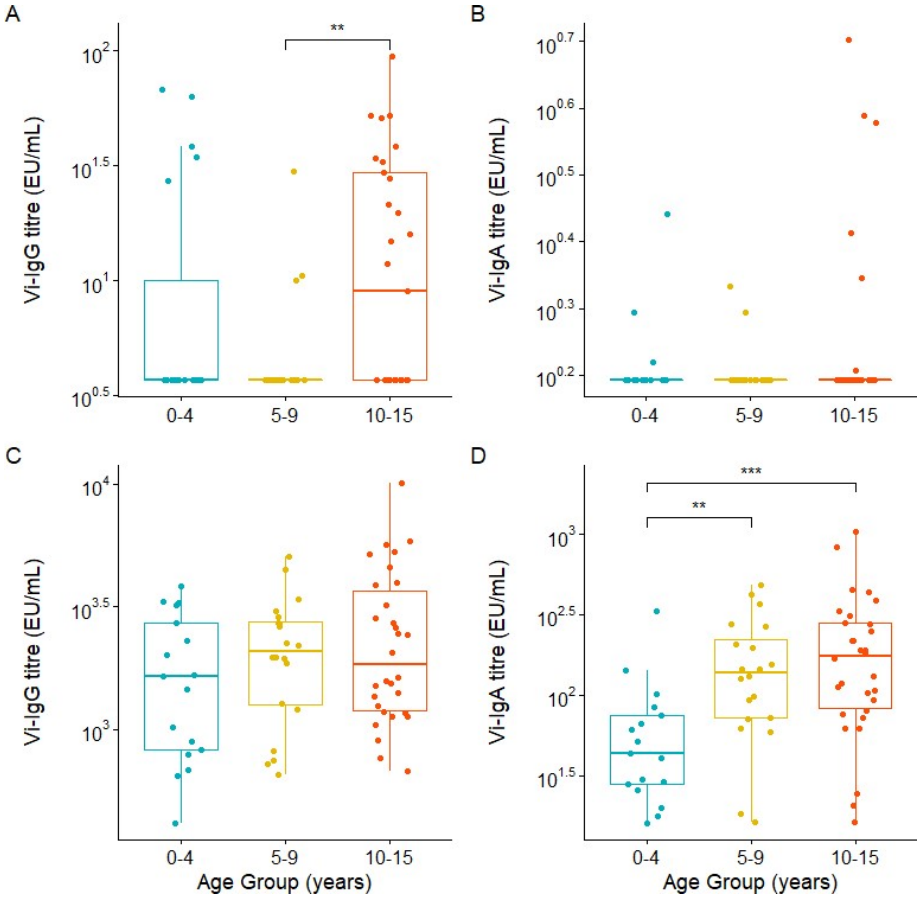


Figure A.9: Vi-IgG and Vi-IgA titres pre and 28 days post-vaccination in Nepalese children A-B) Vi-IgG and Vi-IgA titres were measured across age groups of children aged 0-4 (n = 21), 5-9 (n = 24) and 10-15 years of age (n = 32) in Nepal prior to Vi-TCV vaccination and 28 days following Vi-TCV vaccination (C–D). Box plots display the median and interquartile range for each age group. Differences in ELISA titres between age groups were compared using the Wilcoxon test. (∗∗∗ = $p < 0.001$, ∗∗ = $p < 0.01$).

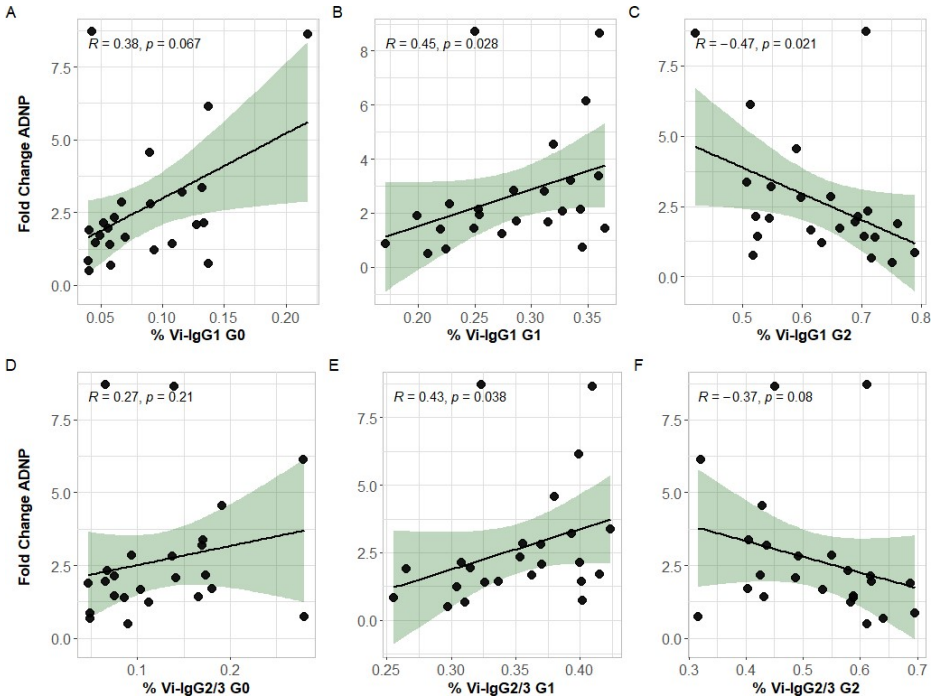


Figure A.10: Fc galactosylation states of Vi-IgG1 and VI-IgG2 with ADNP fold change 28 days following Vi-TCV vaccination A-C) Correlation of the percentage of agalactosylated (G0), monogalactosylated (G1), and digalactosylated (G2) Vi-IgG1 antibodies at D28 with Vi-ADNP phagocytic score between baseline and D28, respectively (n = 24). D-E) Correlation of the percentage of agalactosylated (G0), monogalactosylated (G1),and digalactosylated (G2) Vi-IgG2-3 antibodies at D28 with Vi-ADNP phagocytic score between baseline and D28, respectively. Shown for each graph are the Spearman's Rho, and P-values for significance. The black line shows the linear regression, and the shaded area shows 95 confidence intervals for the model.

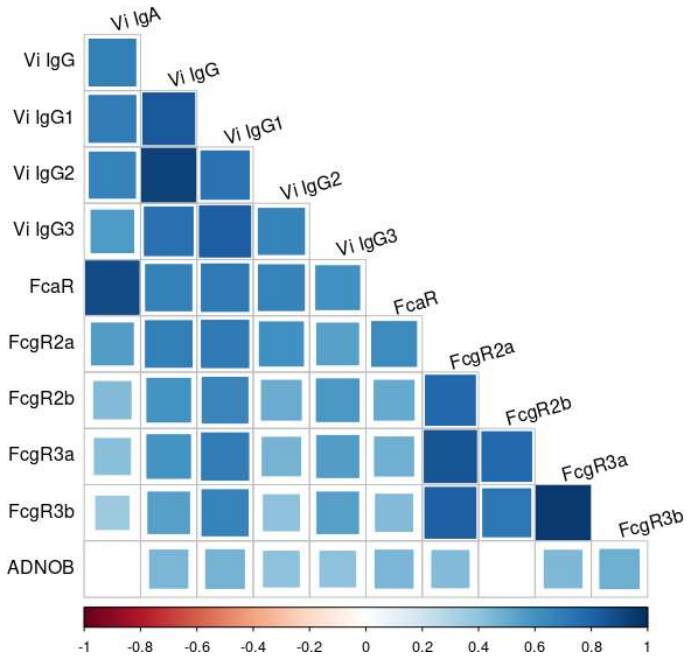


Figure A.11: Correlation of ADNOB with Vi-antibody titres, and Fc-receptor binding 28 days following Vi-TCV or Vi-PS vaccination Pairwise Spearmans rho was used to determine the correlation between antibody measures and ADNOB measured 28-days following VipTCV (n = 37) or Vi-PS (n = 35). The size of each tile is relative to the significance of the correlation, and only significant correlations after adjusted for multiple testing (Bonferroni) are displayed.

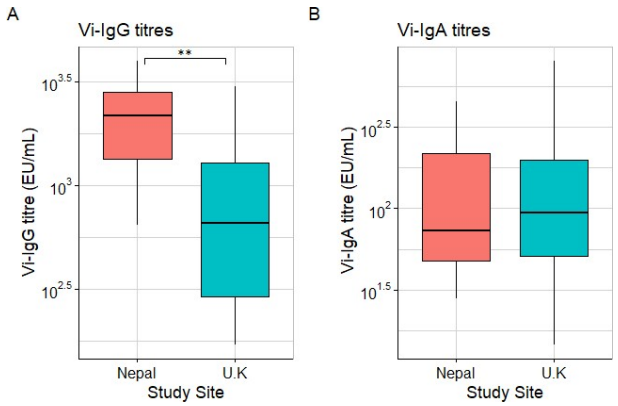


Figure A.12: Vi-IgG and Vi-IgA titres in Nepalese children and U.K adults 28 days following Vi-TCV vaccination Vi-IgG (A) and Vi-IgA (B) titres were measured in plasma from participants in the TyVAC Nepal study (n = 17) and UK adults (n = 16) 28 days post Vi-TCV vaccination. Box plots show the median and interquartile range per group. Differences in ELISA titres between study sites were compared using the Wilcoxon test ($**P < 0.01$).

A.5 GWAS of enteric fever and Vi-vaccination antibody responses

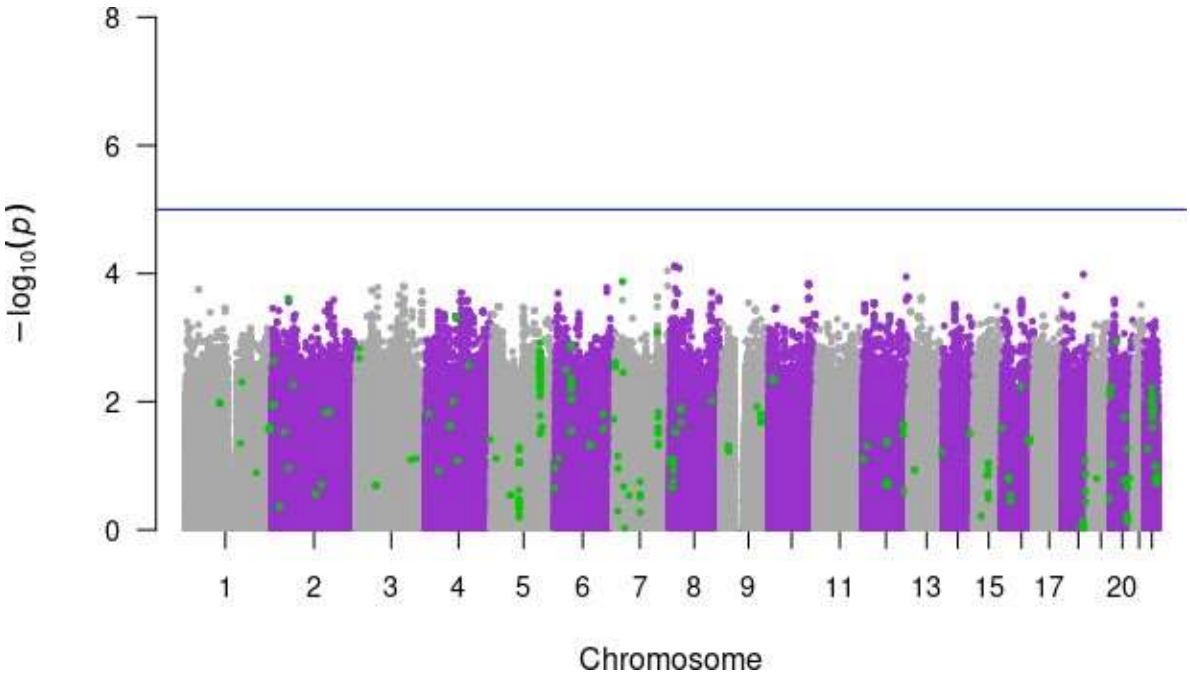


Figure A.13: Preliminary genome-wide association study of enteric fever Participants were challenged orally with S. Paratyphi and monitored for clinical symptoms or microbiological detection (n = 78). The Manhattan plot displays the significance of individual SNPs with the likelihood of receiving a paratyphoid diagnosis. Highlighted in green are the most significant variants associated with typhoid fever following S. Typhi challenge. The line shows the suggestive significance threshold of $P < 1 \times 10^{-4}$.

A.5.1 Genetic susceptibility to typhoid fever

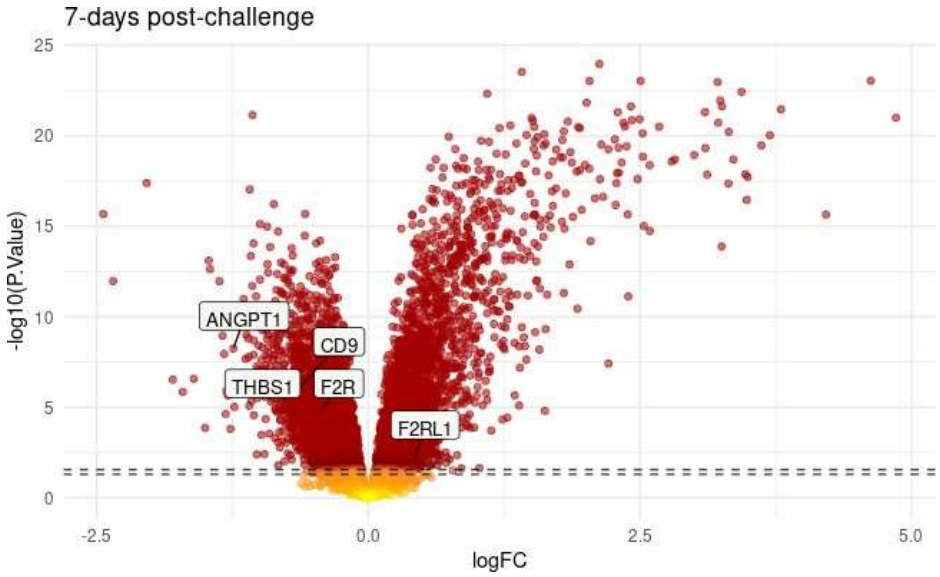


Figure A.14: Differential gene expression between diagnosed TD and non-TD participants Following quality control, RNAseq data was measured between baseline and 7-days following S. Typhi Challenge (n = 49). The volcano plot illustrates the significance of the differential expression of each gene, estimated by Limma, and highlighted are genes involved in platelet formation and coagulation.

A.5.2 GWAS of typhoid vaccine responses

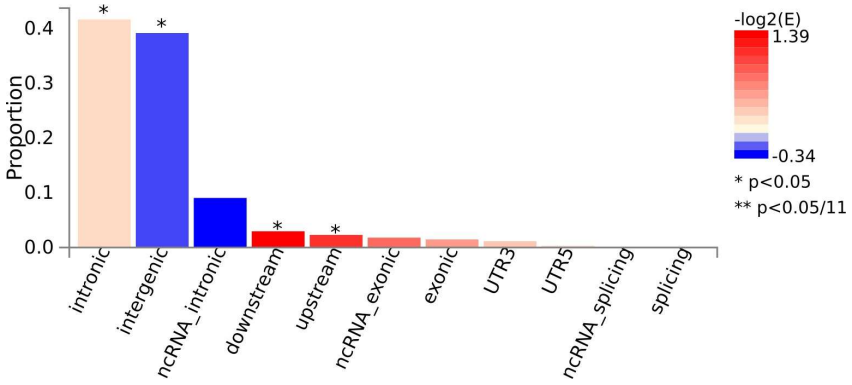


Figure A.15: Features of top SNPs in GWAS of typhoid fever Top GWAS Variants ($P < 1\times 10^{-4}$) were annotated using the Annovar database. Features labelled with * indicates significant enrichment ($adj.P < 0.05$) in our data compared with random sampling.

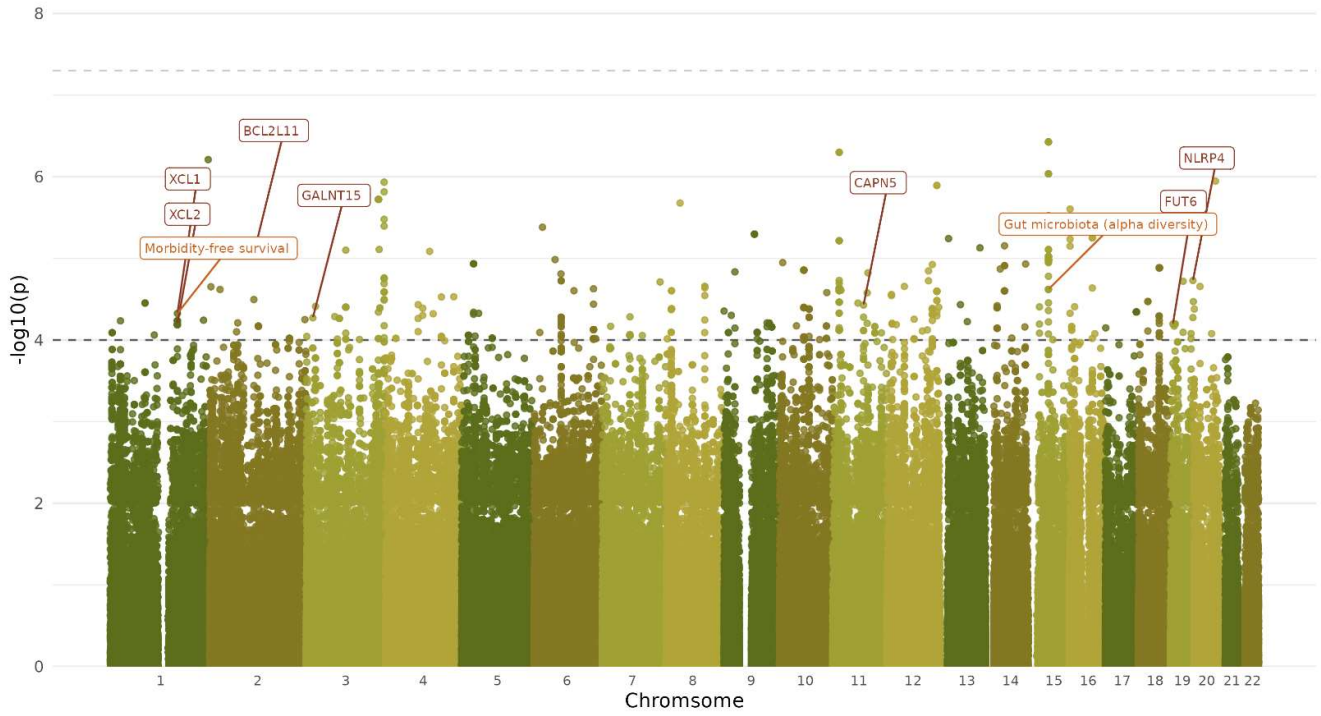


Figure A.16: GWAS of Vi-IgG responses 28 days following vaccination 28-days following vaccination with either Vi-TCV or Vi-PS, Vi-IgG antibody tires were measure by ELISA. The dashed line represents the suggestive significance used in the study ($P < 1 \times 10^{-4}$), highlighted are genes involved the immune response ($n = 72$).

A.5.3 GWAS summary tables

Study Cohorts

Parent study	Strain	Conditions	Genotyping platform and batch	nTD/TD	GWAS n
VAST (Vi-vaccination study)	*S.* Typhi Quailes	Placebo, Vi-PS,Vi-TCV (1:1:1)	Affymetrix GSA V.3 (3)	51/45	96
PATCH (re-challenge)	*S.* Typhi Quailes, S. Paratyphi A	Heterologous re-challenge vschallenge	Affymetrix GSA V.3 (3)	9/15	24
TyGER (Typhoid toxinstudy)	*S.* Typhi Quailes, Typhoid-toxin KO	WT v TN-(1:1)	Affymetrix GSA V.1 (2)	9/26	35
T2 (Typhoid oralvaccine trial)	*S.* Typhi Quailes	Placebo, MZH09 Ty21a(1:1:1)	Illumina OmniExpress-24 v1.0 (1)	31/36	67
T1 (Typhoid-Dose Escalation)	*S.* Typhi Quailes	High Dose Low Dose	Illumina OmniExpress-24 v1.0 (1)	8/18	26
Total (n)				108/140	248

Table A.2: Enteric GWAS cohorts

Study	Strain	Conditions	RNA-seq platform	TD /nTD	GWAS (n)	DGE (Baseline)	DGE (D7-TD)
VAST (Vi-vaccination study)	*S.* Typhi Quailes	Placebo, Vi-PS, Vi-TCV (1:1:1)	Illumina HiSeq 200 V4 Wellcome TrustSanger-Institute	51/45	96	50	24
TyGER (Typhoid toxin study)	*S.* Typhi Quailes, Typhoid-toxin KO	WT v TN-(1:1)	Illumina HiSeq 200 V4 BDI	9/26	35	31	25
Total (n)					101	81	49

Table A.3: Enteric RNA-Seq cohorts

Vi-IgA GWAS-DEGs

dbSNP	Chr	Ref	Alt	p.GWAS	eQTL.Gene	Tissue	p.eQTL	Log2Fc	adj.p. DGE
rs112504005	17	C	CA	8.7e-06	TNFSF13	Fairfax 2014 naive	1.25e-13	0.148	0.0260
rs73345419	14	C	T	2.8e-05	WARS	EQTLGen-Cis	1.26e-05	0.312	0.0261
rs11078693	17	A	G	7.16e-05	ASGR2	EQTLGen-Cis	1.19e-05	0.247	0.0121

Table A.4: SNPs detected in the GWAS of Vi-IgA 28-days post-vaccination with a $P\ value < 1e^{-4}$ were examined for previously published eQTL associations. eQTL data was filtered based on an $FDR < 0.05$, and displayed are the raw p-values. Genes identified with prior eQTL associations were examined for differential gene expression from baseline to 24h post-vaccination. DEG *P*-values were adjusted using the Benjamini-Hochberg correction.

Typhoid GWAS-DEGs

dbSNP	Chr	Ref	Alt	p.GWAS	eQTL.Gene	Tissue	p.eQTL	Log2Fc	adj.p.DGE
rs6025970	20	C	T	$7.9e-05$	PPP4R1L	Whole Blood	0.00062	0.4	$4.3e-05$
rs9302118	15	T	C	$2.1e-05$	SPG11	Macrophage Salmonella	0.00088	0.35	$6.5e-07$
rs9302118	15	T	C	$2.1e-05$	GATM	Macrophage IFNg	$2.1e-16$	−0.53	0.0027
rs2138063	12	T	C	$5e-05$	PLEKHA5	SalmonellaeQTLGen cis	$3.4e-14$	−0.67	$8.5e-07$
rs3001124	9	C	G	$7.7e-06$	TXN	eQTLs Macrophage IFNg	$9.5e-06$	0.49	$1.4e-05$
rs2546161	5	G	A	$3.9e-06$	ZBED3	SalmonellaB-cell CD19	0.00015	−0.9	$4.6e-18$
rs2546161	5	G	A	$3.9e-06$	WDR41	BIOS eQTL	$7.6e-11$	0.53	$7.9e-14$
rs2546161	5	G	A	$3.9e-06$	F2RL1	T-cell CD4	0.00021	0.6	$7e-05$
rs3770115	2	G	A	$6.1e-05$	ITGA4	eQTLGen cis eQTLs	$2.6e-89$	0.43	0.015

Table A.5: SNPs detected in the GWAS of typhoid fever with a $P\ value < 1e^{-4}$ were examined for previously published eQTL associations and differential gene expression between participants who were diagnosed with typhoid versus undiagnosed, from baseline-D7 or time of diagnosis. Displayed are the top ten GWAS-eQTL variants, along with the associated eQTL. eQTL data was filtered based on an $FDR < 0.05$, and displayed are the raw p-values. Gene gene expression DEG *P*-values were adjusted using the Benjamini-Hochberg correction.

CHIM Study Name	Publication
VAST	Efficacy and immunogenicity of a Vi-tetanus toxoid conjugate vaccine inthe prevention of typhoid fever using a controlled human infection model
PATCH	Homologous and heterologous re-challenge with Salmonella Typhi and Paratyphi in a randomised controlled human infection model
TyGER	Investigation of the role of typhoid toxin in acute typhoid fever in a human challenge model
T1	An outpatient, ambulant-design, controlled human infection model using escalating doses of Salmonella Typhi challenge
T2	Using a Human Challenge Model of Infection to Measure Vaccine Efficacy -A Randomised, Controlled Trial Comparing Typhoid Vaccines M01ZH09 with Placebo and Ty21a

Table A.6: Summary of enteric human challenge studies and publication

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