# Safety and Immunogenicity of the iNTS-GMMA Vaccine

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# COVID-19 Impact Statement

My DPhil project is based around the safety and immunogenicity of a novel vaccine against invasive non typhoidal Salmonella . Whilst my planned project was originally to include the safety and immunogenicity clinical trial data from day 0 to 350, after both primary course (first and second vaccine) and booster (third vaccine), the COVID-19 pandemic significantly delayed the start of the SALVO (Salmonella Vaccine Study in Oxford) trial and necessitated a change in the structure of my thesis to accommodate the subsequent time constraints. In particular, the COVID-19 pandemic resulted in a delay in vaccine manufacture, review of study materials by Research Governance, Ethics & Assurance Team as COVID-19 studies were prioritised which led to a subsequent delay in regulatory approvals required for study initiation. As a result, study enrolment finally began in May 2022, with a delay of up to 18 months from the initial project start date.

During the COVID-19 pandemic I formally suspended my DPhil for two terms. In the first of these terms, I was a clinical research fellow on the COVID-19 vaccines studies (COV01, COV02 and COV04) working on both set-up, study documents and as a study doctor. In the second suspended term I returned to clinical practice at Charing Cross Hospital working as an Infectious Diseases ST7 during the second COVID-19 pandemic wave.

Prior to study initiation I worked on study documentation and set up of the iNTS-GMMA vaccine clinical trial in addition to the development and optimisation of assays including the GMMA-based ELISA, functional antibody assays and T cell proliferation assays. Given the delay in study initiation I began parallel projects examining the active GMMA vaccine

components. The first of these projects was a proteomic analysis of the GMMAs and predicted immunogenicity of GMMA proteins. This project forms the basis of Chapter 5 of my thesis. Identified proteins were produced and tested with early iNTS-GMMA vaccine samples to confirm immunogenicity. This work forms sections of Chapter 6 and 7. The second project was to identify if cross-reactive antibody and T cell response to the GMMAs were present across *Salmonella* serovars using samples from *Salmonella* challenge studies at the Oxford Vaccine Group. This project forms sections of Chapter 6 and 7. With the initiation of the SALVO study I began my primary project. However, due to the delay my data points were able to progress up to D84 after the last participant to be vaccinated and included safety points and sample collection up day 28 after the primary vaccination course (second vaccine). I then processed these samples over a wide range of assays (serum IgG and IgA GMMA ELISA, oral fluid IgA GMMA ELISA, GMMA-based and OAg-based ADMP, GMMA-based ADNKA, GMMA-based T cell proliferation assays) that I had developed forming the basis of Chapter 4 of my thesis. The collection of SALVO samples allowed me to develop the proteomics project with confirmatory antibody and T cell experiments, as well as an investigation of immunodominant and cross-reactive antigens.

To date my work has resulted in one first author openBMJ publication (*Salmonella Vaccine Study in Oxford (SALVO) trial: protocol for an observer-participant blind randomised placebo-controlled trial of the iNTS-GMMA vaccine within a European cohort*[1], first author, Chapter 2.2 and 3.2), a first author poster presentation (*Initial Proteomic Analysis of the Active Component of the iNTS-GMMA Vaccine Candidate*, *VASE 2022, USA,* Chapter 5.3) and an oral presentation at an international conference (*Initial safety results from the*

*SALVO trial investigating the iNTS-GMMA vaccine*, *first author/presenter, ECCMID 2023, Copenhagen*, Chapter 3.3), planned first author publications based on chapters in this thesis and the results of the SALVO clinical trial. The iNTS-GMMA vaccine safety data from Chapter 3.3 was also included along with immunogenicity data *not* included in this thesis in an international presentation for which I am one of the authors, titled: *Initial immunogenicity and safety data from a first-in-human randomised controlled trial of an invasive non-typhoidal Salmonella GMMA vaccine: the SALVO trial, Typhoid & Other Invasive Salmonellosis, Kigali, Rwanda 2023*.

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# Abstract


## Safety and Immunogenicity of the iNTS-GMMA Vaccine

Invasive non-typhoidal *Salmonella* (iNTS) is a common cause of bacteraemia in sub-Saharan Africa, where it particularly affects children under five. In this thesis, I outline SALVO (*Salmonella* Vaccine Study in Oxford), a first-in-human phase I randomised placebo-controlled trial investigating the safety and immunogenicity of the iNTS-GMMA vaccine at two dose levels, full dose (40μg O-Antigen) and lower dose (10.6μg O-Antigen) over a three-dose schedule at month 0, 2 and 6. The vaccine is based on outer membrane vesicles (OMVs) from *Salmonella* Enteritidis (SEn) and *Salmonella* Typhimurium (STm), which have been genetically modified to increase the production and reduce the toxicity of these OMVs (called Generalised Modules of Membrane Antigens, GMMAs).

The full dose iNTS-GMMA vaccine exhibited an acceptable safety profile up to 28 days after the second vaccination within the limited time points and blinded data assessed. Confirmation of the safety of the vaccine is pending remaining time points and assessment of unblinded data. The full dose iNTS-GMMA vaccine elicited significantly elevated SEn/STm GMMA immunogenicity at day 14-84 following the first vaccination compared with baseline and placebo. All SEn/STm GMMA, IgG and IgA, antibody-dependent monocyte phagocytosis (ADMP), GMMA specific CD3+CD4+ and CD3+CD8+ memory T cells were significantly elevated at day 28 after the first vaccination compared

with baseline. Sera obtained following *S.* Typhi and *S.* Paratyphi infection contained functional antibodies (ADMP) to SEnGMMA and STmGMMA. Likewise, the iNTS-GMMA vaccine stimulated functional antibodies (ADMP) to *S.* Paratyphi. O-antigen was the immunodominant antigen within GMMAs to sera following natural infection, iNTS-GMMA vaccination, *S.* Typhi and *S.* Paratyphi infection.

Overall, I demonstrate that the full dose iNTS-GMMA vaccine appeared well tolerated (within the limited blinded data assessed) and stimulates a significant multi-faceted immune response up to day 28 following the second vaccination, suggesting the continued advancement of a potential new vaccine against iNTS.

# Introduction

## 1.1 Introduction

Non-typhoidal *Salmonella* (NTS) is an enteric pathogen endemic throughout the world. The Global Burden of Disease (GBD) study estimated 95.1 million cases of NTS gastroenteritis in 2017[2]. Faecal-oral transmission via contaminated food and water is the cause in most instances of infection. Clinical manifestations of gastroenteritis include fever, diarrhoea and abdominal discomfort, in contrast to typhoidal *Salmonella* (*S.* Typhi and *S.* Paratyphi), the cause of enteric fever, which is characterised by a severe systemic illness with 14.3 million cases and 136,000 deaths per year[3]. However, circumstances can arise in which NTS disseminate beyond the gut into the systemic circulation leading to an invasive disease similar to enteric fever called invasive non-typhoidal *Salmonella* (iNTS). Investigating these circumstances has been the mainstay of epidemiological and laboratory research focused on iNTS and remains a crucial step towards developing an effective vaccine. Compared with NTS gastroenteritis and enteric fever, the number of cases of iNTS is fewer at 535,000 per year[2]. However, with 77,500 deaths yearly, the case fatality rate of iNTS exceeds that of enteric fever[2 3].

The burden of iNTS in sub-Saharan Africa, including the association with the under-five age group, HIV, malaria, sickle cell and other immunodeficiencies, has been well described[4-6]. However, the aetiology of these associations continues to be investigated[5]. The emergence of a virulent *Salmonella* Typhimurium ST313 and antimicrobial resistance

are important factors to consider explaining the cause of the comparatively high disease burden in sub-Saharan Africa representing 78.8% of all global cases[2].

Whilst the benefit of a vaccine is clear, there are several obstacles to overcome, not least the lack of an immunological correlate of protection and ambiguity over the immunodominant antigen[7]. By accelerating an iNTS vaccine, data may be garnered for future vaccines and immunology research.

This chapter aims to provide an overview of iNTS, including epidemiology, risk factors and the rationale and immunological basis for a vaccine for this disease. Finally, it outlines the current progress of vaccine development with an emphasis on the iNTS-GMMA vaccine, one of the first vaccine candidates to progress to phase I clinical trials and the core component of this thesis.

## 1.2 Epidemiology

### 1.2.1 NTS vs iNTS

The global burden of disease study estimated there were 93.8 million cases and 155,000 deaths due to NTS gastroenteritis in 2005[8]. All regions of the world were affected, with WHO Western Pacific Region (WPRO), including China, Japan and Australia, experiencing the highest disease burden at 53,610,000 cases and WHO sub-Saharan Africa Region (AFRO) reporting 2,458,000 instances[8]. The latter was likely an underestimate due to the limited availability of diagnostics. Twelve years later, the 2017 GBD study estimated a similar number of cases, reporting 95.1 million worldwide[2]. Sub-Saharan Africa currently

has the highest estimated burden of invasive disease, with 78.8% of the 535,000 global cases compared with 21,500 cases in the WPRO region[8].

## 1.2.2 Geographical Distribution: Sub-Saharan Africa

Non-typhoid *Salmonella* represents one of the most common bacteraemias in sub-Saharan Africa[9]. In this setting, disease follows a bimodal age distribution, with children under five years and adults between 25-45 years being most affected[2]. At under six months of age, fewer cases are seen because of immunity provided by maternal antibodies. However, as maternal antibodies wane, cases begin to rise until 36 months of age, when the immune system is beginning to mature, and cases start to decline again. A second rise in cases is observed in young adults, which has not been replicated in other regions of the world[2]. An immature T cell-dependent B cell response may contribute to the elevated cases in the under-five age group. Other associations, including HIV, malaria, anaemia and malnutrition, may contribute to the increased incidence in both age groups[4-6]. In contrast to NTS, the distribution of *S.* Typhi cases is different showing an increasing incidence with age up to ten years[3]. The development of long-standing immunity or the association with immunosuppression with iNTS might help explain this difference in disease burden.

A multicentre study involving 13 sub-Saharan African countries, including Burkina Faso, Ethiopia, Ghana, Kenya and Senegal, identified 13,431 febrile patients between 2010-2014; iNTS was the second most common bacteraemia [94 (17%) cases], after *Salmonella* Typhi [135,(24%)]. The other bacteria isolated were *Staphylococcus aureus* [70 (12%)], *Escherichia coli* [47 (8%)] and *Streptococcus pneumoniae* [42 (8%)]. Of the 94 iNTS

bacteraemia, *Salmonella* Typhimurium (STm)[40%] was the most common serovar, followed by *Salmonella* Enteritidis (SEn)[12%] and *Salmonella* Dublin[11%][9]. A meta-analysis of 22 studies in the same region identified 2,051 cases of bacteraemia in adults and 3,527 in children. Of these total cases, 1,643 (29.1%) were *Salmonella* species and 58.4% of these were iNTS[10]. In Malawi, 9298 admissions and 2789 blood cultures were identified from all medically admitted adults with a fever over a period of 12 months. The most common bacteria identified was iNTS at 37%, followed by *S. pneumoniae* at 30%. In addition, of those who consented to HIV testing, 92% were found to be HIV positive. The majority of iNTS isolates at 128/164 were STm, followed by SEn at 20/164[11]. In the Democratic Republic of Congo, 180 iNTS blood cultures were identified between 2007 and 2011, of which 184 (79%) were STm[12]. When looking at children <4 years of age, admitted with fever between 2014 and 2016 in Malawi, positive blood cultures identified 99/643 (15.4%) iNTS cases, of which STm was again the most common (16/99), followed by *Salmonella* Typhi (13/99)[13]. In Mali, 657 iNTS bacteraemia were isolated between 2002-2014, most of which were identified as NTS [SEn(224; 35.5%) and STm (221; 32.2%)]. Of these cases, 80.5% were in the under-five age group, with 65.1% in the <36 month old[14]. In Ghana, from 472 children admitted to hospital, 111/472 had a positive blood culture. The most common isolate was *Salmonella* spp., with 24 cases (14/24, SEn and 6/24, *S.* Typhi[15]). Overall iNTS remains one of the most common bacteraemia is sub-Saharan Africa, with a significant overlap in geographical distribution with *S.* Typhi.

In the developed world, iNTS is considerably rarer. One case series in the UK identified only 82 cases from blood cultures of patients admitted at one hospital between 1970 and 1999[16], with 80% of these associated with an extraintestinal focus found in

immunosuppressed individuals. The underlying reasons for sub-Saharan Africa's high case burden have been particularly associated with the emergence of the invasive STm ST313 clade and the co-existing incidence of Malaria, HIV, anaemia, and malnutrition[4 6].

### 1.2.3 NTS Serotypes and Sequence Types

The advent of genome sequencing has facilitated the confirmation of serotypes beyond serological agglutination testing and further sub-classification into *Salmonella* sequence types. Multi-locus Sequence Typing (MLST) has emerged as an essential tool to classify bacteria within the same species by sequencing sections of preserved genes required for cell functioning[17]. Furthermore, whole genome sequencing has facilitated the investigation of important genomic characteristics, phylogenetic analysis and the monitoring of antibiotic resistance[18 19].

The non-typhoidal *Salmonella* group contains > 2,500 serotypes. Of these serotypes STm, SEn and, more recently, *Salmonella* Dublin have emerged as the most common within sub-Saharan Africa[18]. An investigation of 166 iNTS isolates from 10 sub-Saharan African countries found *Salmonella* Typhimurium ST313 66% (110/166) was the most common sequence type, followed by *Salmonella* Enteritidis ST11 18% (30/166), and *Salmonella* Dublin ST10 10% (16/166)[18].

Throughout the world and the United Kingdom, STm ST19 is the most common sequence type and is a typical cause of gastroenteritis. In the UK, STm ST313 has been isolated sporadically. It is typically associated with an imported case of salmonellosis from sub-

Saharan Africa or if UK derived often lacks the typical mobile genetic elements conferring antimicrobial resistance which are present in African strains[20].

Multiple factors may contribute to the ecology of NTS serovars and sequence types in sub-Saharan Africa. One of the most important is antibiotic selection pressure, contributing to the emergence of multidrug-resistant STm ST313 and ST11[19 21 22]. Multidrug resistance (MDR) in NTS is defined as resistance to co-trimoxazole, ampicillin and chloramphenicol[21], with Extensive Drug Resistance (XDR) defined as additional resistance to fluoroquinolones and 3rd generation cephalosporins[19 22].

A study in Kenya found that 61% of iNTS isolates sampled were MDR, with STm identified most frequently (85% of isolates), and 95% of these isolates were ST313. In addition, STm ST11 (23%) and *Salmonella* Dublin (6%) isolates were MDR. Genetic mutations conferring resistance varied significantly across sequence types. ST313 was associated with antimicrobial resistance genes on the Tn21 transposon inserted into the pSLT plasmid[18]. At least one isolate had a blaCTX-M-15 insertion in OmpD conferring resistance to 3rd generation cephalosporins with two further isolates containing a *gyrA* mutation conferring resistance to fluoroquinolones[18]. However, ST313 isolated outside sub-Saharan Africa appears to have a different insert[20 23] which is not associated with significant multidrug resistance. ST11, was associated with several other mechanisms of antimicrobial resistance, including the novel *IncI 1* virulence plasmid associated with the Tn21-like transposon insertion. *Salmonella* Dublin only had one isolate with Tn21 transposon inserted, conferring MDR[18].

Due to the nature of these mobile genetic elements, horizontal transfer of antimicrobial resistance mechanisms occurs across *Salmonella* sp. driving multidrug resistance and the emergence of XDR isolates remains a concern [22 24]. With the lack of availability of diagnostics and late diagnosis due to non-specific symptomatology leading to delayed effective antimicrobial therapy, an effective vaccine is increasingly an urgent public health measure in sub-Saharan Africa.

### 1.2.4 *Salmonella* Typhimurium ST313

Whilst *Salmonella* Typhimurium ST313 is associated with MDR, there is mounting evidence of a genetic and phenotypic shift from a pathogen principally causing gastrointestinal disease to one with an increased predisposition to invasive systemic disease[19 24]. Investigation of the ST313 genome has revealed genomic degradation and pseudogene formation with different genetic mutations but functionally similar characteristics to *Salmonella* Typhi[25]. In addition, the presence of the Tn21 transposon conferring multidrug antibiotic resistance in ST313 lineage I and II is one of the factors which has promoted the expansion of this serotype[19]. Additional genetic factors, including Blantyre Prophage BTP1-BTP5 conferring virulence factors have contributed, with the exact virulence mechanism to be determined[26 27]. One virulence factor, the ST313-td, has been shown to contribute to ST313 infection, with function again yet to be determined[28].

Genomic analysis of multiple historic isolates across Africa, including from Malawi, Kenya, Mozambique, Uganda, Democratic Republic of Congo, Nigeria and Mali, identified the emergence of three closely related STm ST313 lineages, I, II and III. Lineage I emerged approximately 52 years ago, coinciding with the beginning of the HIV epidemic. ST313

spread from Malawi to Kenya, DRC, Uganda, Mozambique, and to the rest of sub-Saharan Africa[19]. In the early 2000s, lineage II was seen to be replaced by lineage I, with the acquisition of the *cat* gene conferring chloramphenicol resistance, an antibiotic used frequently at the time[19 24]. In recent years the emergence of a lineage III in Malawi has been associated with reduced antimicrobial resistance with the absence of the Tn21 transposon similar to UK ST313[20] but more genotypic and phenotypic features conferring adaption to a human host including a reduced biofilm formation and gut colonisation caused by a reduction in catalase and nitrate reductase activity respectively[27].

STm ST313 also demonstrates evidence of an association with and emerging adaption to the HIV-positive and the immunocompromised host. The co-emergence and spread of HIV and ST313 suggests an association[19], with the peak in young adults believed to be partly associated with an increasing incidence of HIV infections[4 6]. A study found that iNTS was particularly associated with HIV infection (Odds Ratio 8.2) compared with *Salmonella* Typhi bacteraemia (Odds Ratio 0.07)[10]. In addition, studies have shown a decline in iNTS associated with a reduction in HIV due to the availability of ART and public health measures designed to minimise the impact of malaria and malnutrition[5 6 29]. Evidence of immunosuppression as a factor in NTS bacteraemia is also seen in the developed world where identification of iNTS in patients prompts an investigation for immunosuppression and HIV testing[16]. In addition, increasing evidence of a shift in transmission from animal-to-humans to human-to-human, as is the case for *S.* Typhi , is being observed[30].

*Salmonella* Typhimurium multi-locus sequence type 313 has emerged as a cause of invasive *Salmonella* in sub-Saharan Africa. Amongst the ecology of *Salmonella* serovars

and serotypes in this region, factors such as multi-drug resistance and immunosuppression due to HIV, malaria and malnutrition have facilitated the gradual emergence and phenotypic characteristics of ST313[5 6 31].

### 1.2.5 HIV, Malaria, Malnutrition and Sickle Cell Disease

In one study, 18% of children diagnosed with iNTS were also seropositive for HIV[32]. The immunological rationale for the effect seen in HIV is multifactorial. Impaired macrophage and neutrophil activation and function due to a reduction in CD4+ T helper(Th)-1 and Th-17 cells, and consequent reduction in IFN gamma production [4 33 34], as well as a reduction in T cell dependent B cell antibody production and function[35], contribute to susceptibility to iNTS. Whilst there is an association between HIV and iNTS, it is not the only risk factor.

A decline in iNTS has been associated with decreased malaria in Malawi[5] and The Gambia[36]. The association with malaria has been described as far back as the 1920s[37], however more recently, a study in Gambian children found a higher incidence of malaria in NTS bacteraemia compared with other bacteraemia[38]. Another study investigating iNTS compared separate regions in the same country associated with either high or low endemicity of malaria and identified a greater incidence of iNTS in areas of high malaria incidence[39]. Malaria is believed to impair both the phagocytic respiratory oxidative burst and complement function, resulting in impaired antibody-mediated killing. Malaria also stimulates immature neutrophil release, and reduced recruitment of neutrophils by inducing IL-10[40 41], as well increasing gut permeability[42]. Malnutrition is well known to impair both B and T cell responses as well as gut mucosal defence against micro-

organisms[43]. As a result, a decline in malnutrition and malaria was associated with reduced cases of iNTS[5].

Sickle cell disease is a known risk factor for invasive bacterial diseases, particularly those caused by NTS[44]. Several mechanisms of impaired immune function have been described, including splenic infarction and dysfunction, opsonisation, T cell subset dysfunction and an increase in gut permeability caused by small vessel disease[42 45].

### 1.2.6 Children < 5 years

Functional antibodies targeting iNTS can affect bacterial killing through activation of the classical pathway of complement and subsequent membrane attack complex assembly or by opsonisation, facilitating phagocytosis and oxidative burst-mediated intracellular killing. In children under five years of age, the age-dependent acquisition of functional antibodies is associated with protection from iNTS[46]. The highest peak of disease is between 6 months to 2 years of age, with the incidence declining after that. The peak coincides with a gap in antibody levels, with protection from placentally transferred maternal antibodies waning by six months and the development of detectable functional antibodies from natural exposure by 16 months of age[46]. A study in Malawi found detectable functional antibodies peaked at 35 months. In addition, this study found STm-specific CD4+ Th cells peaked at 14 months of age, which preceded the functional antibody response, suggesting elevated frequency of specific CD4+ Th cells alone was not protective, but downstream effector function of these cells was required for immunity[46].

### 1.2.7 Immunocompromised

Other than immunosuppression caused by HIV, malaria and sickle cell disease, rarer conditions that are associated with defective killing of intracellular pathogens by phagocytes such as chronic granulomatous disease and Mendelian susceptibility to mycobacterial disease (MSMD) predispose to iNTS[6 47].

## 1.3 Non-Typhoidal *Salmonella* Immunopathology

### 1.3.1 Luminal Intestine and Microbiota

Non-typhoidal *Salmonella* is commonly transmitted by ingestion of contaminated food or water. Specific host pathways have been identified which are stimulated by NTS, promoting its growth in the intestine. The organisms are adapted to survive and replicate within the gut lumen, initiating inflammation of the intestinal epithelium[48-50]. A complex cascade of cytokines, macrophages, neutrophils and T cells mediates this local inflammation. The intestinal cell release of IL-22 stimulates the release of Lipocalin-2 from the same cells[51 52], which inhibits other bacteria in the microbiota by absorbing iron required for their growth. Reactive oxygen species released by activated intestinal epithelial cells into the lumen converts by-products of respiration into the metabolite tetrathionate, necessary for *Salmonella* growth[53].

## 1.3.2 Localised Infection and Gastroenteritis

### Type 3 Secretion System and SPI-1

*Salmonellae* use a Type III Secretion System (T3SS), a protein structure that translocates effector proteins directly from the bacterial cytoplasm into the host cell. The T3SS consists of effector proteins determined by two distinct groups of genetic components, *Salmonella* Pathogenicity Island-1 (SPI-1) and *Salmonella* Pathogenicity Island-2 (SPI-2)[54-56]. SPI-1 contains effector proteins which allow bacteria to invade the intestinal epithelia by acting on the host cell cytoskeleton. This occurs in addition to the usual antigen sampling by professional microfold cells or dendritic cells [54 57]. For most NTS infections, the immune system controls local infection, resulting in self-limited gastroenteritis. The resulting inflammation contributes to an environment that promotes the growth of NTS in the intestinal lumen[51 52 53].

### Role of Cytokines, Macrophages and Neutrophils

Two cytokine pathways have been discovered, which are vital in the host immune response to *Salmonella*.

I) The IL-18 / IL-12 / IFN gamma axis: Macrophages and dendritic cells secrete IL-12 and IL-18 which lead to activation of T cells and NK cells resulting in the release of IFN gamma that further enhances macrophage intracellular killing activity of *Salmonella*, which has acquired mechanisms to survive within macrophages[58 59].

II) The IL-23 / IL-17 axis: in which IL-23 stimulates T helper 17 (Th17), NK and Gamma Delta cells to release IL-17, which induces the release of chemokines that recruit

neutrophils to the infection site[59 60]. Neutrophils are one of the mediators of inflammation and crucial in the control of *Salmonella* bacteria[56].

### 1.3.3 Systemic Dissemination and iNTS

#### Type 3 Secretion System and SPI-2

After crossing the epithelial barrier, non-typhoidal *Salmonellae* encounter and infect resident macrophages in the submucosal space or Peyer's patches, surviving within macrophage vacuoles called *Salmonella* Containing Vacuoles (SCV)[56]. A complex balance is maintained within infected macrophages between bacterial killing (oxidative burst and lysozyme formation) / antigen-presenting mechanisms and inhibitory *Salmonella* virulence factors, which facilitate bacterial growth and survival[56]. Crucially, the ability of *Salmonellae* to survive within these vacuoles correlates with its ability to induce systemic disease in animal models[61]. SPI-2 contains up to 28 effector proteins acquired by horizontal gene transfer with protein variations between serovars[55]. The proteins *SSeF* and *SSeG* have been shown to bring together the host cell's Golgi apparatus and SCV allowing growth nutrients to be directly taken up by *Salmonella*[55]. *SifA* has been shown to maintain the SCV membrane and inhibits the lysozyme enzymes, preventing macrophage intracellular antimicrobial action[55]. *SSeK2* and *SSeK3* inhibit caspase and macrophage apoptosis, allowing *Salmonella* to survive[55 62]. Effector proteins such as *sodCI* and *sodCII* promote superoxide dismutase to inhibit reactive oxygen intermediates and respiratory bursts[63], inhibiting DNA damage and preventing bacterial death.

Survival within macrophages is critical to dissemination and allows replication and transportation of intracellular bacteria and extracellular transit to the mesenteric lymph nodes, spleen and liver. Once within the reticuloendothelial system, the organism can disseminate in the bloodstream, giving rise to a clinical syndrome of invasive disease with bacteraemia, systemic symptoms and secondary foci of infection[56]. It remains unclear why some infections result in local self-limiting disease whilst others cause invasive disease. As described, immunodeficiency states, including those caused by HIV, malaria, malnutrition, sickle cell and chronic granulomatous disease, subvert the normal immune response, predisposing to iNTS[4 6].

## 1.4 Non-Typhoidal *Salmonella* Vaccine Immunology

### 1.4.1 Intracellular vs Extracellular Lifecycle of *Salmonella* Infection

During an invasive infection, non-typhoidal *Salmonella* exist both in the intracellular space of polymorphonuclear cells and macrophages, in the gut, blood and tissue, and in the extracellular space in the blood and between cell-to-cell dissemination. Data from multiple studies[40 46 64-66] highlight the importance of cell and antibody-mediated immunity in the adult population and the precise role of antibody-mediated immunity in children < 5 years old[46]. The observation that iNTS infections are less common in immunocompetent individuals after age five highlights that immunity to disease is possible[2]. Whilst antibody-mediated immunity appears crucial, impairment of cell-mediated immunity leads to an increased risk of invasive infection[35 66]. Indeed, the rationale for a vaccine is thus to induce both antibody-associated immunity to prevent extracellular dissemination and cell-mediated immunity to prevent or clear intracellular reservoirs of *Salmonella*[67 68].

Some studies have highlighted the primacy of antibody-associated immunity[69 70], with the implication that dissemination mainly occurs by the extracellular spread of *Salmonella* and that there are lower intracellular bacteria counts in association with high antibody titres[67].

### 1.4.2 Mucosal Immune Response

Once bacteria enter the intestinal epithelium and are phagocytosed by dendritic cells and macrophages, these antigen-presenting cells travel to the mesenteric lymph nodes and present antigens to B and T cells. Mesenteric lymph nodes contain antigen-specific IgA-producing B cells, which are then activated and transit to the gut-associated lymphoid tissue to release secretory IgA[71]. IgA is the second most abundant immunoglobulin in serum where it exists in a monomeric form[72], with increasing evidence of a role in antigen neutralisation and downstream Fc-mediated effector function[73]. At mucosal surfaces, IgA exists in a dimeric form, consisting of two monomeric IgA attached by a j-chain polypeptide and a secretory component which it acquires as it translocates from the epithelial cell into the gut lumen[72]. Once in the lumen, secretory IgA binds to bacteria, neutralising binding to epithelial cell surfaces and preventing it from invading the epithelial cells. Secretory IgA constitutes a significant component of the mucosal immune responses along with resident, mucosal antigen-specific IgA memory B cells, and non-specific IgA secreting B cells in the gut lymphoid tissue following primary infection. The memory B cell populations in the gut are characteristically short-lived compared with their lymph node-based equivalents[74]. A vaccine that stimulates a significant mucosal IgA response with persistent IgA-secreting memory B cells would provide an initial adaptive immune response at the site of bacterial invasion following secondary infection. This

could give a degree of attenuation or protection against NTS gastroenteritis in addition to iNTS which would be a significant boon to any iNTS vaccine.

### 1.4.3 Innate Immune Response

The innate response has evolved to recognise conserved pathogen-associated molecular patterns (PAMPs), via pattern recognition receptors (PRRs) resulting in activation of innate cells such as monocytes and macrophages[49]. Gram-negative bacteria contain multiple PAMPs, including lipopolysaccharides, flagellin and lipoproteins that stimulate the innate immune cells. This is essential in extracellular bacterial clearance and release of cytokines that stimulate a pro-inflammatory response which is crucial to initiate a cascade of pathways which activate the adaptive response[75]. In addition to cytokine release, macrophages and dendritic cells present antigens to B cells and peptides within MHC II complexes to T cells to initiate an antigen-specific response. However, *Salmonella* has developed mechanisms via the SPI-2 effector proteins to inhibit antigen presentation by maintaining survival within SCV, as described earlier[55 76]. Whilst the innate response is essential in the initial clearance of infection and stimulating the adaptive response, it may also cause many of the symptoms and signs of NTS disease such as fever[77]. In the context of a vaccine, too strong an innate response may lead to side effects and reactogenicity, rendering the vaccine unusable.

### 1.4.4 B Cell and Antibody-Mediated Immune Response

Recent evidence has demonstrated the importance of antibodies against invasive non-typhoid *Salmonella*. Mouse studies have shown that antibodies produced by B cells are

essential for protection in STm challenge[78]. The data from human studies is limited as previously described.

Antibody quantity and functional capacity are critical in the humoral response. Antibodies can directly neutralise antigens or can stimulate downstream effector responses. The Fc region of the antibody controls these downstream responses. The specific effector cell can vary and depends on the Fc region, including modifications such as glycosylation, and the Fc receptor type expressed on the effector cell surface[79]. Effector functions include opsonisation and stimulation of phagocytosis by macrophages and monocytes, activation of the complement cascade leading to the development of a membrane attack complex and bacterial death and stimulation of NK cells. An assessment of this broad functional antibody response is called systems serology[79].

B cell responses can be functionally classified into T cell -independent or -dependent. In a T cell-independent response, activation may occur by direct B cell receptor binding to the antigen or antigen trapped on the surface of antigen-presenting cells (APC) [80 81]. In children aged under 5, B cell responses to oligosaccharides are typically T cell-independent[80 81], as is the initial response up to twenty-one days following a primary *Salmonella* infection[82]. However, to achieve greater functionality including B cell memory and long-lived plasma cell response a T cell dependent response is required. However, for T cells to provide co-stimulatory signals to the B cell, they must first be primed via interaction of the T cell receptor with specific peptides (predominantly) in the MHC II complex[57]. An iNTS candidate vaccine whose target demographic is children under five years would be required to stimulate a T cell-dependent antibody response. This type of

response is the basis of conjugate-based vaccines, which consistently outperform the polysaccharide-only vaccines, as seen in Typhoid conjugate[83] and pneumococcal conjugate vaccines[84].

### 1.4.5 T Cell Immune Response

Unlike B cells which recognise conformational epitopes on large macromolecules such as proteins or carbohydrates via surface Ig, T cell surface receptors recognise linear peptide epitopes presented in the context of MHC on antigen presenting cells. Multiple types of T cells have been described. The two most important are the CD3+CD4+ helper cells and CD3+CD8+ cytotoxic cells. As described earlier, the CD3+CD4+ Th cells are essential as co-stimulators of B cells and activate the process of affinity maturation and class switching[82]. In addition, they stimulate CD3+CD8+ cytotoxic (Tc) cells, which in turn recognise and promote the death of infected cells contributing to the clearance of intracellular *Salmonella*. However, the impact of CD8 Tc cells on immune response to NTS infection needs to be further clarified[85]. In addition, multiple CD3+CD4-CD8- cells contribute to the immune response, including gamma delta cells, and mucosal associated invariant T-cells (MAIT) cells[75 86 87].

The importance of rarer T cells in *Salmonella* response has been investigated. T helper 17 (Th17) cells have been shown to produce IL-17, which activates downstream cytokine pathways leading to the recruitment of neutrophils to the local site of infection[75]. Disruption of this pathway in mouse models has been shown to increase the infectivity of *Salmonella*[88]. MAIT cells are innate T cells that release IFN gamma, particularly in response to intracellular organisms[87 89]. They are activated by co-stimulatory vitamin B2,

with STm ST313 having been found to evade these cells by increased production of metabolites in an overexpressed riboflavin pathway[87].

## 1.5 *Salmonella* Antigens

### 1.5.1 Carbohydrate Antigens

Possible targets for functional antibodies include O-antigen (OAg)[7 90], outer membrane protein antigens (OmpD)[91], flagellar antigens [92], and SseB[93 94].

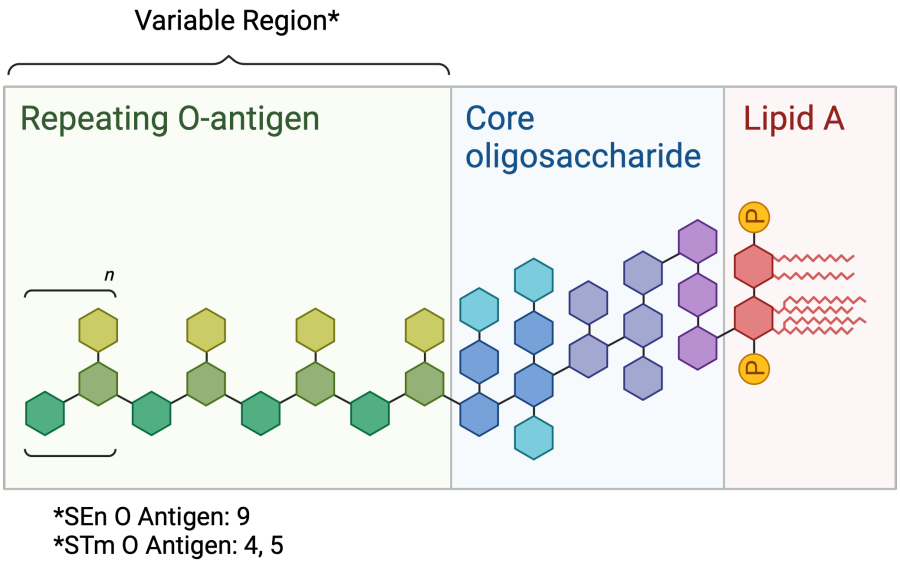



*Figure 1 Structure of (O-Antigen) Lipopolysaccharide (Created with BioRender.com)*

OAg together with lipid A forms part of the lipopolysaccharide component of the outer membrane of *Salmonella* and, is the first point of contact with the host immune system, making it an important immune target[95]. It functions as a virulence factor, with multiple studies showing attenuation of virulence in bacteria with mutations impairing OAg synthesis[96 97]. OAg structure can vary depending on the individual oligosaccharide units, which form the basis of Kauffman-White serotyping of serovars commonly used in microbiology laboratories. STm is associated with O:4,5 whilst SEn is associated with O:9[7]

[98]. Anti-OAg titres correlate with serum bactericidal assay titres consistent with immunity in children[46]. Furthermore, mice immunised with OAg conjugate vaccine generated sera with increased bactericidal antibody activity against NTS *in vitro*. When these mice were challenged with NTS they later showed a reduction of bacterial burden in the spleen and liver[99]. Another study showed higher levels of bactericidal antibodies in mice and rabbits injected with *Salmonella* Typhimurium OAg[100]. However, one paper in HIV positive adults found high levels of Anti-OAg IgG to be inhibitory, in contrast to porin antibodies which were bactericidal[64].

An area requiring clarification is the antibody response to iNTS in individuals living with HIV. Very high O antigen antibody titres are found in a subpopulation of people living with HIV, whose serum was inhibitory to complement-mediated cell killing. In these individuals, antibodies to porins were significant and high O-antigen antibody titres prevented the formation of the complement membrane attack complex required for cell-mediated killing[65].

## 1.5.2 Protein Antigens

Although targets for functional antibodies include principally the O-antigen (OAg) component of the outer membrane lipopolysaccharide (LPS)[90 99], other antigens such as the outer membrane protein antigens (OMP Ag)[91] and flagella antigen (FliC)[46] may also be important. A study examining serum from Malawian children suggested that serum bactericidal activity correlates with the detectable levels of IgG to lipopolysaccharide (LPS) but not to outer membrane protein (OMP) or flagella proteins[46]. However,

antibodies against the FliC flagellar protein and membrane-bound porin proteins such as OmpD were protective in mouse models[20,21].

Although flagellar (H) antigens stimulate a significant innate response[101], the ability of *Salmonella* to down-regulate flagellum expression, may reduce the value of this antibody target[102].

The most common outer membrane protein (OMP) antigens described are the porins: OmpC[103], OmpD[91], and OmpF[103]. OmpD, in particular, has been investigated as a possible vaccine component and has been shown to induce antibodies capable of limiting *Salmonella* Typhimurium infection in mouse studies[99 104]. As proteins, porins could be targets of both B and T cells, or provide conjugation to polysaccharide targets.

Whilst outer membrane proteins are being investigated as antigens in *Salmonella*, there is an ongoing debate about the role of surface versus intracellular protein antigens in *Salmonella* immunogenicity. Surface proteins have been described as necessary in stimulating T cells due to the ability of *Salmonella* to remain intact within SCV in macrophages, making the surface proteins the most accessible[105]. However, studies investigating T cell responses to a range of *Salmonella* serovars found intracellular antigens such as PhoN, Cdt and HlyE to stimulate a more robust T cell response[106].

Further, theoretical antigen targets include the effector proteins of the type 3 secretion system, including either proteins from SPI-1 contributing to intestinal invasion or SPI-2 contributing to survival within macrophages and dissemination[54 55 94].

## 1.6 *Salmonella* Vaccines

### 1.6.1 Licensed Animal Vaccines

Whilst there are no current licensed human NTS vaccines, multiple well-established *Salmonella* vaccines are licensed for animals. *Salmonella* vaccines have been investigated for use in cattle[107], swine[108], sheep[109] and laying hens[110]; with a purpose to improve livestock's health and prevent transmission of *Salmonella* to humans. *Salmonella* vaccines used in laying flocks during the 1980s resulted in a substantial decrease in cases of reported *Salmonella* infections, proving that using vaccines in animals can inhibit transmission to humans and reduce infections[111]. These currently licensed NTS livestock vaccines use live attenuated or inactivated organisms[112] (see table 1) which whilst being reactogenic, are effective and cheaper to produce than subunit vaccines[113].

| Name Of Vaccine | Target Species | Vaccine Type | Active Constituent |
|---|---|---|---|
| AviPro Salmonella Duo | Chickens | Live Attenuated | SEn, Sm24/Rif12/Ssq |
| | | | STm Nal2/Rif9/Rtt |
| AviPro Salmonella Vac E | Chickens | Live Attenuated | SEn, Sm24/Rif12/Ssq |
| AviPro Salmonella Vac T | Chickens | Live Attenuated | STm Nal2/Rif9/Rtt |
| Nobilis Salenvac | Chickens | Inactivated | Formalin Killed SEn |
| Bovilis Bovivac S | Cattle | Inactivated | Formalin Killed S. Dublin S342/70 |
| | | | Formalin Killed STm S341/70 |
| Nobilis Salenvac T | Chickens | Inactivated | SEn PT4 |
| | | | STm DT104 |
| Nobilis SE | Chickens | Live Attenuated | SEn CAL10 Sm+/Rif+/Ssq- |
| Nobilis Salenvac | Chickens | Inactivated | SEn PT4 |
| | | | STm DT104 |
| | | | S. Infantis strain A, S03499-06 |
| Gallimune Se + St | Chickens | Inactivated | SEn PT4 |
| | | | STm DT104 |
| Cevac Salmovac | Chickens | Live Attenuated | SEn, 441/014 |
| Salmoporc | Pigs | Live Attenuated | STm, 421/125 |
| Biosuis Salm | Pigs | Inactivated | STm |
| | | | S. Derby |
| | | | S. Infantis |

*Table 1 Licensed Veterinary Salmonella Vaccines (taken from Veterinary Medicines Directorate Product Information Database[112])*

## 1.6.2 Human Vaccines Under Development

There are multiple human iNTS vaccines at various stages of development. Three vaccine candidates have progressed to phase I first in human clinical studies[114 115], and more are in pre-clinical testing[116]. The aim of these vaccines is in the prevention of invasive disease rather than NTS gastroenteritis.

The first non-typhoid *Salmonella* vaccine candidate to enter a first-in-human study was in 2001[114]. WT05, produced by *Microscience Ltd*., recruited 18 participants in a dose escalation study, with nine participants receiving an oral attenuated *Salmonella* Typhi and nine participants receiving an oral attenuated *Salmonella* Typhimurium at a dose of $10^7$, $10^8$, $10^9$ Colony Forming Units (CFU). Both were attenuated with mutations in *aroC* and *ssaV*, with *ssaV* associated with a reduction in the function of the type III secretion system. Of the nine participants who received the *Salmonella* Typhimurium oral agent, only the three participants in the $10^9$ CFU group had a significant IgG antibody response. However, these participants experienced prolonged shedding, and further development of this candidate has not proceeded.

The next vaccine candidate to enter clinical trials was almost two decades later. This trivalent conjugate vaccine was produced by the University of Maryland and consists of *S.* Enteritidis and *S.* Typhimurium O-antigen conjugated with FliC flagellar antigen, and *S.* Typhi Vi conjugate is yet to be published but entered a first-in-human phase I clinical trial shortly before the COVID-19 pandemic[115]. The third vaccine candidate to enter clinical trials, the iNTS-GMMA vaccine is the subject of this thesis.

Other vaccines in pre-clinical development include an oral live attenuated vaccine (CVD 1931, CVD 1994), a bivalent OAg conjugate vaccine (COPS, CRM197), protein vaccines (Flagella and OmpD)[116 117] and the Multiple Antigen Presenting System (MAPS) which facilitates the presentation of multiple *Salmonella* antigens on a pneumococcal protein carrier[118].

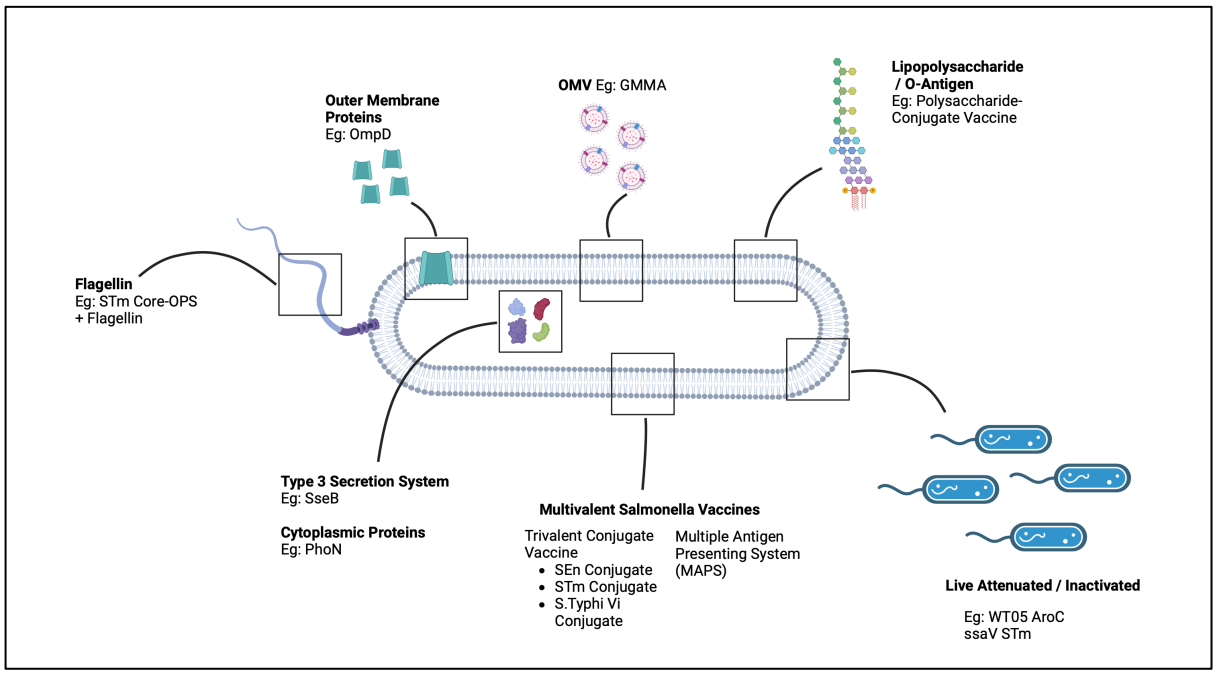



*Figure 2 Non-typhoid Salmonella vaccines under investigation[115 116 117 118] (Created with BioRender.com)*

## 1.7 The iNTS-GMMA Vaccine

### 1.7.1 Background

The iNTS-GMMA vaccine consists of the outer membrane vesicles of the two most common serovars to cause iNTS, *Salmonella* Enteritidis and *Salmonella* Typhimurium[7]. Outer membrane vesicles (OMVs) are blebs naturally released from the outer membrane

of most Gram-negative organisms. These OMVs have several possible functions, including immune decoys, delivery packages for effector molecules, quorum sensing, and waste removal from the bacteria[119 120]. OMVs contain many of the outer membrane's antigens, and thus lends themselves to being used as a vaccine. As vesicles, they contain no cellular machinery and cannot replicate within the host[119-121]. They contain outer membrane proteins which could facilitate the stimulation of the T cell dependant B cell responses to OAg via linked recognition[119]. OMVs contain minimal cytoplasmic proteins[122] and therefore, may facilitate further understanding of the relative immunogenicity of outer membrane versus intracellular proteins. In addition, further research into OMVs is being conducted investigating their use as delivery packages for non-native antigens or therapeutics[123 124].

### 1.7.2 Manufacture of nOMV, dOMV and GMMAs

The culture conditions of Gram-negative bacteria at the point of OMV production can modify the protein composition of OMVs. A study looking at OMV composition when generated under multiple bacterial culture conditions identified different protein compositions, particularly type 3 secretion system effector proteins in conditions similar to macrophage vacuoles[125].

The generalised modules for membrane antigens (GMMAs) are OMVs. However, these GMMAs differ from natural OMVs in their production and modification. The two origin strains of SEn and STm, have been genetically modified to increase OMV production by modification of the *tolR* gene and detoxify lipid A by modification of *msbB* and *pagP* genes, causing a structural change from the more reactogenic hexa- and hepta-acylated

forms to the less reactogenic penta-acylated lipid A[7 126]. It is hoped that this modification of lipid A reduces vaccine toxicity and reactogenicity. To manufacture, the genetically modified bacterial strains are cultured at scale with the high-yield OMV filtered and collected[7 126].

Multiple alternative methods exist to extract OMVs, each with its own drawbacks. These methods include filtering spontaneous OMVs (sOMV), which results in low yield; detergent extracted OMV, resulting in loss of LPS (dOMV); bacterial membrane destabilisation (nOMVs); and finally using antibiotics[127].

### 1.7.3 Current OMV Vaccines

The only OMV vaccines licensed are for the prevention of Meningococcal B infection, and include, Bexsero, MenBVac in Norway, MenNZB in New Zealand, and VA-MENGOC-BC in Cuba. These vaccines are safe and have an acceptable reactogenicity profile[128]. Of note, there was a reduction in immunogenicity in children under five, with a multi-dose regimen required to improve immunogenicity[128] in part explaining the multi-dose vaccine regimen used in subsequent *Shigella* GMMA trials[129 130 131].

There have been four proof-of-concept clinical trials of a *Shigella sonnei* GMMA vaccine; three phase 1 trials in Europe (two 3-dose vaccine regimen and one booster study)[129 130] and one phase 2a trial in Kenya (one 2-dose vaccine regimen)[131]. In the initial phase 1 trial in France, an observer-blind, randomised dose escalation study, the vaccines induced a median antibody response higher than the non-vaccinated participants. The phase 1 trial in the UK, another observer-blind, randomised trial using the lower dose of 0.29µg OAg

/5µg protein, showed minimal antibody response in agreement with the French study at this dose. The vaccines were well tolerated in both studies, and no participant experienced SAEs or AEs leading to withdrawal. The most common solicited adverse event was injection site pain which was mild to moderate. Of the unsolicited vaccine-related AE, there were 8 cases of neutropenia, all transient and asymptomatic[121]. The study in France looked at boosting already primed versus vaccine naïve participants and found the vaccine was well tolerated with boosted *S. sonnei* LPS IgG antibody showing a higher peak in the previously primed group[130]. The phase 2a trial in Kenya showed an increase of 2.10- and 4.43-times antibody response in the lower dose and higher dose vaccine respectively at 28 days following first vaccination compared with day 0 (baseline). There were 10 cases of neutropenia. One subject experienced 24 hours of severe fatigue five days after vaccination. One subject experienced myalgia and fever from 6 hours to day two post-first vaccine, with no further episodes post-second and third[131]. Finally, a *Shigella* challenge study testing the *Shigella* GMMA vaccine found no efficacy at the vaccine dose tested with an endpoint of *Shigella* gastroenteritis and not invasive disease[132]. The study demonstrated an increase LPS IgG levels but had multiple limitation including participants selected with high baseline *Shigella* LPS IgG in both groups prior to receipt of vaccine or placebo. The antibody levels including the type of immune response (mucosal) required to prevent gastroenteritis is unclear and may have contributed.

Further outer membrane vesicle vaccines are in early pre-clinical testing, including for *Neisseria gonorrhoea*[133], *Acinetobacter baumannii*[134], and *Pseudomonas aeruginosa*[135].

### 1.7.4 Vaccine Rationale

The primary aim of the iNTS-GMMA vaccine is in preventing iNTS disease. The iNTS-GMMA vaccine represents the first occasion two OMV components from different bacterial serovars have been combined to produce a multivalent vaccine. As a result, immunogenicity against both components are being tested. The iNTS-GMMA vaccine is quantified based on O-antigen content, the assumed immunodominant antigen. However, protein antigens present in the GMMAs, including but not limited to flagellin, OmpD, OmpA, OmpC, and OmpF, may evoke a broader antibody response. The proteomic composition of the active GMMA vaccine components is yet to be established and will be determined as part of this thesis. Therefore, the linked recognition of proteins within the GMMAs may stimulate a T cell-dependent B cell response of particular importance in children under five and may subsequently stimulate a longer lasting protective antibody response in this age group. In addition, the active GMMA components have been formulated on Alhydrogel, an aluminium hydroxide suspension, shown in animal studies to reduce toxicity whilst maintaining immunogenicity[136].

## 1.8 iNTS Vaccine Strategy

The target of an iNTS vaccine is primarily in the most affected demographic, children under five in sub-Saharan Africa. As discussed, iNTS disease is associated with HIV, malaria and malnutrition[4-6]. As seen in multiple studies, preventive and prompt treatment measures for these conditions may reduce the risk of iNTS disease[29]. However, completely eradicating these diseases is unlikely to occur in the foreseeable future. Therefore, adults

with HIV or sickle cell disease in sub-Saharan Africa and the developed world, may also benefit from an iNTS vaccine.

However, to produce a more cost-effective vaccine against iNTS, the development of a multivalent *Salmonella* vaccine would combine activity against the common serovars causing iNTS and *S.* Typhi or *S.* Paratyphi, making the vaccine more deployable and viable[137]. The first intramuscular iNTS vaccine trial to enter phase I is a trivalent vaccine incorporating components of NTS and *S.* Typhi[115].

## 1.9 *Salmonella* Vaccine Study in Oxford (SALVO)

The planned advancement of the iNTS-GMMA vaccine begins with the *Salmonell*a Vaccine Study in Oxford (SALVO). This study is the initial major interventional component of the Vacc-iNTS consortium, which includes a second phase I study in a country of high endemicity and sero-epidemiological studies[138 139]. The Vacc-iNTS project aims to develop the iNTS-GMMA vaccine in two phase I studies, the first in a European country (SALVO) and the second in a country of high endemicity (sub-Saharan Africa). The project consists of a multi-site sero-epidemiology study to clarify the burden of disease in sub-Saharan Africa and identify sites for phase II and III studies[138]. The Vacc-iNTS consortium includes a number of academic and commercial institutions throughout the world including the University of Oxford and GSK Vaccines Institutes for Global Health (GVGH)[139 140].

SALVO is a first-in-human phase I randomised-placebo controlled study of the iNTS-GMMA vaccine at two dose levels: the lower dose consisting of 5.3 µg OAg SEn GMMA and 5.3 µg STm GMMA; and the full dose consisting of the 20 µg OAg SEn GMMA and 20

µg STmGMMA. The primary objective is evaluating the safety and tolerability of the iNTS-GMMA vaccine. The secondary aim is quantifying the immunogenicity of the iNTS-GMMA as measured by O: 4, 5, 9 IgG ELISA. Additional exploratory objectives include an expansion of immunogenicity testing to include investigation of mucosal immunity, functional antibody, and B and T cell responses.

The set-up, management and sample analysis up to D28 following the primary vaccine course (first and second vaccine) formed the foundation of this thesis. Following this initial phase I trial, a second phase I in a country of high iNTS endemicity[139] is planned in conjunction with a third study in children and adults[141]. Ultimately a trivalent vaccine together with a *S*. Typhi Vi conjugate[137] component, which separately has proven high vaccine efficacy[142] is foreseen within the further development pipeline of iNTS-GMMA. To progress any iNTS vaccine, efficacy must be determined once safety and tolerability have been established. Given the incidence of iNTS, a field study in sub-Saharan Africa would require a multicentre study involving a large sample size. An alternative approach would be the identification of a correlate of protection and efficacy via controlled human infection (CHIM) studies with a smaller sample size, similar to *S.* Typhi vaccine development[143]. There are no established CHIM studies for iNTS but one study is in development[144 145].

## 1.10 Aims and Objectives of this thesis

This thesis aims to evaluate the safety and immunogenicity of the lower dose and full dose iNTS-GMMA vaccine in a first-in-human phase I study following first and second

vaccination, and up to 28 days following the second vaccine. The objectives of this thesis are:

1. The design, set-up, initiation and initial management of the iNTS-GMMA vaccine trial (SALVO study) up to day 28 after second vaccination (Chapter 2 and 3).
2. The evaluation of the safety and tolerability of the iNTS-GMMA vaccine at two dose levels: lower dose and full dose iNTS-GMMA vaccine; up to day 28 after second vaccination (Chapter 3).
3. Evaluation of the serum IgG and IgA antibody response following first and second vaccination up to day 28 after second vaccination using a validated SEnGMMA and STmGMMA IgG and IgA ELISA (Chapter 4).
4. Evaluation of the functional antibody response following first and second vaccination up to day 28 after second vaccination using antibody dependant monocyte phagocytosis (ADMP) and NK activation (ADNKA) assays (Chapter 4).
5. Evaluation of the mucosal immune response following first and second vaccination by measuring absolute oral fluid IgA using a SEnGMMA and STmGMMA IgA ELISA (Chapter 4).
6. Evaluation of the T cell memory response following first and second vaccination using a GMMA-based T cell proliferation assay (Chapter 4).
7. Evaluation of the proteomic components of the iNTS-GMMA vaccine (Chapter 5).
8. Identification of immunogenic homologous *Salmonella* proteins conserved across *Salmonella* serovars (Chapter 5).

9. Evaluation of serum IgG and IgA antibody response to SEnGMMA and STmGMMA, generated following infection with non-typhoid *Salmonella* using a validated SEnGMMA and STmGMMA IgG and IgA ELISA (Chapter 6).
10. Evaluation of serum IgG and IgA cross-reactive antibodies to SEnGMMA and STmGMMA, generated following infection with *S.*Typhi and *S.* Paratyphi using a validated SEnGMMA and STmGMMA IgG and IgA ELISA (Chapter 6).
11. Evaluation of serum IgG and IgA functional primary and cross-reactive antibody response to SEnGMMA and STmGMMA, generated following infection with *S.*Typhi, *S.* Paratyphi or non-typhoid *Salmonella* using antibody dependant monocyte phagocytosis (ADMP) and NK activation (ADNKA) assays (Chapter 6).
12. Evaluation of the functional cross-reactivity of the iNTS-GMMA vaccine generated antibodies beyond non-typhoid *Salmonella* (Chapter 6).
13. Identification of primary immunodominant IgG and IgA SEnGMMA and STmGMMA specific antigen following iNTS-GMMA vaccination or non-typhoid *Salmonella* infection (Chapter 6).
14. Identification of the cross-reactive IgG and IgA SEnGMMA and STmGMMA specific antigen following *S.* Typhi and *S.* Paratyphi infection (Chapter 6).
15. Identification of protein antigens in the GMMA which may contribute to the direct and cross-reactive antibody response (Chapter 6).
16. Evaluation of the GMMA-specific monocyte activation by the expression of IL-1 and IL-6 (Chapter 7).
17. Evaluation of the cross-reactive responses of *S.* Typhi and *S.* Paratyphi PBMCs post-infection using SEn / STm GMMA-specific T cell proliferation assays (Chapter 7).

18. Identification of proteins in the GMMAs which may contribute to the direct $CD3^+CD4^+$ and $CD3^+CD8^+$ response following iNTS-GMMA vaccination (Chapter 7).

This thesis will test the following hypotheses:

1. The iNTS-GMMA vaccine is safe and tolerable at two dose levels: lower dose and full dose; up to day 28 after second vaccination (Chapter 3).
2. The iNTS-GMMA vaccine stimulates an IgG and IgA antibody response above baseline and placebo recipients following first and second vaccination up to day 28 after second vaccination (Chapter 4).
3. The iNTS-GMMA vaccine stimulates a mucosal immune response above baseline and placebo recipients following first and second vaccination up to day 28 after second vaccination as measured by an oral fluid IgA GMMA ELISA (Chapter 4).
4. The iNTS-GMMA vaccine stimulates functional antibody responses as measured by ADMP and ADNKA, above baseline and placebo recipients, following first and second vaccination up to day 28 after second vaccination (Chapter 4).
5. The iNTS-GMMA vaccine and proteins therein facilitate antigen linked recognition and generate memory T cells ($CD3^+CD4^+$ and $CD3^+CD8^+$) following iNTS-GMMA vaccination (Chapter 4).
6. SEn and STm GMMA particles contain outer membrane proteins that are conserved across multiple *Salmonella* serovars (Chapter 5).
7. Identified *Salmonella* homologous GMMA proteins are immunogenic as determined by computational methods (Chapter 5) and confirmed by immunological assays (SEnGMMA / STmGMMA IgG ELISA and T cell proliferation assays) [Chapter 6].

8. SEn and STm GMMA particles bind cross-reactive functional antibodies generated following *S.* Typhi and *S.* Paratyphi infection which may suggest a modified (boosted or interference) in the functional antibody response to these Salmonella serovars following iNTS-GMMA vaccination (Chapter 6).
9. SEn and STm GMMA particles stimulate functional antibody responses to Typhoidal *Salmonella* serovars suggesting a possible role as a component in a cross-protective pan-*Salmonella* vaccine (Chapter 6).
10. The O-antigen component of the SEn and STm GMMA and corresponding bacterial serovars is the primary immunodominant and cross-reactive antigen (Chapter 6).
11. The protein components of the SEn and STm GMMA contribute to the antibody response to the SEn and STm GMMA (Chapter 6).
12. Memory T cells ($CD3^+CD4^+$ and $CD3^+CD8^+$) to SEn and STm GMMA particles and proteins therein are generated following infection with *S.* Typhi or *S.* Paratyphi (Chapter 7).

### 1.10.1 Safety of the iNTS-GMMA Vaccine

The initial objective of this thesis is the set up and management of the iNTS-GMMA vaccine trial (SALVO Study), followed by an interim assessment of the safety and tolerability of the iNTS-GMMA vaccine at the two dose levels. The safety of the iNTS-GMMA vaccine at lower and full doses up to 28 days following the second vaccination forms the first results chapter of this thesis.

### 1.10.2 Exploratory Immunogenicity Analysis of the iNTS-GMMA Vaccine

Exploratory immunological analysis of the iNTS-GMMA vaccine consists of the development, optimisation and application of multiple assays investigating the broad immune response to the primary course of the vaccine (table 2).

| **Sample** | **Analysis** |
|---|---|
| Serum | SEnGMMA and STmGMMA IgG and IgA ELISA, Antibody Dependant Monocyte Phagocytosis (ADMP), Antibody Dependant NK Activation (ADNKA) |
| PBMC | T Cell proliferation assay |
| Oral Fluid | SEnGMMA and STmGMMA IgA ELISA |

*Table 2 Immunoassays and Sample Type*

### 1.10.3 Identification of Antigens within the GMMA vaccine component

The presumed immunodominant antigen within the active GMMA component is the O-antigen. As part of this thesis, the identification of possible antigens within the GMMA is undertaken using proteomic analysis and computational prediction of the immunogenicity of GMMA proteins. A selection of proteins were manufactured and tested in ELISAs and T-cell proliferation assays to determine immunogenicity. Confirmation of O-antigen as the immunodominant antigen within the GMMAs is undertaken using serum pre-adsorbed with O-antigen tested on GMMA IgG ELISAs.

### 1.10.4 Determination of *Salmonella* cross-reactivity to the active GMMA vaccine component

The final component of this thesis is an assessment of broad *Salmonella* sp. cross-reactivity to the active iNTS-GMMA vaccine components to determine if antibody, CD3+CD4+ and CD3+CD8+ cells following *S.* Typhi and *S.* Paratyphi infection cross-react with the GMMAs. Cross-reactivity might indicate interference or boosting of specific or cross-serovar immunogenicity.

# Material and Methods

## 2.1 Introduction

This chapter describes the methods for the investigation of the safety and immunogenicity of the iNTS-GMMA vaccine as part of the *Salmonella* Vaccine Study in Oxford (SALVO) study. The proteomic analysis and selected protein production are also described. Finally, methods and development of the exploratory immunological assays are outlined.

## 2.2 *Salmonella* Vaccine Study in Oxford (SALVO)

### 2.2.1 Overview

SALVO is a first-in-human placebo-controlled participant-observer blind trial aiming to evaluate the safety and immunogenicity of the iNTS-GMMA vaccine. This study will be conducted at the Centre for Clinical Vaccinology and Tropical Medicine, Oxford University recruiting participants in the Thames Valley area.

The study will recruit 30-42 participants into one of three groups. There is a planned dose escalation from a 'lower' 10.6µg iNTS-GMMA vaccine to a 'full' 40µg iNTS-GMMA Vaccine between groups 1 and 2, depending on a safety review by the Data and Safety Monitoring Committee(DSMC). Groups 1 and 2 will primarily enrol 6 participants randomised 1:1 to

receive a vaccine or placebo, with group 3 enrolling 18 randomised 2:1 to receive a vaccine or placebo (figure 3).

The placebo consists of Alhydrogel, an aluminium compound which has been shown to reduce the reactogenicity of GMMA vaccines in animal studies[146]. A placebo-controlled study allows the direct comparison of adverse events and immunogenicity between active and non-active components providing greater strength in the study results.

| Objective | | Outcome measure |
|---|---|---|
| Primary | To determine the safety and tolerability of the iNTS-GMMA vaccine | Clinical review and participant recording of solicited, unsolicited adverse events, serious adverse events, withdrawals, and laboratory parameters (haematology/biochemistry) |
| Secondary | To investigate immunogenicity of the iNTS-GMMA vaccine | Measurement of serovar specific O-antigen by ELISA before and after vaccination |
| Exploratory | To further characterise the immune response to vaccination | Exploratory immunological analyses including functional antibody assays and antigen-specific memory B cell responses and T cell responses before and after vaccination |

*Table 3 SALVO Study Objectives taken from Salmonella Vaccine Study in Oxford (SALVO) Trial: Protocol for an Observer-Participant Blind Randomised Placebo-Controlled Trial of the iNTS-GMMA Vaccine within a European Cohort[1]*

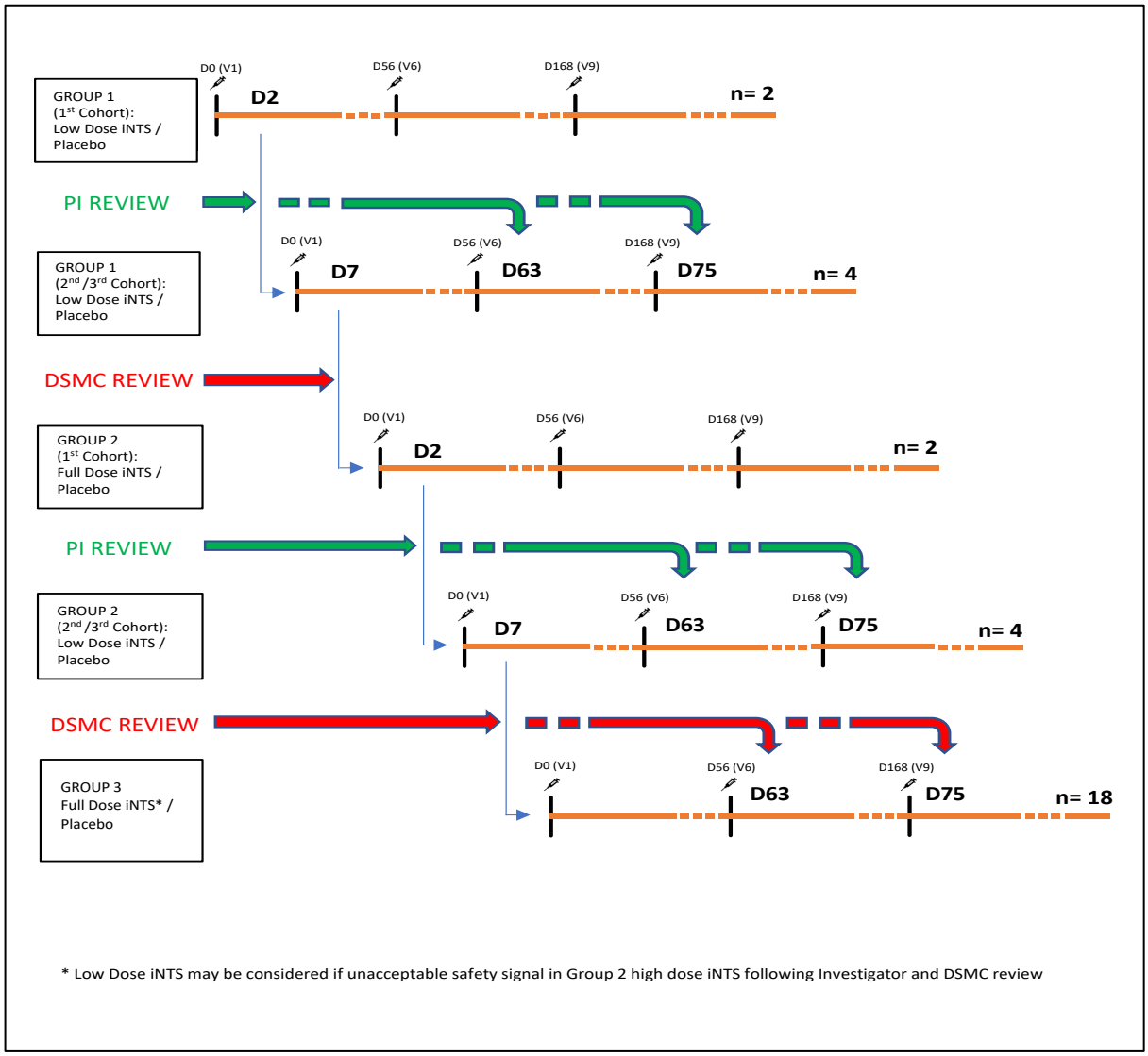



*Figure 3 SALVO dose escalation, internal safety and DSMC reviews; vaccine administration at V1(D0), V6 (D56) and V9 (D168) (taken from Salmonella Vaccine Study in Oxford (SALVO) Trial[1])*

There are 13 face-to-face visits including screening, with the vaccine or placebo administered at baseline, 2 and 6 months. The total duration for a participant in the trial is 12 months (figure 4).

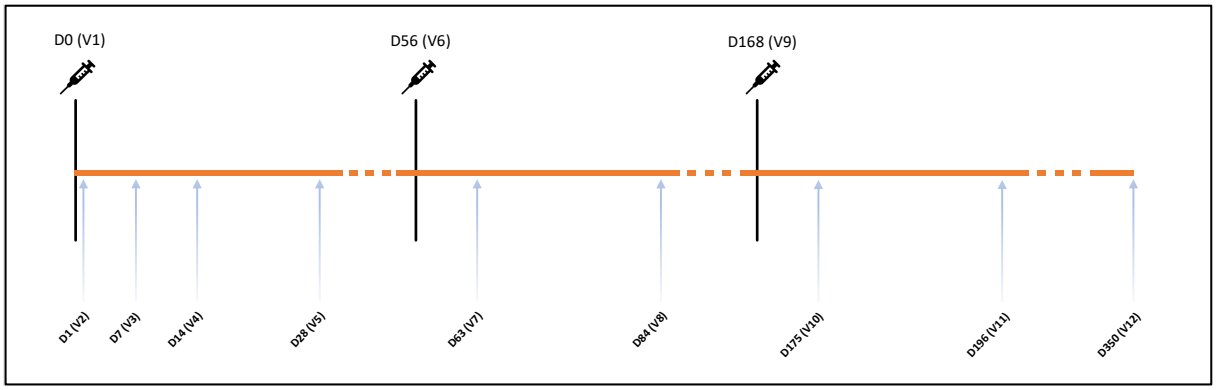



*Figure 4 SALVO vaccine and visit schedule (taken from Salmonella Vaccine Study in Oxford (SALVO) Trial[1])*

## 2.2.2 Primary Objective: Safety of the iNTS-GMMA Vaccine

The primary objective of the study is the safety of the iNTS-GMMA vaccine at two dose levels, lower dose and full dose. Solicited and unsolicited adverse events (AE), medically important adverse events (those requiring healthcare worker contact), serious adverse events (SAE), and suspected unexpected serious adverse events (SUSAR) are collected according to the scheme in table 4. Each adverse event is assessed for causality to the investigational product and placebo (table 5, SALVO Protocol[1], 11.2), and severity is graded from 1-5 (table 6-9, SALVO Protocol[1], 11.3, appendices A-D).

| SALVO Safety Data | Collection details | |
|---|---|---|
| Solicited Adverse Events | Day 0 to 7 following each vaccination | |
| | *Local* | *Pain*<br>*Tenderness*<br>*Redness*<br>*Swelling*<br>*Hardness* |
| | *Systemic* | *Abdominal Pain*<br>*Chills*<br>*Diaarhoea*<br>*Fatigue*<br>*Feverishness*<br>*Headache*<br>*Joint Pain*<br>*Loss of Appetite*<br>*Malaise*<br>*Muscle Pain*<br>*Nausea*<br>*Rash*<br>*Temperature*<br>*Vomiting* |
| Unsolicited Adverse Events | Day 0 to 28 following each vaccination | |
| Medically Important AEs* | Outside of D0-D28 Unsolicted AE window following vaccination | |
| SAEs / SUSARS | Throughout Trial Period | |

*Table 4 Scheme for the collection of adverse events*

| | | |
|---|---|---|
| Not related | • No temporal relationship to vaccine administration or other study procedure, ***and***<br>• Alternative aetiology (clinical, environmental or other intervention), ***and***<br>• Does not follow pattern of recognised response to vaccine administration or other study procedure. | |
| Related | Possible | • Reasonable temporal relationship to vaccine administration or other study procedure, ***or***<br>• Event not readily explained by alternative aetiology (clinical, environmental or other interventions), ***or***<br>• Similar pattern of response to that seen to vaccine administration or other study procedure. |
| | Probable | • Reasonable temporal relationship to vaccine administration or other study procedure, ***and***<br>• Event not readily produced by alternative aetiology (clinical, environment, or other interventions), ***or***<br>• Known pattern of response with vaccine administration or other study procedure. |
| | Definite | • Reasonable temporal relationship to vaccine administration or other study procedure, ***and***<br>• Event not readily produced by alternative aetiology (clinical, environment, or other interventions), ***and***<br>• Known pattern of response to vaccine administration or other study procedure. |

*Table 5 Assessment of causality for adverse events (taken from Salmonella Vaccine Study in Oxford (SALVO) Trial Protocol[1])*

| Adverse event | Grade | Definition (in degrees Celsius) |
|---|---|---|
| Temperature | 0 | < 37.6 |
| | 1 | 37.6 – 38.0 |
| | 2 | 38.1 – 39.0 |
| | 3 | > 39 |

*Table 6 Severity grading for temperature (taken from Salmonella Vaccine Study in Oxford (SALVO) Trial Protocol[1])*

| Adverse event | Grade | Definition |
|---|---|---|
| Any symptom | 0 | Absence or resolution of symptom |
| | 1 | Awareness of symptom but tolerated; transient or mild discomfort; little or no medical intervention required |
| | 2 | Discomfort enough to cause limitation of usual activity; some medical intervention or therapy required |
| | 3 | Significant interference with daily activity |
| | 4 | Emergency department visit or hospitalisation |
| | 5* | Fatality |

*Table 7 General adverse event grading for symptoms and medical events (taken from Salmonella Vaccine Study in Oxford (SALVO) Trial Protocol[1])*

| Observation | Grade 1 | Grade 2 | Grade 3 |
|---|---|---|---|
| Oral temperature (°C) | 37.6 – 38.0 | 38.1 – 39.0 | >39 |
| Tachycardia (beats/min) | 101-115 | 116-130 | >130 |
| Bradycardia (beats/min) | 50-54 | 45-49 | <45 |
| Systolic hyper-tension (mmHg) | 141-150 | 151-155 | >155 |
| Diastolic hyper-tension (mmHg) | 91-95 | 96-100 | >100 |
| Systolic hypotension (mmHg) | 85-89 | 80-84 | <80 |

*Table 8 Assessment of severity of observation adverse events (taken from Salmonella Vaccine Study in Oxford (SALVO) Trial Protocol[1])*

| Parameter | Grade 1 | Grade 2 | Grade 3 | Grade 4* |
|---|---|---|---|---|
| **Haemoglobin: decrease from baseline value (g/l)** | ≤ 15 | 16-20 | 21-50 | >50 |
| **White cell count: elevated ($10^9$/L)** | 11–15 | 16–20 | 21–25 | >25 |
| **White cell count: depressed ($10^9$/L)** | 2.5-3.5 | 1.5-2.4 | 1.0-1.4 | <1.0 |
| **Neutrophil count ($10^9$/L )** | 1.5-2.0 | 1.0-1.4 | 0.5-0.9 | <0.5 |
| **Platelets ($10^9$/L)** | 125-140 | 100-124 | 25-99 | <25 |
| **Sodium: hyponatraemia (mmol/L)** | 132–134 | 130–131 | 125–129 | <125 |
| **Sodium: hypernatraemia (mmol/L)** | 146 | 147 | 148–150 | >150 |
| **Potassium: hyperkalaemia (mmol/L)** | 5.1–5.2 | 5.3–5.4 | 5.5–5.6 | >5.6 |
| **Potassium: hypokalaemia (mmol/L)** | 3.3–3.4 | 3.1–3.2 | 3.0 | <3.0 |
| **Urea (mmol/L)** | 8.2–8.9 | 9.0–11 | >11 | RRT |
| **Creatinine (µmol/L)** | 132-150 | 151-176 | 177-221 | >221 or RRT |
| **ALT and/or AST (IU/L)** | 1.1–2.5 x ULN | >2.6–5.0 x ULN | 5.1-10 x ULN | >10 x ULN |
| **Bilirubin, with increase in LFTs (µmol/L)** | 1.1–1.25 x ULN | 1.26–1.5 x ULN | 1.51–1.75 x ULN | >1.75 x ULN |
| **Bilirubin, with normal LFTs (µmol/L)** | 1.1–1.5 x ULN | 1.6–2.0 x ULN | 2.1–3.0 x ULN | >3.0 x ULN |
| **Alkaline phosphatase (IU/L)** | 1.1–2.0 x ULN | 2.1–3.0 x ULN | 3.1–10 x ULN | >10 x ULN |
| **Albumin: hypoalbuminaemia (g/L)** | 28–31 | 25–27 | <25 | Not applicable |
| **C-reactive protein** | >10-30 | 31-100 | 101-200 | >200 |

*Table 9 Assessment of adverse events for laboratory adverse events (taken from Salmonella Vaccine Study in Oxford (SALVO) Trial Protocol[1])*

### 2.2.3 Secondary Objective: Immunogenicity of the iNTS-GMMA Vaccine

The secondary objective of the study is the assessment of immunogenicity of the iNTS-GMMA vaccine as measured by an O: 4, 5, 9 antigen ELISA at time points D0, D28, D56, D84, D168, D196 and D350. This is planned to be performed by GSK Vaccine Institute for Global Health.

### 2.2.4 Exploratory Objectives: Further Immunogenicity analysis of the iNTS-GMMA vaccine

The exploratory objectives consist of an analysis of the broader immune responses to the iNTS-GMMA vaccine and include an analysis of functional antibody, B cell and T cell responses. Further details of the samples and exploratory analyses being performed are included in table 10. As part of this thesis, immune response following the primary iNTS-GMMA vaccine (Vaccine 1, D0 and 2, D56) up to Vaccine 2 + 28 days is assessed using GMMA-based ELISAs, functional antibody assays, antibody-dependent monocyte phagocytosis (ADMP), antibody-dependant NK activation (ADNKA) and T cell proliferation assays. The method and material for these assays are outlined in this chapter.

| Sample | Analysis |
|---|---|
| Serum | GMMA IgG ELISA, Antibody Dependant Monocyte Phagocytosis (ADMP), Antibody Dependant NK Activation (ADNKA) |
| PBMC | T Cell proliferation assays |
| Oral Fluid | OMV, O:4, 5, 9 IgG and IgA ELISA |

*Table 10 Sample collection and planned analysis*

## 2.2.5 Screening, Eligibility and Enrolment

Participants are screened and enrolled according to the eligibility criteria set out in table 11. Potential volunteers are directed to a pre-screening questionnaire and, if provisionally eligible, undergo telephone screening followed by in-person screening where formal consent, vital signs, physical examination, blood, urine and pregnancy (in female participants) tests are undertaken to complete the assessment of eligibility.

| | |
|---|---|
| Inclusion criteria | Willing and able to give informed consent for participation in the study |
| | Aged between 18-55 years inclusive |
| | In good health as determined by:<br>- Medical history<br>- Physical examination<br>- Laboratory assessment<br>- Clinical judgement of the investigators |
| | Willing to use highly effective contraception from one month prior to receiving the first vaccine and for the duration of the study (Females) |
| | Able to attend the scheduled visits and to comply with all study procedures, including internet access for the recording of diary cards |
| | Willing to allow his or her General Practitioner and/or Consultant, if appropriate, to be notified of participation in the study |
| | Willing to provide their national insurance number or passport number to be registered on The Over-Volunteering Prevention System (TOPS) |
| Exclusion criteria | History of significant organ/system disease that could interfere with the trial conduct or completion in the clinical judgement of the investigators. |
| | Have any known or suspected impairment or alteration of immune function. |
| | Study significant abnormalities on screening investigations, that are either unlikely to resolve or do not resolve on repeat testing |
| | Prior history of receipt of an oral typhoid vaccine (e.g.: Ty21a) within the last three years or a paratyphoid vaccine (as part of a clinical trial) |
| | Prior history of participation in a Typhoid or Paratyphoid controlled human infection study |
| | Receipt of a live vaccine within 4 weeks prior to vaccination or a killed vaccine within 7 days prior to vaccination |
| | Plan to receive any vaccine other than the study vaccine within 4 weeks after any study vaccination (except for COVID-19 vaccines) |
| | History of allergy or anaphylaxis to a previous vaccine or vaccine components |
| | Receipt of immunoglobulin or any blood product transfusion within 3 months of study start |
| | Participation in another research study involving an investigational product or that which may compromise the integrity of the study |
| | Inability, in the opinion of the Investigator, to comply with all study requirements including likelihood of successful venepuncture during the trial |
| | Female participants who are pregnant, breastfeeding/lactating or planning pregnancy during the course of the study |
| | Weight less than 50kg or a BMI < 18.4 kg/$m^2$ or a BMI > 40 kg/m2 |
| | Any other significant disease or disorder which, in the opinion of the Investigator, may:<br>- Put the participants at risk because of participation in the study<br>- Influence the result of the study<br>- Impair the participant's ability to participate in the study |

*Table 11 SALVO Eligibility Criteria (taken from Salmonella Vaccine Study in Oxford (SALVO) Trial Protocol[1])*

### 2.2.6 Drug and Safety Monitoring Committee (DSMC) Safety Review (Up to D84)

The DSMC will review the safety data at three formal time points up to D84. The first following seven days of safety data after the first six participants are vaccinated in group 1 (lower dose iNTS-GMMA vaccine versus placebo) to confirm progression to group 2 (full dose iNTS-GMMA vaccine versus placebo) and the vaccination of the second cohort of six participants. The second DSMC safety review will take place following seven days of safety data after the six participants in group 2 receive their first vaccination to decide on progression to group 3 (full dose iNTS-GMMA vaccine versus placebo). The third safety review will take place seven days after the second vaccination in group 3. These time points are summarised in figure 3.

### 2.2.7 COVID-19 Provisions

The study will take place towards the end of the COVID-19 pandemic. At this time, the unpredictability of both the longevity and progression of the pandemic required multiple safety and scientific validity provisions to be put in place. These are outlined in the SALVO Protocol[1], including the provision to allow for participant COVID-19 vaccination should they become eligible and offered this vaccine, and modification of study procedures should participants develop COVID-19.

## 2.3 Proteomic analysis to define the protein antigenic repertoire of SEn and STm GMMA

### 2.3.1 Materials and Reagents

| Key Reagents | Company |
|---|---|
| SEnGMMA | GVGH |
| STmGMMA | GVGH |
| Salmonella Enteritidis stock (CMCC4314; GVGH1835) | GVGH |
| Salmonella Typhimurium stock (D23580; GVGH1842) | GVGH |
| LB Broth Powder | Sigma |
| Phosphate Buffered Saline Tablets | Sigma |
| Lysozyme | Thermo Scientific |
| Pierce BCA Protein Assay Kit* | Thermo Scientific |
| EasyPep Mini MS Sample Prep Kit** | Thermo Scientific |

*Table 12 Materials and reagents for peptide preparation prior to mass spectrometry*

*Pierce BCA Protein Assay kit containing:
- Bovine serum albumin standard (BSA)
- Reagent A
- Reagent B

** EasyPepMini MS Sample Prep kit containing:
- Lysis Solution
- Reduction Solution
- Alkylation Solution
- Digestion Solution
- Elution Solution
- Wash Solution A
- Wash Solution B
- Nuclease
- Trypsin/Lys-C Protease
- Clean-up column

## 2.3.2 Reagent Preparation

Luria-Bertani (LB) Broth:

20g Luria Bertani powder (*Sigma-Aldrich, Dorset, UK;* L3522-250G) was dissolved in 1L of sterile water and autoclaved to sterilise according to the manufacturer's instructions.

1x Phosphate Buffered Saline (PBS):

2x PBS (*Sigma-Aldrich, Dorset, UK;* P2272) tablets were dissolved in 400mL of sterile water and autoclaved to sterilise according to the manufacturer's instructions.

## 2.3.3 Bacterial and GMMA Preparation

*Salmonella* Enteritidis (SEn) [CMCC4314; GVGH1835] and S*almonella* Typhimurium (STm) [D23580; GVGH1842] were thawed and cultured in Luria-Bertani (LB) Broth overnight for 18-24 hours at 37°C in a CO2 shaking incubator. 1mL at $5x10^8$ bacterial CFU/mL of each strain was centrifuged at 16,000g for 5 minutes, and the pellet was retained for lysis.

SEn GMMA and STm GMMA were thawed, centrifuged at 6000rpm for 5 minutes, and re-suspended in 1xPBS to a concentration of 300 µg/ml. Each sample was then centrifuged at 6000 rpm for 5 minutes, and the pellet was retained for lysis.

## 2.3.4 Bacterial and GMMA Lysis

Bacterial and GMMA lysis proceeded as per EasyPep™ Mini MS Sample Prep Protocol (*Thermofisher Scientific*)[147]. Lysis Buffer (200µL), 50mg/mL Lysozyme Solution (2µL) [*Thermofisher Scientific, Massachusetts, USA;* A40006] and Universal Nuclease (2µL)

[*Thermofisher Scientific, Massachusetts, USA;* A40006] was added to each sample pellet in turn. Each sample was centrifuged at 16,000g for 5 minutes with the supernatant retained and made up to 1mL using EasyPep Lysis Buffer. Protein concentrations were measured using a Pierce BCA Protein Assay Kit (*Thermofisher Scientific, Massachusetts, USA;* 23227) according to the manufacturer's instructions.

## 2.3.5 Peptide digestion and clean-up

Two methods of peptide digestion and clean-up were tested with the intention of using the most appropriate single method for further biological replicates.

*Method 1: Deactivation of bacteria and GMMA to produce lysate material for on-site peptide digestion and clean up*

All reagents used for this method were from the EasyPep™ Mini MS Sample Kit (*Thermofisher Scientific, Massachusetts, USA;* A40006), and the process was as per the EasyPep™ Mini MS Sample Prep Protocol (*Thermofisher Scientific*)[147]. Protein samples 200 µL at 1mg/mL (bacteria and GMMAs) were reduced and alkylated using 5µL of Reduction Solution (*Thermofisher Scientific, Massachusetts, USA;* A40006) and 50µL of Alkylated Solution (*Thermofisher Scientific, Massachusetts, USA;* A40006). The samples were then heated to 95°C for 10 mins to complete reduction and alkylation. Each protein sample was digested with 50µL Trypsin/Lys-c Protease solution (*Thermofisher Scientific, Massachusetts, USA;* A40006) and incubated at 37°C for 3 hours, at which time 50µL Digestion Stop Solution (*Thermofisher Scientific, Massachusetts, USA;* A40006) was added. Each peptide sample was centrifuged at 1500g for 2 minutes within the peptide clean-up

column and washed once with 300µL Wash A Solution (*Thermofisher Scientific, Massachusetts, USA;* A40006) [centrifuged at 1500g for 2 minutes] and twice with Wash B Solution (*Thermofisher Scientific, Massachusetts, USA;* A40006) and finally with Elution Solution with retention of the peptide containing eluate. The cleaned peptide solution was then transferred to the Target Discovery Institute for mass spectroscopy.

*Method 2: Deactivation of bacteria and GMMA to produce lysate material for off-site digestion and clean-up*

Protein samples 200 µL at 1mg/mL were heated to 95°C for 10 minutes and transported to collaborators (R. Fischer and S. Flannery) at The Target Discovery Institute for downstream peptide digestion and clean-up.

## 2.3.6 Mass Spectrometry Protein Identification

Three biological replicates were processed at 200ng of peptide mix per sample. The peptides were separated on a 1h nanoLC (*SCIEX, USA*) gradient and analysed on the Q Exactive mass spectrometer (*Thermo Scientific, USA*). Samples were run together to reduce batch effect. Results were then analysed in MaxQuant (*Max-Planck-Institute of Biochemistry, Germany*)[148] using *S*. Typhimurium (strains D23580, SL1344) and *S*. Enteritidis (strain P125109) reference databases to identify and quantify unique proteins present by Label-Free Quantification (LFQ) Intensity. Abundance was assigned to each protein, as measured by log10 Label-Free Quantification (LFQ) intensity using MaxQuant[148] for raw LFQ values and R *(R Core Team, Vienna, Austria)*[149] / R Studio *(Version 2023.03.0+386, RStudio Team, Boston, USA)*[150] for determining log10 values.

Collaborators (R. Fischer and S. Flannery) performed Mass Spectroscopy and MaxQuant[148] analysis at the Target Discovery Institute, University of Oxford.

### 2.3.7 Data Analysis

Raw data files in excel format were analysed as outlined in *Chapter 5.2.*

## 2.4 Protein Production

Protein production was performed by collaborators at the University of Oxford. The protein production method courtesy of Dr Young Kim is included in Appendix A.

In brief, genes for the selected proteins were (OmpA, OmpC, OmpF, OmpD, SlyB, FkpA, LptA, YnCE, CdtB, HlyE and Flagellin) inserted into expression plasmids in *E. coli* BL21(DE3) pLysS strains. Once cultured overnight at 37°C in LB broth, the bacterial cells were harvested and pelleted. Cells were lysed, sonicated and resuspended in 50 mM Tris HCl buffer (pH 8) containing 150 mM NaCl and 10 mM imidazole. The supernatants were then filtered in a 0.45 μm membrane and purified by affinity chromatography.

## 2.5 Non-SALVO Sera, PBMCs and Monoclonal Antibodies

### 2.5.1 Pooled Non-Typhoid *Salmonella* Sera and Monoclonal Antibodies

Three sets of pooled sera were used in the development of the SEn and STm GMMA IgG ELISA and functional antibody assays. The most important serum used for development of the standard curve in all ELISAs, and functional antibody assays was the 'pooled (convalescent) serum'. This serum consisted of pooled convalescent sera from 10 sub-Saharan African patients taken between 14 days and 3 months after NTS bacteraemia (the identification one of the ten samples used in the pooled sera was changed from NTS to *S.* Typhi following whole genome sequencing; samples from the SETA study[151]). Two further sera included pooled Malawian (high endemicity[152 153]) and pooled UK (low endemicity[20]) serum which was a control serum obtained from healthy adult donors.

| Sera / Antibody | Details |
|---|---|
| SEn-specific serum | Single Convalescent Serum 14 days following *Salmonella* Enteritidis Bacteraemia, High O:9 antibody level (SETA Study[151]) |
| STm-specific serum | Single Convalescent Serum 14 days following *Salmonella* Typhimurium Bacteraemia, High O:9 antibody level (SETA Study[151]) |
| Pooled (convalescent) Serum | Pooled Sera 14 days to 3 months following NTS Bacteraemia – combination of SEn / STm / Unspecified NTS Sera (SETA Study[151]) |
| Pooled Malawian Serum | Pooled Non-specific Sera from Healthy Adults in Malawi (high endemicity[152 153]) |
| Pooled UK Serum | Pooled Non-specific Sera from Healthy Adults in UK (low endemicity[20]) |
| Rabbit Polyclonal Salmonella Antibody | Unconjugated Rabbit IgG polyclonal (*S.* Enteritidis, *S.* Typhimurium, *S.* Heidelburg) [*Thermofisher Scientific, Massachusetts, USA*; PA1-7244] |

*Table 13 Pooled sera and polyclonal antibody used in ELISA and functional antibody optimisation*

### 2.5.2 *S.* Typhi and *S.* Paratyphi A Sera and PBMCs from Controlled Human Infection Studies (CHIM)

Baseline (D0) and D28 post *S.* Typhi and *S.* Paratyphi A bacteraemia samples from UK volunteers in controlled human infection studies were identified. Sera and peripheral blood mononuclear cells (PBMCS) from P1[154], PATCH[155] and VAST[83] studies, were used to investigate antibody, monocyte activation and T-cell cross-reactivity in Chapters *6* and *7.*

### 2.5.3 Standard and Quality Controls for SEn and STm Assays

Pooled *Salmonella* sp. convalescent sera, quality controls, and positive and negative controls were used as the standard serum across all assays. These sera were obtained from the SETA study[151]. Different controls were used for SEn and STm assays (table 14 and 15).

| Standards and Quality Controls - SEn | | NTS Convalescent Serum | Dilutions |
|---|---|---|---|
| Quality Controls | Upper Limit | 40535V1 | 1:2000 |
| | | 11569V1 | 1:2000 |
| | Lower Limit | 40155V3 | 1:2000 |
| | | 10903V4 | 1:1000 |
| Positive Control | | 11451V1 | 1:2000 |
| Negative Control | | 40155V1 | 1:2000 |
| | | 40155V3 | 1:2000 |

*Table 14 Standards and quality controls (Sample IDs) for SEn assays*

| Standards and Quality Controls - STm | | NTS Convalescent Serum | Dilutions |
|---|---|---|---|
| Quality Controls | Upper Limit | 40535V1 | 1:2000 |
| | | 10903V4 | 1:1000 |
| | Lower Limit | 40155 V3 | 1:2000 |
| | | 11569V1 | 1:2000 |
| Positive Control | | 11451V3 | 1:2000 |
| Negative Control | | 40155V1 | 1:4000 |

*Table 15 Standards and quality controls (Sample IDs) for STm assays*

## 2.6 GMMA Enzyme-Linked Immunosorbent Assay (ELISA)

### 2.6.1 Materials and Reagents

| **Key Reagents** | **Company** |
|---|---|
| SEnGMMA | GVGH |
| STmGMMA | GVGH |
| 96 Well Nunc-Immuno Maxisorp Plate | Sigma |
| Pooled Malawian Serum | N/A |
| SEn Convalescent Sera | N/A |
| STm Convalescent Sera | N/A |
| Phosphate Buffered Saline Tablets | Sigma |
| Bovine Serum Albumin | Sigma |
| Tween20 | Sigma |
| Casein | Thermo Scientific |
| Carbonate-bicarbonate buffer | Sigma |
| TMB Solution (Highest Sensitivity) | AbCam |
| 450nm TMB Stop Solution | AbCam |
| Anti-Human IgG (γ-chain specific) peroxidase antibody | Sigma |
| Anti-Human IgA (alpha-chain specific) peroxidase antibody | Sigma |

*Table 16 Materials and Reagents for ELISAs*

### 2.6.2 Reagent Preparation

Carbonate-Bicarbonate Buffer:

2x Carbonate-bicarbonate capsules *(Sigma-Aldrich, Dorset, UK;* C3041) were emptied in 200mL of sterile water according to the manufacturer's instructions.

1% Bovine Serum Albumin (BSA) in 1x PBS:

4g Bovine Serum Albumin (*Sigma-Aldrich, Dorset, UK;* A7030) was added to 400mL of 1x PBS.

1xPBS – 5% PBS Tween:

50x PBS tablets was added to 1L of sterile water. 5mL Tween20 (*Sigma-Aldrich, Dorset, UK;* P1379) was added to the 10x PBS to create the stock. The stock solution was diluted in sterile water 1:10 to create 1xPBS – 5% PBS Tween for use.

## 2.6.3 Method

Nunc Maxisorp™ 96-well flat-bottom plates (*Thermofisher Scientific, Massachusetts, USA;* 243656) were coated overnight with 50µL per well of 3µg/mL SEn GMMA or STm GMMA in carbonate-bicarbonate buffer at pH 9.6. Plates were washed five times using 200µL PBS-Tween per well and blocked with 100µL Casein (*Thermofisher Scientific, Massachusetts, USA;* 37528) per well for 1 hour at 25°C. Plates were then washed five times using 200µL PBS-Tween per well. 50µL of test serum diluted, standard serum (serial dilution), quality controls (upper and lower limit), negative and positive controls in 1% Bovine Serum Albumin (BSA) in 1x PBS added to designated wells in duplicates and incubated for 2 hours at 25°C. Plates were then washed five times using 200µL PBS-Tween per well and 50µL of secondary antibody added:

- IgG [Anti-Human IgG (γ-chain specific) peroxidase antibody [*Sigma-Aldrich, Dorset, UK;* I3382], diluted at 1:5000 in Casein was added to each well and incubated for 1 hour at 25°C.
- IgA [Anti-Human IgA (alpha-chain specific) peroxidase antibody [*Sigma-Aldrich, Dorset, UK;* A0295], diluted at 1:2500 in Casein, was added to each well and incubated for 1 hour at 25°C.

Plates were then washed five times using 200µL PBS-Tween per well, and 100µL 3,3',5,5'-Tetramethylbenzidine (TMB) substrate (*Abcam, Cambridge, UK;* ab171523) was added to

each well. Plates were allowed to develop in the dark, and 100µl stop solution was added at ~10 mins. Plates were read at OD450 using a BioTek ELx808 Absorbance Microplate Reader (*BioTek Instruments, Vermont*; USA).

### 2.6.4 Statistical Analysis

Raw data was exported to Microsoft Excel (*Microsoft, Washington, USA*), and statistical analysis was performed using Prism version 10.0.0. (*Graphpad™, Boston, USA*). P-values <0.05 were considered significant.

## 2.7 Protein Enzyme-Linked Immunosorbent Assay (ELISA)

### 2.7.1 Materials and Reagents

Identical materials and reagents were used for the Protein ELISAs. Proteins were produced as per Section 2.6 / Appendix A: OmpA, OmpC, OmpF, OmpD, SlyB, FkpA, LptA, YnCE, CdtB, HlyE and Flagellin.

### 2.7.2 Reagent Preparation

Reagent preparation as previously described.

### 2.7.3 Method

Nunc Maxisorp™ 96-well flat-bottom plates were coated overnight with 50µL per well of 2µg/mL of each protein in carbonate-bicarbonate buffer at pH 9.6. Plates were washed five times using 200µL PBS-Tween per well and blocked with 100µL Casein per well for 1 hour at room temperature. Plates were then washed five times using 200µL PBS-Tween per well. 50µL of test serum diluted, standard serum (serial dilution), quality controls (upper and lower limit), negative and positive controls in 1% Bovine Serum Albumin (BSA) in PBS added to designated wells in duplicates and incubated for 2 hours at room temperature. Plates were then washed five times using 200µL PBS-Tween per well. 50µL of secondary antibody was then added dependant on IgG or IgA assay:

For IgG ELISA: Anti-Human IgG (γ-chain specific) peroxidase antibody, diluted at 1:5000 in Casein, was added to each well and incubated for 1 hour at room temperature.

For IgA ELISA: Anti-Human IgA (alpha-chain specific) peroxidase antibody, diluted at 1:2500 in Casein, was added to each well and incubated for 1 hour at room temperature.

Plates were then washed five times using 200µL PBS-Tween per well, and 100µL 3,3',5,5'-Tetramethylbenzidine (TMB) substrate was added to each well. Plates were allowed to develop in the dark and 100 µl stop solution was added at 10 mins. Plates were read at OD450 using a BioTek ELx808 Absorbance Microplate Reader.

## 2.7.4 Statistical Analysis

Raw data was exported to Microsoft Excel (*Microsoft, Washington, USA)*, and statistical analysis was performed using Prism version 10.0.0. (*Graphpad$^{TM}$, Boston, USA*). P-values <0.05 were considered significant.

## 2.8 Antibody Dependent Monocyte Phagocytosis (ADMP)

### 2.8.1 Materials and Reagents

| Key Reagents | Company |
|---|---|
| SEnGMMA | GVGH |
| STmGMMA | GVGH |
| Salmonella Enteritidis stock (CMCC4314; GVGH1835) | GVGH |
| Salmonella Typhimurium stock (D23580; GVGH1842) | GVGH |
| SEn O:9 Antigen | GVGH |
| STm O:4, 5 Antigen | GVGH |
| 1µm red FluoSpheres™ carboxylate-modified microspheres | Thermo Scientific |
| 1µm blue FluoSpheres™ carboxylate-modified microspheres | Thermo Scientific |
| DMTMM | Sigma |
| PD-10 Sephadex desalting column | Cytiva |
| Phosphate Buffered Saline Tablets | Sigma |
| Bovine Serum Albumin | Sigma |
| Tween20 | Sigma |
| LB Broth Powder | Sigma |
| Vibrant Dil | Thermo Scientific |
| Atto NHS Ester | Sigma |
| THP-1 Cells | TIB-202, *ATCC* |
| RPMI 1640 | Sigma |
| Fetal Bovine Serum | Gibco |
| Penicllin and Streptomycin | Gibco |
| L-Glutamine | Gibco |
| Mercaptoethanol | Gibco |
| 96-well round bottom plate | Thermo Scientific |
| 4% Paraformaldehyde | Thermo Scientific |

*Table 17 Materials and Reagents for Antibody-Dependent Monocyte Phagocytosis (ADMP) Assay*

### 2.8.2 Reagent Preparation

R10: 60mL from a 500mL RPMI-1640 (*Sigma-Aldrich, Dorset, UK; R5886-500ML*) was discarded and replaced with 50mL Fetal Bovine Serum (FBS)[*Sigma-Aldrich, Dorset, UK; F7524-500ML*], 5mL Penicillin / Streptomycin (*Thermofisher Scientific, Massachusetts,*

*USA; 15070063*), 5mL L-Glutamine (*Thermofisher Scientific, Massachusetts, USA; 25030081*) and 500µg Mercaptoethanol.

## 2.8.3 Method

### OAg-Bead Conjugation

O:4 and O:5 antigens (*Salmonella* Typhimurium, STm) and O:9 antigen (*Salmonella* Enteritidis, SEn) were separately conjugated to 1µm red FluoSpheres™ carboxylate-modified microspheres (*Thermofisher Scientific, Massachusetts, USA;* F8816) using the method outlined in Schlottmann et al, 2006[156]. In short, 80µL of 200mg/mL 4-(4,6-Dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMTMM)[Sigma-Aldrich*, Dorset, UK;* 74104-1G-F] diluted in distilled water was added to 1mL of each of SEn and STm O-antigens. Samples were incubated on a rolling incubator at room temperature for 40-60 minutes. DMTMM-modified O-antigen was separated and collected using a PD-10 Sephadex desalting column (*Cytiva, Massachusetts, USA; 17085101*). 500µL of purified DMTMM-modified O-antigen was then sonicated with 10µL and 100µL of the FluoSphere™ to create separate stock solutions of 10µL and 100µL SEn and STm O-Antigen conjugated Fluospheres™. Samples were vortexed and incubated overnight in the dark at room temperature on a shaker incubator. Samples were then washed twice with 2mL PBS and centrifuged at 13000rpm for 2 minutes and re-suspended in 1ml 1% BSA in PBS and stored at 4°C until use.

## Bacterial Culture

SEn and STm bacteria were cultured in LB Broth overnight for 16-18 hours, washed and re-suspended in PBS to achieve a concentration of $1x10^6$ cells/mL.

## Vibrant Dil Staining

Bacteria and GMMAs were independently incubated with 1mg/mL Vibrant Dil (*Thermofisher Scientific, Massachusetts, USA;* V22885*)* at a ratio of 5µL:400µL $1x10^6$ cells/mL bacteria and 2.5µL:400µL 0.3mg/mL GMMA for 20 minutes and then washed three times and re-suspended in PBS. GMMAs and bacteria were centrifuged for 5 minutes at 6000rpm and 13000rpm, respectively.

## Atto 488 NHS Ester Staining

Bacteria and GMMAs were independently incubated with 2mg/mL Atto NHS Ester (*Sigma-Aldrich, Dorset, UK;* 72464-1MG-F) at a ratio of 7µL:400µL $1x10^6$ cells/mL bacteria and 3.5µL:400µL 0.3mg/mL GMMA for 60 minutes and then washed three times and re-suspended in PBS. GMMAs and bacteria were centrifuged for 5 minutes at 6000rpm and 13000rpm, respectively.

## THP-1 Cell Preparation

THP-1 (*ATCC, Virginia, USA;* TIB-202) cells were cultured in R10 at 37°C and passaged to maintain a cell concentration $2x10^{5}$ cells/mL.

## ADMP Assay

The ADMP assay was modified from the method described in Ackerman et al (2011)[157]. 10µL/well of target antigen (SEn/STm OAg-Bead, SEn/STm GMMA, SEn/STm bacteria) was incubated at 37°C for 2 hours with 100uL/well of diluted serum/plasma in a 96-well round bottom plate. Control wells were positive control, negative control, quality controls and wells with no serum/plasma with stained antigen, unstained antigen and/or no antigen. After incubation, the plates were centrifuged at 2000 rpm for 10 minutes and re-suspended 200µL/well with THP-1 cells at $1.25x10^5$ cells/mL. The samples were then incubated overnight for 16-18 hours at 37°C. The plates were centrifuged at 500g for 5 minutes and fixed with 100µL/well 4% paraformaldehyde (PFA)[ *Santa Cruz Biotechnology, Dallas, Texas;* SC281692] in the dark at room temperature for 15 minutes. Samples were then washed and re-suspended in 100µL/well 1% PBS. Plates were stored at 4°C in the dark until flow cytometric analysis.

## 2.8.4 Flow Cytometric Analysis

Flow cytometric analysis was performed using a BD LSRFortessa™ Cell Analyser (*BD, New Jersey, USA*) and BD FACSDiva™ (*BD, New Jersey, USA*). FCS files were exported and analysed using BD FlowJo™ (*BD, New Jersey, USA*). Each sample was gated according to the ADMP gating strategy (figure 5).

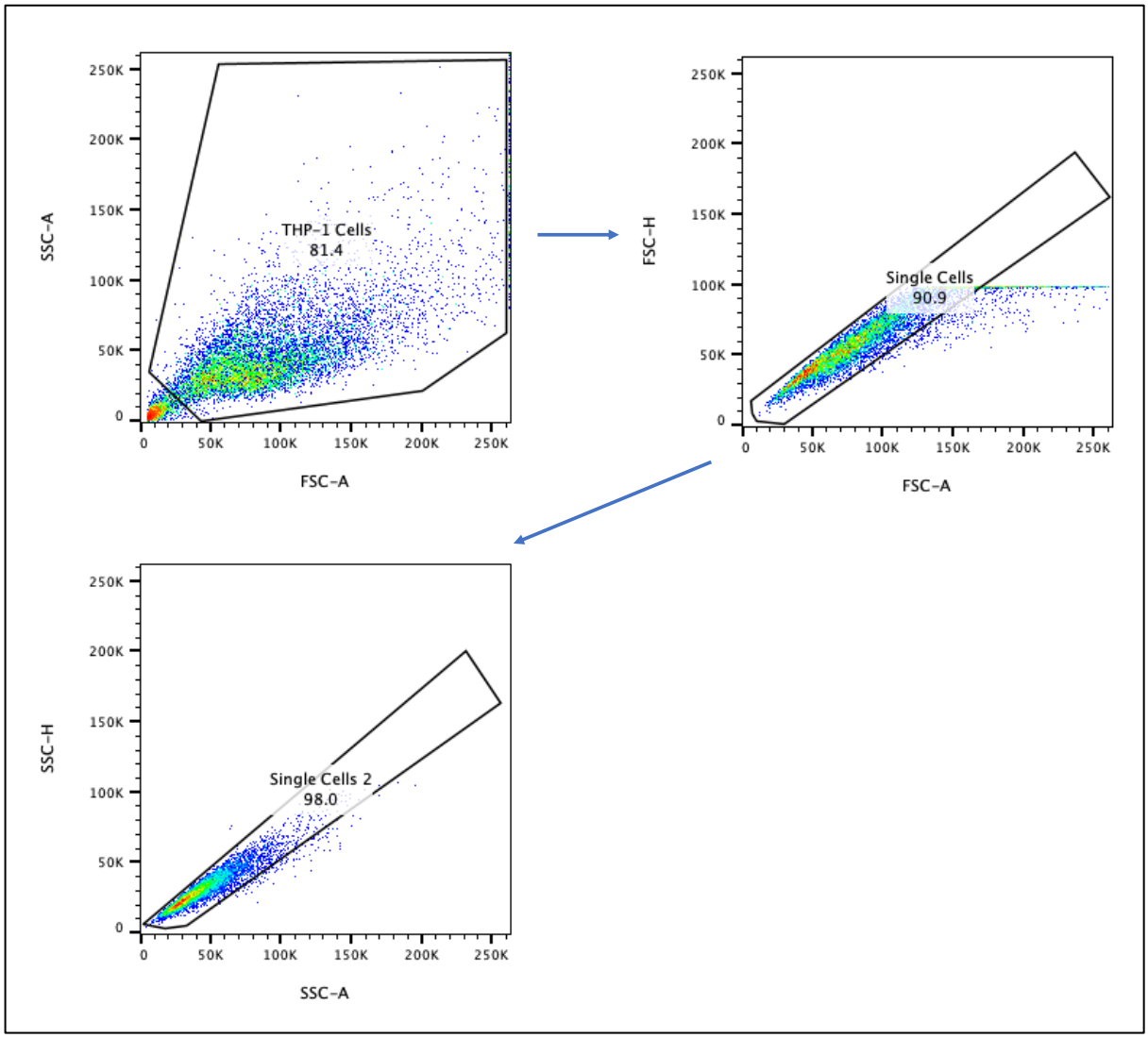



*Figure 5 Gating Strategy for Antibody-Dependent Monocyte Phagocytosis Assay*

Median fluorescence intensity (MedFI) was the measure used to quantify THP-1 phagocytosis for the GMMA-based ADMP. The phagocytic score was the measure used to quantify THP-1 phagocytosis for the OAg bead-based ADMP. The phagocytic score is calculated by % bead positive THP-1 cells x mean fluorescence intensity[157]. The phagocytic score could not be used as an output measure for the GMMA-based ADMP due to varying sizes of GMMA, leading to a skew in the final output measure.

## 2.8.5 Statistical Analysis

Raw data were exported to Microsoft Excel (*Microsoft, Washington, USA*), and statistical analysis was performed using Prism version 10.0.0. (*Graphpad™, Boston, USA*). P-values <0.05 were considered significant.

## 2.9 Antibody Dependent NK Activation (ADNKA)

### 2.9.1 Materials and Reagents

| Key Reagents | Company |
|---|---|
| SEnGMMA | GVGH |
| STmGMMA | GVGH |
| 96 Well Nunc-Immuno Maxisorp Plate | Sigma |
| Phosphate Buffered Saline Tablets | Sigma |
| Bovine Serum Albumin | Sigma |
| Tween20 | Sigma |
| Casein | Thermo Scientific |
| Carbonate-bicarbonate buffer | Sigma |
| RPMI 1640 | Sigma |
| Fetal Bovine Serum | Gibco |
| Penicllin and Streptomycin | Gibco |
| L-Glutamine | Gibco |
| Mercaptoethanol | Gibco |
| Dulbecco's Modified Eagle's Medium | Sigma |
| AutoMACS Running Buffer | Miltenyi Biotec |
| Dulbecco's Phosphate Buffered Saline | Sigma |
| 96-well V bottom plate | Greiner Bio-One |
| CompBead Anti-Mouse Ig, Negative Control Compensation | BD |
| Cytofix/Cytoperm Fixation/Permeabilisation | BD |
| PE Mouse Anti-Human CD107a | BD |
| BV786 Mouse Anti-Human CD56 | BD |
| APC-Cy7 Mouse Anti-Human CD5 | BD |
| Fixable Viability Stain 780 | BD |
| GolgiStop Protein Transport Inhibitor (Containing Monensin) | BD |
| Brefeldin A | BD |
| Natural killer cell line retroviral transduced to express human CD16, PTA-8836 | ATCC |
| Alpha minimum essential medium | Gibco |
| Horse Serum | Sigma |
| Myo-inostol | Sigma |
| Folic Acid | Sigma |
| Non-essential Amino Acids (100x) | Gibco |
| Sodium Pyruvate (100mM) | Gibco |
| Human Interleukin-2 | Roche |
| Benzonase Nuclease | Sigma |

*Table 18 Materials and reagents for the Antibody Dependent NK Activation (ADNKA) Assay*

## 2.9.2 Reagent Preparation

NK Culture Media: 126mL from 500mL Alpha Minimal Essential *(Gibco, Thermofisher Scientific, Massachusetts, USA;* 12561056*)* was discarded and replaced with 50mL FBS, 50mL Horse Serum (Sigma-Aldrich*, Dorset, UK;* H1270-500mL), 5mL Myo-Inositol (Sigma-Aldrich*, Dorset, UK;* I5125*)*, 62.5µL Folic Acid (Sigma-Aldrich*, Dorset, UK;* F8758*)* , 1mL 2-mecaptoethanol, 5mL L-Glutamine, 5mL non-essential amino acid (*Gibco, Thermofisher Scientific, Massachusetts, USA;* 11140050*)*, 5mL Pyruvate (*Gibco, Thermofisher Scientific, Massachusetts, USA;* 11360070*)* and 5mL Penicillin/Streptomycin.

ADNKA Media: 50mL from a 500mL Dulbecco's Modified Eagle's Medium (Sigma-Aldrich*, Dorset, UK;* D6546*)* was discarded and replaced with 50mL FBS and 5mL L-Glutamine.

## 2.9.3 Method

The ADNKA assay was modified from the method described in Barrett et al (2021)[158].

### NK Cell Preparation

NK Cells (*ATCC, Virginia, USA;* PTA-8836 NK cells expressing CD16 with a transfected green fluorescent protein) were thawed in NK culture media with 2µL Benzonase (Sigma-Aldrich*, Dorset, UK;* E1014-25KU*)*. The cells were washed in NK culture media (centrifuged at 125g for 10 minutes and resuspended) to 300,000 cells/mL. 1:100 IL-2 (Roche, Basel, Switzerland; 11011456001) was added to the NK cells and incubated at 37°C in a $CO_2$ incubator and expanded over 7-8 days. Cells were then passaged every 3-4 days using 50mL NK culture media and 500µL IL-2 to maintain the cell line.

## ADNKA Assay

Nunc Maxisorp™ 96-well flat-bottom plates were coated overnight with 50µL per well of 3µg/mL SEn GMMA or STm GMMA in carbonate-bicarbonate buffer at pH9.6. Plates were washed five times using 200µL PBS-Tween per well and blocked with 100µL Casein (*Thermofisher Scientific*) per well for 1 hour at 25°C. Plates were then washed five times using 200µL PBS-Tween per well and 50µL of samples (including positive, negative and quality controls) in 1% Bovine Serum Albumin (BSA) in PBS added to designated wells and incubated for 2 hours at 25°C. 1% BSA in PBS was used as a negative control. Plates were then washed five times using 200µL PBS-Tween per well.

The NK cell line was transferred to ADNKA Media, with the addition of Brefeldin A (10µg/mL)[*BioLegend, San Diego, USA;* 420601], GolgiStop (4µl/6mL) [*BD, New Jersey, USA;* 554724], and CD107 marker (47.5µL/mL) [*BioLegend, San Diego, USA;* 328607]. NK Cells (without CD107 marker) were plated initially as one of the controls, followed by NK Cells (with CD107 marker) at 200µl per well and incubated at 37°C for 5 hours. After incubation, the NK cells were transferred to a 96-well v-bottom plate (*Corning, New York, USA;* 10462012), centrifuged at 300g for 10 minutes, and re-suspended with 100µL/well of 1xPBS. The plate was centrifuged at 300g for 10 minutes and re-suspended with 100µL Viability Staining (Diluted at 1 in 10000 in 1xPBS)[*BD, New Jersey, USA;* 565388]. After 10 minutes of incubation in the dark at room temperature were washed (300g for 10 minutes) with 100µL/well running buffer and finally resuspended in 100µL/well with Cytofix/Cytoperm [*BD, New Jersey, USA;* 555028]. After 20 minutes of incubation at 4°C, the samples were washed with 100µL/well running buffer and resuspended to a final

volume at 150uL/well Running Buffer (*Miltenyi Biotech, North Rhine-Westphalia, Germany;* 130-091-221). Plates were stored at 4°C in the dark until flow cytometric analysis. Compensation was calculated using NK Cell (B530/30, GFP), CD107a (YG586/15, PE), Viability Stain (R780/60, FVS780) and CD56 (V780/60, BV786) )[ *BD, New Jersey, USA;* 564058].

### 2.9.4 Flow Cytometric Analysis

Flow cytometric analysis was performed using a BD LSRFortessa™ Cell Analyser (*BD, New Jersey, USA*) and BD FACSDiva™ (*BD, New Jersey, USA*). FCS files were exported and analysed using BD FlowJo™ (*BD, New Jersey, USA*). Each sample was gated according to the ADNKA gating strategy (figure 6). The percentage of CD107 positive cells was used to quantify activated NK Cells.

### 2.9.5 Statistical Analysis

Raw data were exported to Microsoft Excel (*Microsoft, Washington, USA*), and statistical analysis was performed using Prism version 10.0.0. (*Graphpad™, Boston, USA*). P-values <0.05 were considered significant.

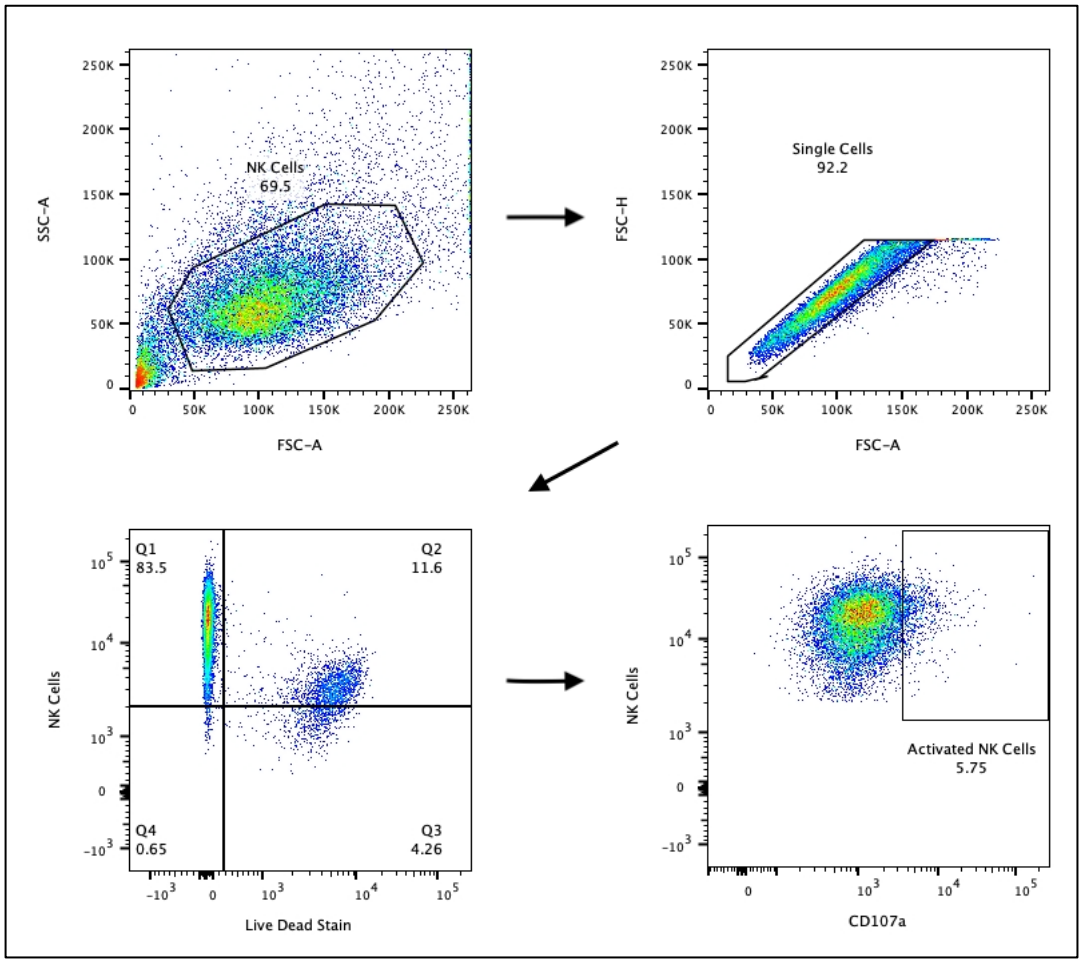



*Figure 6 Gating Strategy for Antibody Dependent NK Activation Assay*

## 2.10 GMMA Activation of Innate Immune System (Monocytes)

### 2.10.1 Materials and Reagents

| **Key Reagents** | **Company** |
|---|---|
| SEnGMMA | GVGH |
| STmGMMA | GVGH |
| 50mL Leucosep | Greiner |
| 15mL Lymphoprep | Stemcell Tech |
| AutoMACS Rinsing Solution | Miltenyi Biotec |
| RPMI 1640 | Sigma |
| Fetal Bovine Serum | Gibco |
| Penicllin and Streptomycin | Gibco |
| L-Glutamine | Gibco |
| Mercaptoethanol | Gibco |
| ACK Lysis Buffer | Thermo Scientific |
| Dulbecco's Phosphate Buffered Saline | Sigma |
| 96-well round bottom plate | Thermo Scientific |
| Brefeldin A | BD |
| *E. coli* 0111:B4 Lipopolysaccharide (LPS-EB Ultrapure) | Invivogen |
| Phosphate Buffered Saline | Sigma |
| LIVE/DEAD Fixable Aqua Dead Cell Stain | Thermo Scientific |
| 4% Paraformaldehyde | Thermo Scientific |
| Permeabilization Buffer | Thermo Scientific |
| CompBead Anti-Mouse Ig, Negative Control Compensation | BD |
| CD1c Brilliant Violet 711 | BD |
| CD14 PE/Cyanine7 | BD |
| CD16 APC/Fire 750 | BD |
| CD141 PerCP/Cyanine5.5 | BD |
| IL-6 APC | BD |
| IL-12/IL-23 p40 PE | BD |
| TNF alpha Brilliant Violet 605 | BD |
| CD33 Brilliant Violet 650 | BD |
| IL-1 Alexa Fluor 647 | BD |
| Heat Inactivated Human Sera AB Positive, Sterile-Filtered | Sigma |

*Table 19 Materials and reagents for the Monocyte Activation Assay*

## 2.10.2 Reagent Preparation

R10 with 5% Human Sera (instead of 10% FBS): 35mL from a 500mL RPMI-1640 was discarded and replaced with 25mL Fetal Bovine Serum (FBS), 5mL Penicillin / Streptomycin, 5mL L-Glutamine and 500µg Mercaptoethanol.

## 2.10.3 Method

### PBMC Isolation

Fresh whole blood (collected from *Salmonella* naïve donors) was decanted into a pre-prepared 50mL Leucosep (*Greiner, Kremsmünster, Austria; 227288)* tube containing 15mL Lymphoprep (*Stemcell Technologies, Vancouver, Canada; 07851*) and centrifuged at 1000g for 15 minutes. The PBMC layer was aspirated and washed with autoMACS Rinsing Solution (*Miltenyi Biotec, Bergisch Gladbach, Germany; 130-091-222*) by centrifugation at 300g for 10 minutes. The remaining cell pellet was re-suspended in R10 [45mL RPMI 1640 media (*Sigma-Aldrich, Dorset, UK; R5886-500ML*) + 5mL Foetal Calf Serum (*Sigma-Aldrich, Dorset, UK; F7524-500ML*) + 50µL Penicillin/Streptomycin (*Thermofisher Scientific, Massachusetts, USA; 15070063*) + 50µL L-Glutamine (*Thermofisher Scientific, Massachusetts, USA; 25030081*)].

### Leucocyte Isolation

Fresh whole blood was incubated for 5 minutes with ACK Lysis Buffer (*Thermofisher Scientific, Massachusetts, USA; A1049201*) in a ratio 1:9 and then centrifuged at 500g for 5 minutes. The cell pellet was resuspended in Dulbecco's Phosphate Buffered Saline

(*Sigma-Aldrich, Dorset, UK; D8537-500ML*) and centrifuged at 300g for a further 5 minutes. The remaining cell pellet was re-suspended in R10.

## Monocyte and DC Activation Assay

*Stimulation*

Leucocyte/PBMCs were plated into a 96-well round bottom plate at 500,000 cells per well and stimulated with serial dilutions between 0.01 to 100 micrograms/mL of SEnGMMA, STmGMMA in R10 media + Brefeldin A (*BioLegend, San Diego, USA;* 420601) for 5 hours at 37°C *E. coli* 0111:B4 Lipopolysaccharide (LPS-EB Ultrapure) [*InvivoGen, San Diego, California*;*tlrl-3pelps*] and non-stimulated wells served as the positive and negative controls respectively.

*Fixation*

Following incubation, cells were washed with 200µL Phosphate Buffered Saline (PBS) per well (1500rpm for 5 minutes). After further centrifugation at 1500 rpm for 5 minutes, 20µL LIVE/DEAD Fixable Aqua Dead Cell Stain (*Thermofisher Scientific, Massachusetts, USA; L34957*) diluted 1:200 in PBS was added to each well, vortexed and incubated in the dark for 15 minutes. The cells were then washed with 200µL PBS per well (1500 rpm for 5 minutes). Finally, the cells were centrifuged at 1500rpm for 5 minutes and re-suspended with 100µL 4% PFA and 100µL PBS per well. Cells were then incubated in the dark at 4°C overnight for 16-18 hours.

*Staining*

The cells were washed twice in 200µL Permeabilization Buffer (*Thermofisher Scientific, Massachusetts, USA; 00-8333-56*) [2000rpm for 5-10 minutes]. In preparation for application of the antibody cocktail, the cells were centrifuged at 2000rpm for 5 minutes. The 20µL antibody cocktail (dependent on the experiment) was prepared in Permeabilization Buffer and added to each well. After incubation in the dark for 30 minutes at room temperature 200µL permeabilization buffer was added to each well. The cells were then washed with 200µL PBS, and then resuspended in 180µL PBS (2000rpm for 5 minutes). Samples were stored at 2-8°C pending flow cytometric analysis.

| **Antibodies** | **Marker** | **Fluorochrome** | **LSR / Fortessa Detector** |
|---|---|---|---|
| Biolegend | CD1c | Brilliant Violet 711 | V710/50 |
| Biolegend | CD14 | PE/Cyanine7 | YG780/60 |
| Biolegend | CD16 | APC/Fire 750 | R780/60 |
| Biolegend | CD141 | PerCP/Cyanine5.5 | VB710/50 |
| Thermofisher | IL-6 | APC | V450/50 |
| Biolegend | IL-12/IL-23 p40 | PE | YG585/15 |
| Biolegend | TNF-α | Brilliant Violet 605 | V610/20 |
| Biolegend | CD33 | Brilliant Violet 650 | V670/30 |
| Thermofisher | IL-1 | Alexa Fluor 647 | R670/30 |

*Table 20 Example antibody cocktail for innate and monocyte cell activation assay*

## 2.10.4 Flow Cytometric Analysis

Flow cytometric analysis was performed using a BD LSRFortessa™ Cell Analyser (*BD, New Jersey, USA*) and BD FACSDiva™ (*BD, New Jersey, USA*). FCS files were exported and analysed using BD FlowJo™ (*BD, New Jersey, USA*). Each sample was gated according to the gating strategy below (figures 7 and 8).

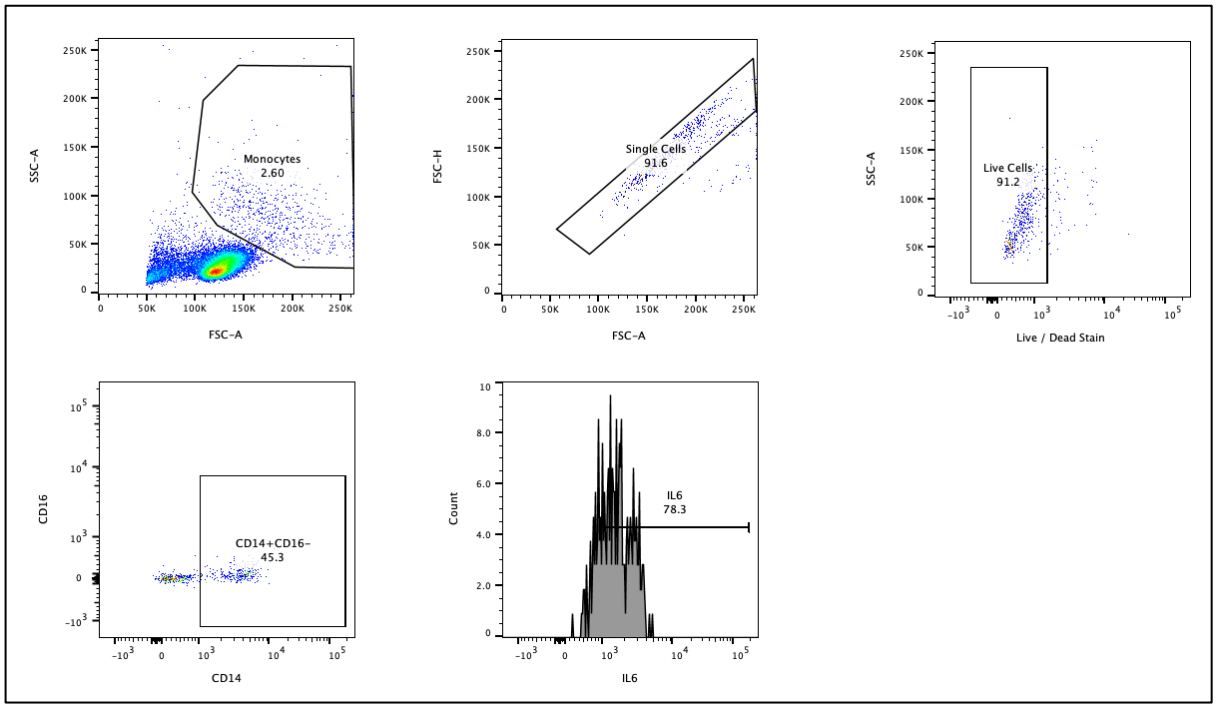



*Figure 7 Gating Strategy for Monocyte Activation Assay from Whole Blood Extraction*

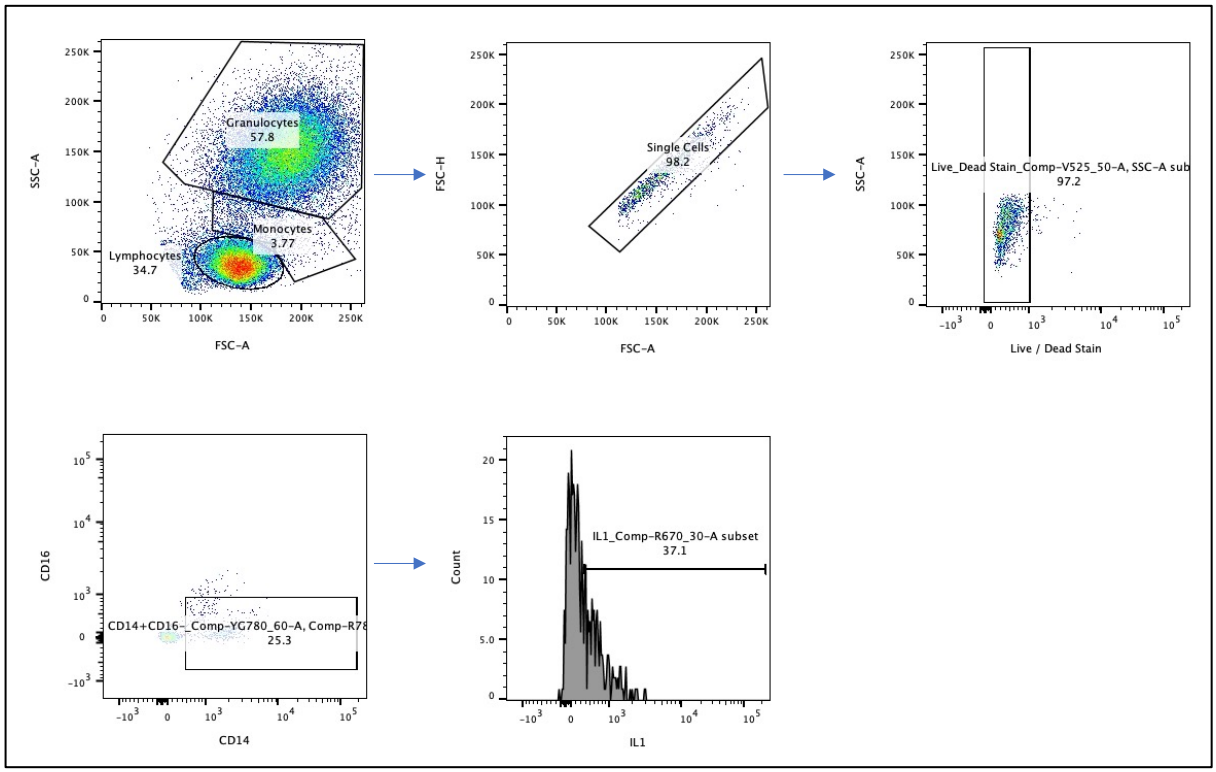



*Figure 8 Gating Strategy for Monocyte Activation Assay from PBMC Extraction*

## 2.11 GMMA and Protein T Cell Proliferation Assay

### 2.11.1 Materials and Reagents

| Key Reagents | Company |
|---|---|
| SEnGMMA | GVGH |
| STmGMMA | GVGH |
| Celltrace™ CFSE | Thermo Scientific |
| Phytohaemagglutinin P (PHA) | Invivogen |
| RPMI 1640 | Sigma |
| Fetal Bovine Serum | Gibco |
| Penicllin and Streptomycin | Gibco |
| L-Glutamine | Gibco |
| Mercaptoethanol | Gibco |
| 96-well round bottom plate | Thermo Scientific |
| Phosphate Buffered Saline | Sigma |
| LIVE/DEAD Fixable Aqua Dead Cell Stain | Thermo Scientific |
| 4% Paraformaldehyde | Thermo Scientific |
| Permeabilization Buffer | Thermo Scientific |
| CompBead Anti-Mouse Ig, Negative Control Compensation | BD |
| CD4 Alexa Fluor 700 | BD |
| CD8 PerCP/Cyanine5.5 | BD |
| CD3 Brilliant Violet 650 | BD |
| Heat Inactivated Human Sera AB Positive, Sterile-Filtered | Sigma |

*Table 21 Materials and reagents for the T Cell Proliferation Assay*

### 2.11.2 Reagent Preparation

Reagent preparation as per previous assays.

### 2.11.3 Method

#### CFSE Staining

PBMCs were washed twice in 5mL PBS (1500 rpm for 5 minutes). The final cell pellet was re-suspended in 2mL PBS. 2mL of diluted Celltrace™ CFSE (*Thermofisher Scientific,*

*Massachusetts, USA;* C34554) [1µL CFSE in 10mL PBS)] was added to the cells, vortexed and incubated in the dark for 10 minutes at room temperature. Staining was quenched with ice cold 10% sterile filtered FBS at 1:1 volume and incubated at 2-4°C for 5 minutes. The sample was then centrifuged at 1500rpm for 10 minutes and re-suspended in 10mL of R10 (5% Human Serum)[*Sigma-Aldrich, Dorset, UK;* H4522]. CFSE labelled cells were washed twice with R10 (5% Human Serum), resuspended in 5mL, and counted.

## Stimulation

CFSE labelled cells were plated onto a 96-well round bottom plate at 200,000-500,000 cells per well, dependent on experiment and stimulated with serially diluted SEn GMMA, STm GMMA, Phytohaemagglutinin P (PHA)[ *InvivoGen, San Diego, USA;* inh-phap], CEFTA (CD4) peptide pool (*Mabtech, Stockholm, Sweden;* 3617-1) positive control or no stimulation dependant on the experiment. Samples were then incubated for 4 days at 37°C. At day 4 the plates were centrifuged at 1500rpm for 3 minutes with 50% well volume replaced and resuspended with R10 (5% Human Serum). The plates were re-incubated for a further 3 days at 37°C.

## Fixation

Cells were washed with 200µL PBS per well (1800rpm for 5 minutes). Following centrifugation at 1500rpm for 5 minutes, 20µL of Aqua Live Dead Stain (diluted at 1:200) was added to each well and incubated in the dark for 15 minutes at room temperature. 200µL PBS per will was added and centrifuged at 1500rpm for 5 minutes. Finally, the wells were re-suspended in 100µL 4% paraformaldehyde (PFA) and 100µL 1% PBS per well.

Cells were then incubated in the dark at 4°C for at least 30 minutes in preparation for staining.

### Surface marker staining

Staining was performed using the same process to the monocyte activation assay and cells stored in the same conditions (section 2.10.3). Staining markers are listed in table 22 below.

| Antibodies | Marker | Fluorochrome | LSR / Fortessa Detector |
|---|---|---|---|
| Biolegend | CD4 | Alexa Fluor 700 | R730/45 |
| Biolegend | CD8 | PerCP/Cyanine5.5 | B710/50 |
| Biolegend | CD3 | Brilliant Violet 650 | V670/30 |

*Table 22 Antibody cocktail for T Cell proliferation assay*

## 2.11.4 Flow Cytometric Analysis

Flow cytometric analysis was performed using a BD LSRFortessa™ Cell Analyser (*BD, New Jersey, USA*) and BD FACSDiva™(*BD, New Jersey, USA*). FCS files were exported and analysed using BD FlowJo™(*BD, New Jersey, USA*). Each sample was gated according to the T cell proliferation assay gating strategy (figure 9). Phytohemagglutinin (PHA) control is used to demonstrate the gating strategy and delineation between $CD3^+CD4^+$ and $CD3^+CD8^+$ proliferating cells.

## 2.11.5 Statistical Analysis

Statistics were performed using Prism version 10.0.0. (*Graphpad™, Boston, USA*). P-values <0.05 were considered significant.

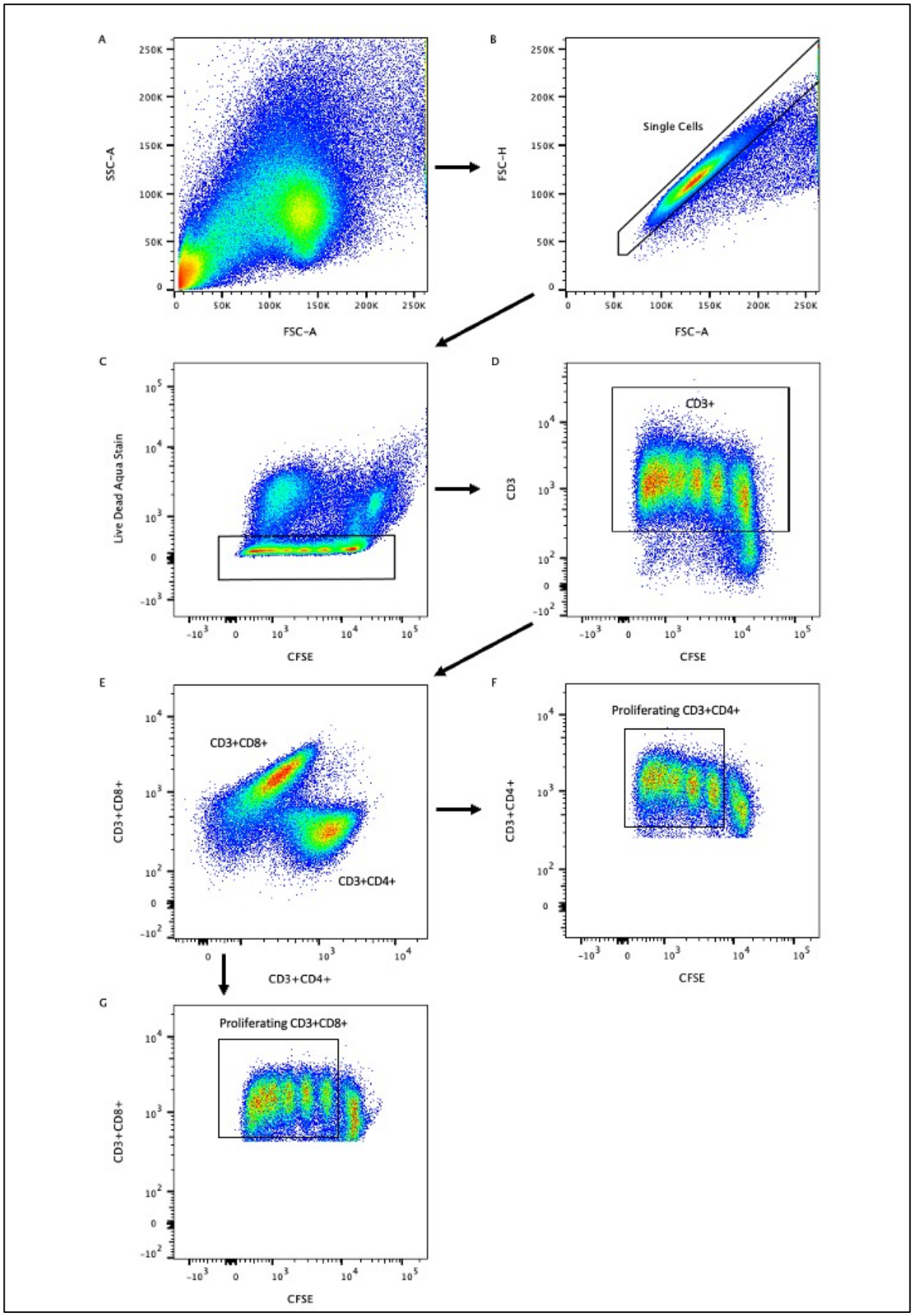



*Figure 9 Example Gating strategy to select proliferating CD3+CD4+ and CD3+CD8+; PHA control. Gating of lymphocyte population (a); single cells, FSC-A vs FSC-H (b); Live proliferating cells (c); CD3+ cells (d); CD3+CD4+ and CD3+CD8+ populations (e); proliferating CD3+CD4+ cells (f); and proliferating CD3+CD8+ cells (g)*

# Safety of the iNTS-GMMA Vaccine

## 3.1 Introduction

### 3.1.1 Invasive Non-Typhoid *Salmonella*

Invasive non-typhoid Salmonellosis (iNTS) is a neglected infectious disease with the highest disease burden in sub-Saharan Africa. Non-specific symptoms and limited availability of diagnostics lead to delays in treatment resulting in high mortality in adults and children[4 159]. There is no licensed vaccine, and the *Salmonella* Vaccine Study in Oxford (SALVO) is investigating one of the first vaccines against iNTS to proceed to human trials. In this phase 1 first-in-human clinical trial, the primary objective is to identify the safety and tolerability of the iNTS-GMMA Vaccine.

### 3.1.2 iNTS-GMMA Vaccine

The iNTS-GMMA vaccine consists of equal parts (by O-antigen content) of the outer membrane vesicles (OMVs) from genetically modified bacteria, including the two most common serovars to cause iNTS in sub-Saharan Africa, *Salmonella* Enteritidis (SEn) and *Salmonella* Typhimurium (STm)[7 160].

These OMVs are known as genetically modified membrane antigens (GMMAs), and contain many outer membrane components, including lipopolysaccharide with O-antigen, membrane proteins and other pathogen-associated molecular patterns (PAMPs)[121 126 161]. Stimulation of the innate immune system following recognition of PAMPs by pathogen recognition receptors (PRRs) stimulates a cascade of inflammatory cytokines, including IL-

6 and TNF alpha which produces both local and systemic side effects[77 126]. The mediators of this response are monocytes and neutrophils, which have a dual role in facilitating the innate response and presenting antigens to the adaptive immune system. This initial innate response mediated by the release of cytokines produces many of the symptoms experienced during acute bacterial infection[77]. Hypersensitivity reactions are less common but can be more severe and include IgE-mediated, immune-complex, and T cell-mediated reactions[162].

One of the essential PRR families is the Toll-like receptors (TLR) which are particularly important in regard to the GMMA. In particular, TLR4 recognises Lipopolysaccharide (LPS), TLR2 recognises lipoproteins, and TLR5 recognises flagellin[126 161]. In GMMAs, one of the most potent molecules identified to stimulate these receptors is the lipid A component of the LPS [126 161]. As such, mutations were introduced in *msbB* and *pagP* genes, which caused a structural change from the more reactogenic hexa- and hepta-acylated forms of LPS to the less reactogenic penta-acylated shown *in vitro* to stimulate less IL-6[126].

The SALVO clinical trial will examine the clinical reactogenicity elicited by the GMMAs and in particular determine whether the modification to the LPS limits the inflammatory cascade which might be stimulated from the lipid A component causing significant adverse reactions in human participants[163]. This thesis will examine the reactogenicity and tolerability of the vaccine up to 28 days after the second vaccinations. An acceptable safety profile will allow the progression of the vaccine trial and follow on trials of the iNTS-GMMA vaccine.

### 3.1.3 Pre-Clinical Safety Analysis

Prior to progression to this first-in-human study pre-clinical safety data has been assessed by the Medicines and Healthcare products Regulatory Agency (MHRA). This data includes the O-antigen based immunogenicity in mice studies, rabbit toxicity studies and in vitro human monocyte activation studies (See *SALVO Protocol*[1]).

### 3.1.4 Relevant Prior Trials

Over 200 participants have received a version of GMMA platform vaccines in clinical trials, with results showing a well-tolerated vaccine, although efficacy is yet to be determined[132] [164]. There have been five clinical trials involving GMMA vaccines against *Shigella,* including two phase I trials[129], one phase I extension trial[130], one phase 2a in an endemic population[131] and phase 2b controlled human infection trial[132]. Across all trials, the most common side effects were local, pain at the injection site. Regarding laboratory adverse events, 10 cases of neutropenia were noted in the first phase I trials, although these were transient with no sequelae[164].

The OMV platform vaccine with the most safety data is the 4CMenB vaccine (Bexsero) which contains a group B meningococcal OMV as the major component and is currently licensed for general use. Bexsero is well-tolerated but known to be reactogenic, causing fever in 69% of infants and injection site pain and headache in adolescents and adults[165].

### 3.1.5 Aims and Objectives

This chapter describes the interim safety and tolerability of the iNTS-GMMA vaccine at two dose levels, full dose iNTS-GMMA and lower dose iNTS-GMMA vaccine 28 days following the primary course (month 0 and month 2), contributing to the assessment of the primary safety objective of the *Salmonella* Vaccine Study in Oxford (SALVO) clinical trial. The specific objectives are outlined below:

1. The design, set-up, initiation and initial management of the iNTS-GMMA vaccine trial (SALVO study) up to day 28 after second vaccination (Chapter 2 and 3).
2. The evaluation of the safety and tolerability of the iNTS-GMMA vaccine at two dose levels: lower dose and full dose iNTS-GMMA vaccine; up to day 28 after second vaccination (Chapter 3).

## 3.2 Methods

### 3.2.1 *Salmonella* Vaccine Study in Oxford (SALVO)

The *Salmonella* Vaccine Study in Oxford (EudraCT Number: 2020-000510-14, Oxfordshire Research Ethics Committee Ref 22/SC/0059), is a first-in-human phase I participant-observer blind randomised placebo-controlled study. The three doses of the iNTS-GMMA vaccine for both lower and full doses is given at month 0, 2 and 6. Thirty-one participants were sequentially enrolled into three groups. Group 1 consists of 6 participants randomised 1:1 to receive the lower dose iNTS-GMMA vaccine vs placebo. Group 2 consists of 6 participants randomised 1:1 to receive the full dose vaccine vs placebo. Group 3 consists of 18 participants randomised to receive the full dose iNTS-vaccine versus placebo.

The sample time points used for the investigation of immunogenicity were chosen based on estimated peak humoral responses and are listed in *Chapter 4 Immunogenicity of the iNTS-GMMA Vaccine*. Samples taken from participants include sera, PBMCs and oral fluid.

Between the first enrolment and the last participant to reach day 28 following the second vaccination, there are two in-house safety reviews and three DSMC reviews.

The data to determine the safety of the iNTS-GMMA vaccine consists of solicited, unsolicited, laboratory and observational adverse events. Solicited adverse events were collected in an e-diary completed by the participants up to 7 days after each vaccination.

Unsolicited adverse events were collected up to 28 days after each vaccination and identified in e-diaries, participant visits or through direct communication with the participants. Outside of these time points only medically imported unsolicited adverse events were collected. Serious adverse events (SAEs) were collected throughout the study period. Observational adverse events were collected at in-person study visits. Laboratory testing including full blood count (FBC), urea and electrolytes (U&E), liver function tests (LFT) and c-reactive protein (CRP) were collected at D0, D7, D28, D56, D63, and D84.

For an outline of the safety time points and triggers and the assessment of causality and severity, please see *Chapter 2.2.*

### 3.2.2 Statistical Analysis

Due to the sample size (n = 30-42), no formal statistical analyses are used in this chapter except for descriptive statistics as per the study protocol. Adverse events graphs in *r* were produced by K. Tanha.

## 3.3 Results

### 3.3.1 Screening and Enrolment

The SALVO study opened for enrolment in May 2022. Between May to November 2022, 127 responses were received following pre-screening using a web-based questionnaire. Of these 127 responses, 71% (90) were successfully contacted by study staff for an eligibility assessment.

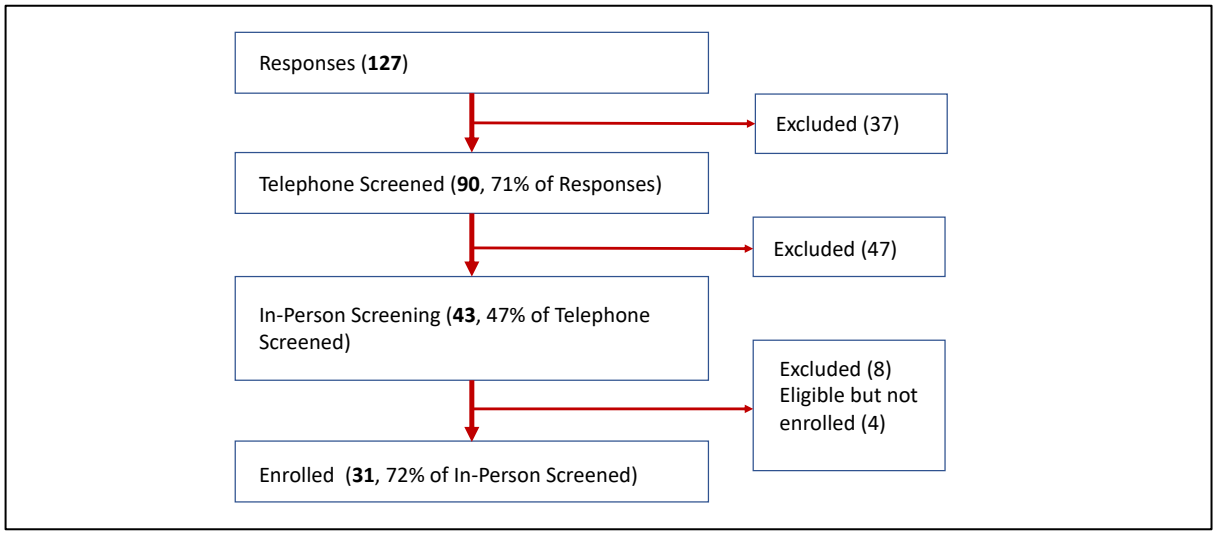



*Figure 10 SALVO screening from responses to enrolment*

Thirty-one participants were enrolled in the SALVO study, with 92 excluded and four eligible but not enrolled due to enrolment completion. Most of the individuals who were excluded were those who were either uncontactable or withdrew consent during the screening process. Reasons for exclusion ranged from iron deficiency anaemia to previous infection with *Salmonella* Typhi (figure 10).

## 3.3.2 Demographics of Enrolled Participants

The 31 participants taking part in the study were skewed towards the male sex, predominantly in their 20s, with an age range between 18-53 across all groups (table 23). Most participants only lived or travelled for a maximum of 2 months outside the UK. Fewer participants travelled to areas of higher non-typhoidal *Salmonella* endemicity, sub-Saharan Africa and SE Asia[2] (table 24).

| | Group 1 | Group 2 | Group 3 |
|---|---|---|---|
| Sex (Female) | 2 /7 (29%) | 2 /6 (33%) | 6 /18 (33%) |
| Age at V1 (Median) | 22 | 46 | 27 |
| Age Range | 20-53 | 26-50 | 18-52 |
| Ethnicity | | | |
| White | 4 (57%) | 5 (83%) | 17 (94%) |
| Other | 3 (43%) | 1 (17%) | 1 (6%) |

*Table 23 Demographics of SALVO enrolled participants*

| Group | Travel Outside of UK > 2months | Regions | | | | | |
|---|---|---|---|---|---|---|---|
| | | Europe | Americas | Africa | SE Asia | Middle East | Oceania |
| 1 | 4 ( 57%) | 3 | 2 | 2 | 0 | 1 | 0 |
| 2 | 2 (33%) | 2 | 0 | 0 | 0 | 0 | 0 |
| 3 | 5 (28%) | 2 | 1 | 0 | 2 | 0 | 1 |

*Table 24 Travel history of enrolled SALVO participants*

## 3.3.3 Study Progression

Groups 1 to 3 completed enrolment in June, September and November 2022, respectively. Day 28 following the last participant to receive the second vaccine was in March 2023. During this period, no SAEs or holding rules were met. One participant withdrew unrelated to the vaccine but agreed to telephone safety follow-up (figure 11).

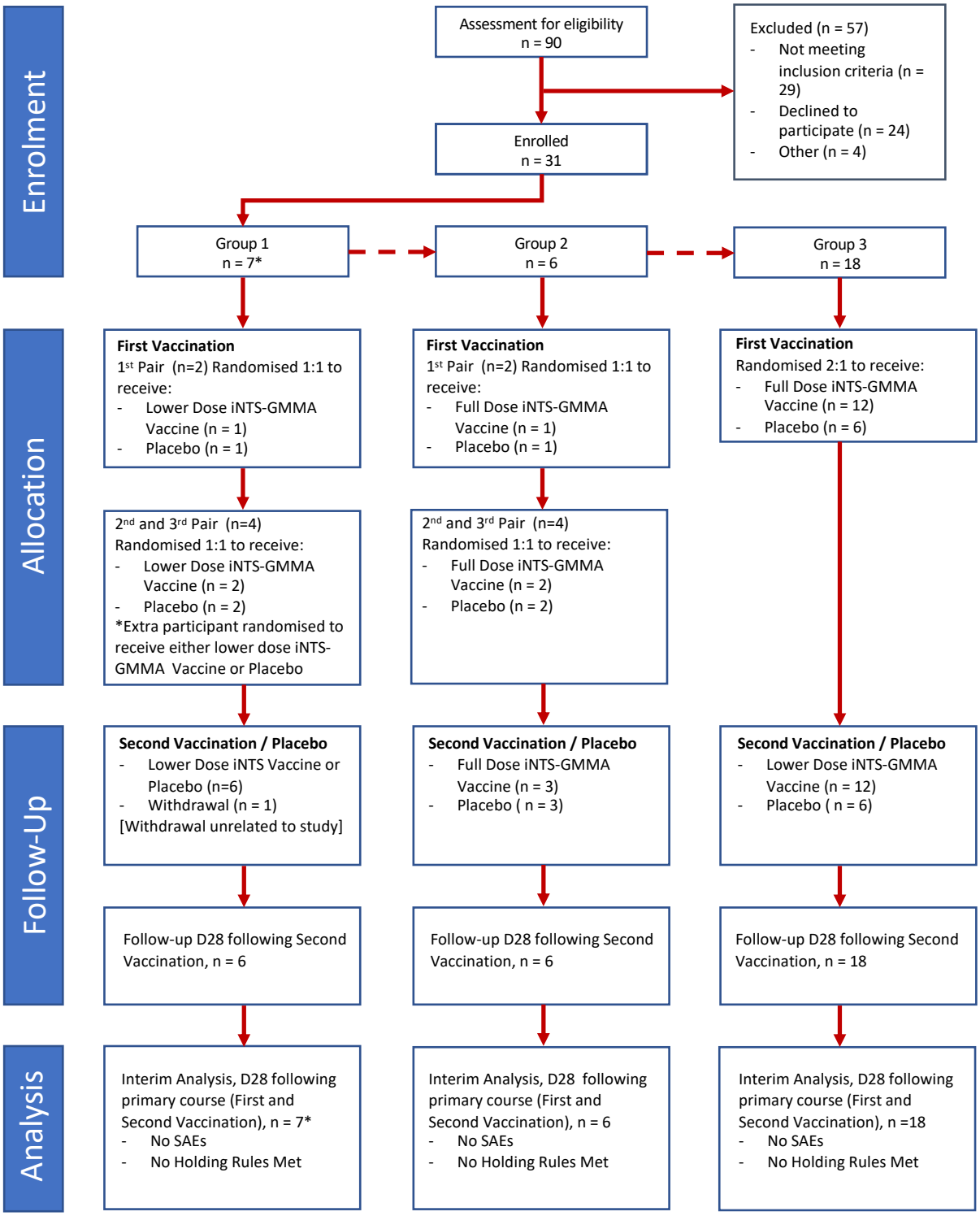



*Figure 11 SALVO consort diagram from D0-D84 (28 days following the second vaccine, primary course). *An additional healthy adult participant was recruited into Group 1 due to an error in group allocation.*

### 3.3.4 Safety of the iNTS-GMMA Vaccine

An interim analysis of **blinded** safety data is presented here as the study is ongoing. The safety data has been combined into lower dose vs placebo (Group 1) and full dose vs placebo (Groups 2 and 3). Trial unblinding will occur after the final analysis of the final assay. The interim analysis includes safety data up to 28 days following second vaccination. Therefore, a limited blinded assessment of the safety of the iNTS-GMMA vaccine will be presented, excluding data following third vaccination and up to day 350.

### 3.3.5 Safety Data in Group 1 (Lower Dose and Placebo) – First and Second Vaccine (Solicited)

#### Systemic Reactogenicity in the 7 Days Following Vaccination

Most solicited systemic reactions were mild to moderate as assessed subjectively by the participant. There were two grade 3 (severe) adverse events in the lower dose/placebo group seven days following the first vaccination and nil following the second vaccination. The first grade 3 event was diarrhoea, possibly related to the vaccine but could have been related to the SARS-CoV-2 infection in this participant (associated with AE 8 in table 26). This participant accounted for many of the aggregated symptoms seen after the first vaccine. The second grade 3 adverse event was muscle pain unrelated to the vaccine (associated with AE 1 in table 26). All symptoms were transient and self-resolved (figure 12 and 13).




*Figure 12 Overall solicited systemic symptoms following the first and second vaccines in group 1 (graph produced by K.Tanha)*




*Figure 13 Systemic solicited symptoms following the first and second vaccination in group 1; symptoms, if present, were transient and self-resolved with simple measures (graph produced by K.Tanha)*

# Local Reactogenicity in the 7 Days Following Vaccination

Injection site symptoms, including pain and tenderness, were the most common side effect, and were transient and self-resolved (figure 14). Few patients experienced redness and swelling following the first vaccination, with more experiencing local inflammation following the second vaccination (figure 15).

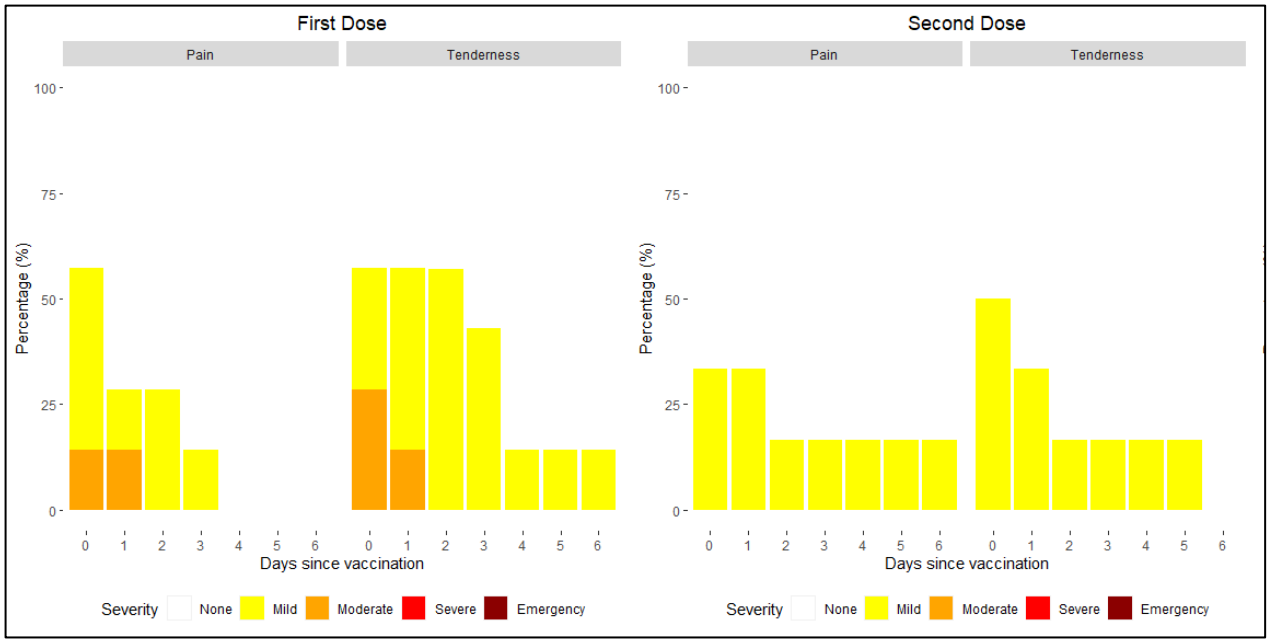



*Figure 14 Local injection site symptoms following vaccination (pain and tenderness) in group 1 (graph produced by K.Tanha)*

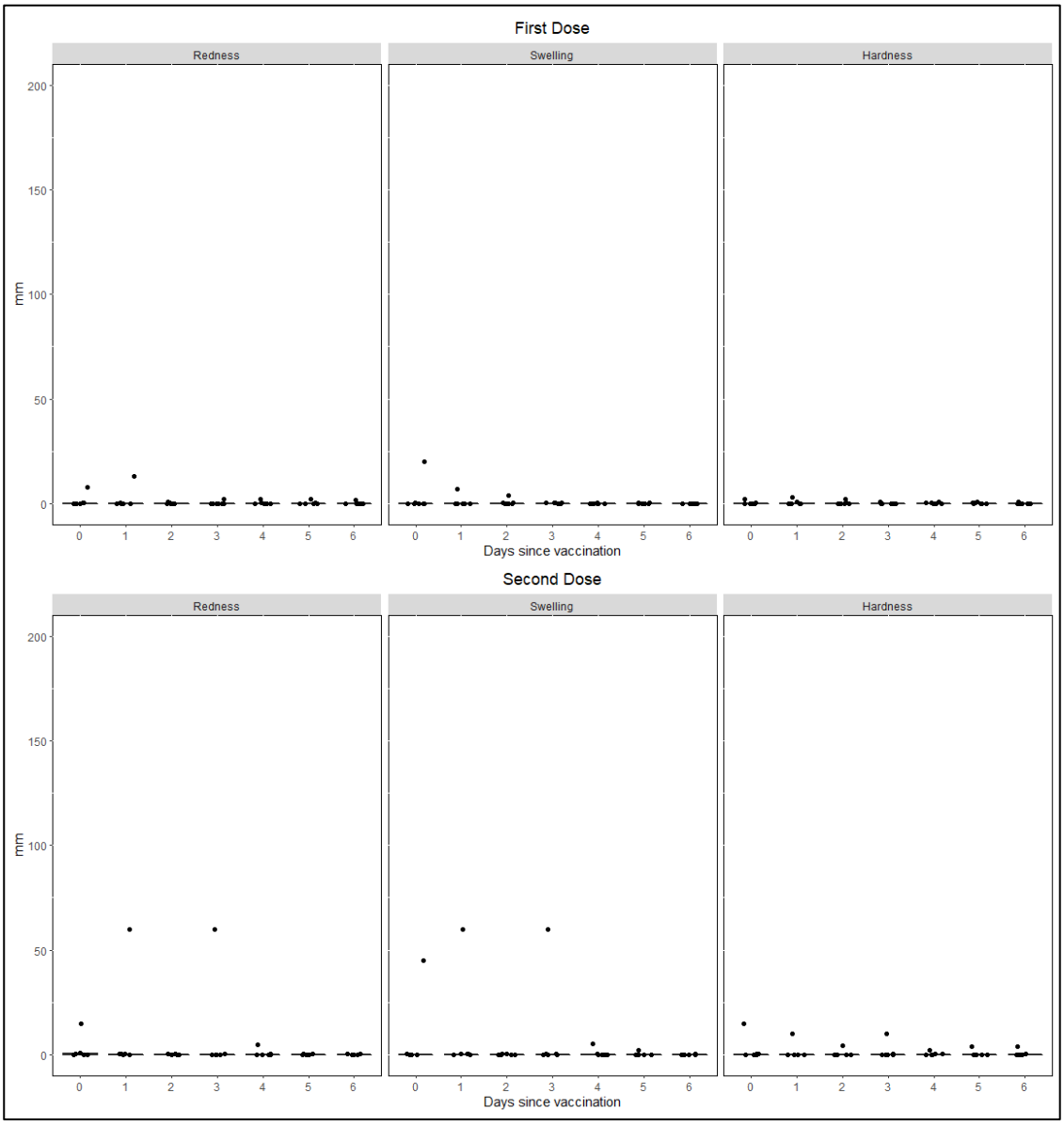



*Figure 15 Local injection site symptoms following vaccination (redness, swelling and hardness) in group 1 (graph produced by K.Tanha)*

# Safety Data in Group 1 (Lower Dose and Placebo) – Laboratory Adverse Events at Day 0, Day 7 and Day 28 Following Vaccination

There were no laboratory adverse events grade 3 or above at D0, D7 and D28 following each vaccination. The most common adverse was a mild drop in haemoglobin which remained at grade 1 throughout and was of no clinical concern (figure 16 and 17).




*Figure 16 Laboratory adverse events (haematological indices) following first and second vaccination in group 1 (graph produced by K.Tanha)*




*Figure 17 Laboratory adverse events (biochemistry indices) following first and second vaccination in group 1 (graph produced by K.Tanha)*

## 3.3.6 Safety Data in Group 1 (Lower Dose and Placebo) – First and Second Vaccine (Unsolicited)

Only six unsolicited adverse events were observed within the first seven days of the lower dose-iNTS vaccine or placebo (blinded data). Only two of these were possibly related to the vaccine or placebo. The participant who developed COVID-19, SAL-026, contributed to many of the symptoms, including a grade 3 episode of diarrhoea. There were two grade 3 unsolicited events in this group, the second of which was traumatic back pain unrelated to the vaccine (table 25).

| *AE Number* | *AE Date* | *Group* | *First Vaccination* | *Day Post First Vaccination* | *Participant ID* | *Preferred Term* | *MedDra Code* | *Related to COVID-19* | *Severity Grade* | *Causality* | *Resolution* |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 28/06/2022 | 1 | 22/06/2022 | 6 | SAL-009 | Back pain | 10003988 | No | 3 | No Relationship | Yes |
| 2 | 14/09/2022 | 1 | 22/06/2022 | 84 | SAL-009 | Upper respiratory tract infection | 10046306 | Yes | 1 | No Relationship | Yes |
| 3 | 06/07/2022 | 1 | 06/07/2022 | 0 | SAL-024 | Muscular weakness | 10028372 | No | 2 | Probable | Yes |
| 4 | 01/08/2022 | 1 | 06/07/2022 | 26 | SAL-024 | Upper respiratory tract infection | 10046306 | No | 1 | No Relationship | Yes |
| 5 | 01/08/2022 | 1 | 06/07/2022 | 26 | SAL-024 | Anaemia | 10002034 | No | 1 | No Relationship | Yes |
| 6 | 06/07/2022 | 1 | 06/07/2022 | 0 | SAL-024 | Hypotension | 10021097 | No | 1 | No Relationship | Yes |
| 7 | 12/07/2022 | 1 | 06/07/2022 | 6 | SAL-026 | Sunburn | 10042496 | No | 2 | No Relationship | Yes |
| 8 | 12/07/2022 | 1 | 06/07/2022 | 6 | SAL-026 | COVID-19 | 10084268 | Yes | 1 | No Relationship | Yes |
| 9 | 09/07/2022 | 1 | 06/07/2022 | 3 | SAL-026 | Diarrhoea | 10012735 | No | 3 | Probable | Yes |
| 10 | 26/07/2022 | 1 | 18/07/2022 | 8 | SAL-013 | Neutropenia | 10029354 | No | 1 | Probable | Yes |
| 11 | 10/08/2022 | 1 | 03/08/2022 | 7 | SAL-030 | Hypertransaminasaemia | 10068237 | No | 1 | No Relationship | Ongoing |
| 12 | 05/10/2022 | 1 | 03/08/2022 | 63 | SAL-030 | Syncope | 10042772 | No | 1 | No Relationship | Yes |

*Table 25 List of unsolicited adverse from D0-D84 Group 1 (lower dose iNTS-GMMA vaccine versus placebo)*

### 3.3.7 Safety Data in Groups 2 and 3 (Full Dose and Placebo) – First and Second Vaccine (Solicited)

#### Systemic Reactogenicity in the 7 Days Following Vaccination

There were more solicited symptoms reported in groups 2 and 3 full dose/placebo group than in group 1 lower dose/placebo which were similarly mild to moderate and self-resolving. Three grade 3 adverse events were noted in this group, all of which were transient and self-resolved, including two episodes of malaise and 1 episode of headache (Figures 18 and 19).




*Figure 18 Overall solicited systemic symptoms following the first and second vaccines in groups 2 and 3 (graph produced by K.Tanha)*

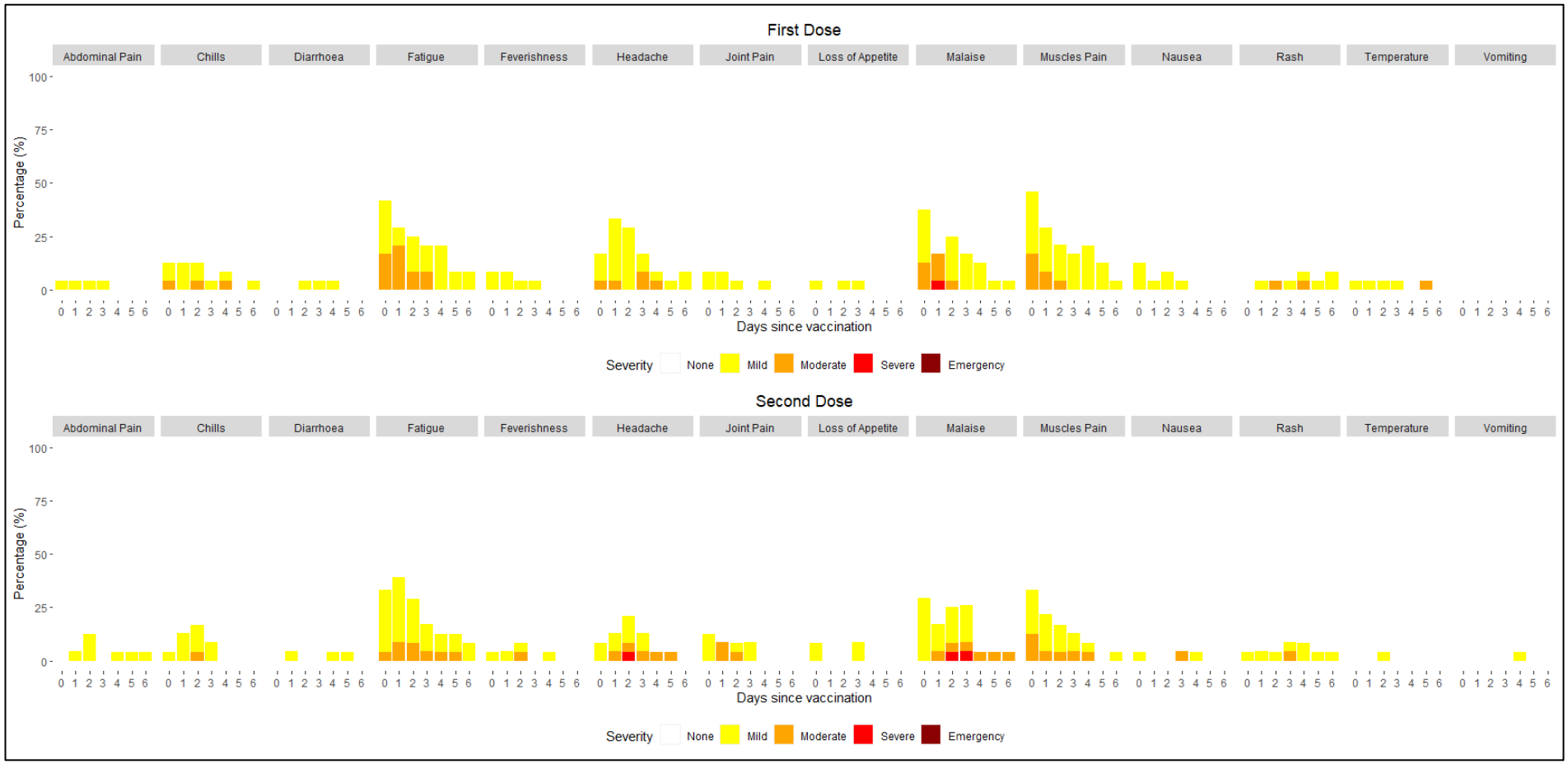



*Figure 19 Systemic solicited symptoms following first and second vaccination in groups 2 and 3; symptoms, if present, were transient and self-resolved with simple measures (graph produced by K.Tanha)*

## Local Reactogenicity in the 7 Days Following Vaccination

Similar to group 1 (lower dose/placebo), local injection site reactions were the most common side effects experienced by participants (figure 20), with more seen in groups 2 and 3. In addition, more participants experienced local inflammation and redness above 5cm. These were managed with simple measures and were self-resolving (figures 20 and 21).

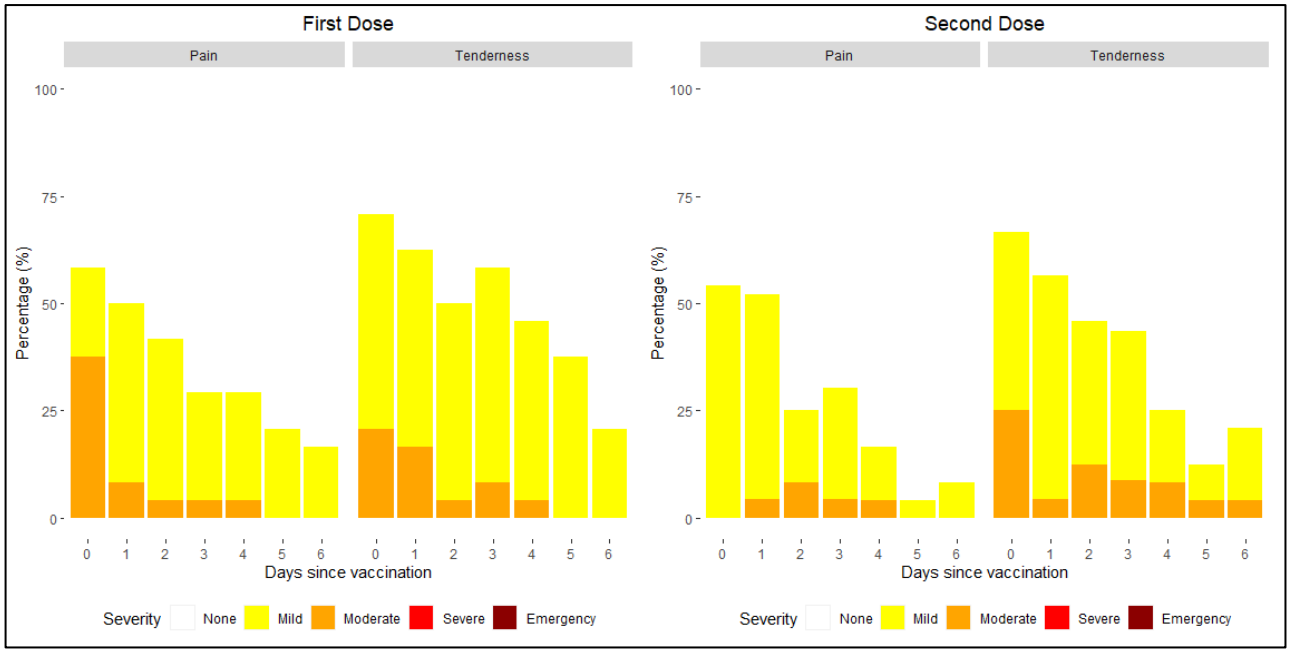



*Figure 20 Local injection site symptoms following vaccination (pain and tenderness) in groups 2 and 3 (graph produced by K.Tanha)*

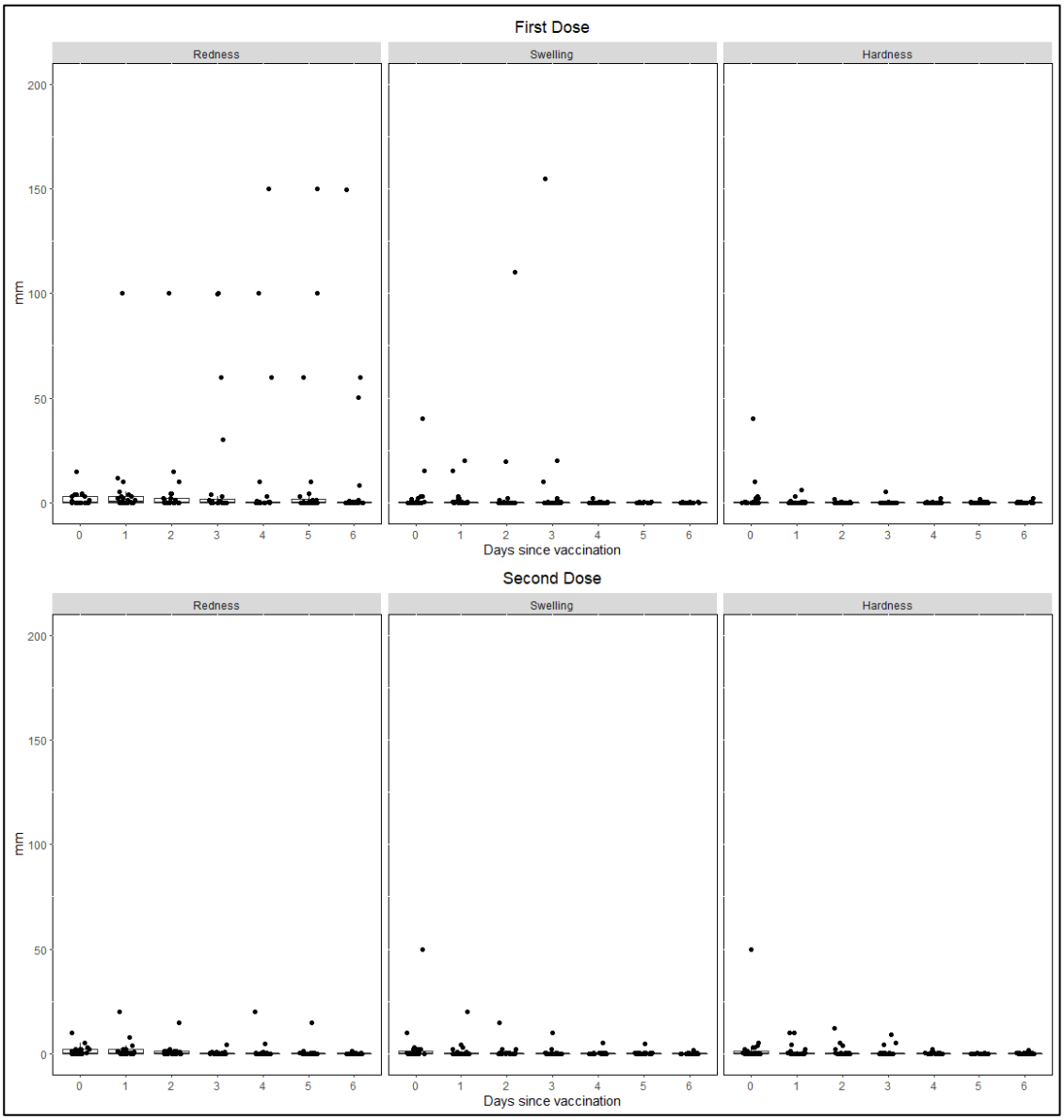



*Figure 21 Local injection site symptoms following vaccination (redness, swelling and hardness) in groups 2 and 3 (graph produced by K.Tanha)*

# Safety Data in Groups 2 and 3 (Full Dose) – Laboratory Adverse Events at Day 0, Day 7 and Day 28 Following Vaccination

There were no laboratory adverse events grade 3 or above at D0, D7 and D28 following each vaccination, with the most common adverse again being a mild drop in haemoglobin which remained at grade 1 throughout and was of no clinical concern (figure 22 and 23).




*Figure 22 Laboratory adverse events (haematological indices) following first and second vaccination in groups 2 and 3 (graph produced by K.Tanha)*




*Figure 23 Laboratory adverse events (biochemistry indices) following first and second vaccination in groups 2 and 3 (graph produced by K.Tanha)*

### 3.3.8 Safety Data in Group 1 (Lower Dose/Placebo) Group 2 and 3 (Full Dose/Placebo) – First and Second Vaccine (D0-D84 Unsolicited)

Only eight unsolicited adverse events were observed within the first seven days of a full dose-iNTS vaccine or placebo (blinded data presented; table 26). Only one is definitely related to the vaccine or placebo. There were four grade 3 unsolicited events in groups 2 and 3, and only one was definitely related to a vaccine or placebo (erythema at the injection site in participant SAL-077). This group had ten upper respiratory tract infections, with two directly related to COVID-19 associated with a positive antigen lateral flow or PCR in addition to one further listed COVID-19 disease. Most upper respiratory infections occurred between October 2022 to February 2023, coinciding with the winter months and the peak in respiratory viruses[166].

| AE Date | Group | First Vaccination | Day Post First Vaccination | Participant ID | Preferred Term | MedDra Code | Related to COVID-19 | Severity Grade | Causality | Resolution |
|---|---|---|---|---|---|---|---|---|---|---|
| 22/08/2022 | 2 | 17/08/2022 | 5 | SAL-034 | Erythema | 10015150 | No | 1 | No Relationship | Yes |
| 12/10/2022 | 2 | 17/08/2022 | 56 | SAL-034 | Back pain | 10003988 | No | 2 | No Relationship | Yes |
| 04/11/2022 | 2 | 08/09/2022 | 57 | SAL-042 | Testicular pain | 10043345 | No | 2 | No Relationship | Yes |
| 22/11/2022 | 2 | 14/09/2022 | 69 | SAL-046 | Haemoglobin decreased | 10018884 | No | 3 | No Relationship | Yes |
| 25/09/2022 | 2 | 22/09/2022 | 3 | SAL-051 | Ear pain | 10014020 | No | 1 | No Relationship | Yes |
| 29/09/2022 | 2 | 22/09/2022 | 7 | SAL-051 | Helicobacter gastritis | 10054272 | No | 3 | No Relationship | Yes |
| 22/12/2022 | 3 | 22/11/2022 | 30 | SAL-003 | Upper respiratory tract infection | 10046306 | No | 1 | No Relationship | Yes |
| 10/02/2023 | 3 | 22/11/2022 | 80 | SAL-003 | Hypertransaminasaemia | 10068237 | No | 1 | No Relationship | Yes |
| 25/10/2022 | 3 | 17/10/2022 | 8 | SAL-028 | Upper respiratory tract infection | 10046306 | No | 1 | No Relationship | Yes |
| 07/12/2022 | 3 | 17/10/2022 | 51 | SAL-028 | Lower respiratory tract infection | 10024968 | No | 3 | No Relationship | Yes |
| 16/12/2022 | 3 | 23/11/2022 | 23 | SAL-032 | Gastroenteritis | 10017888 | No | 2 | No Relationship | Yes |
| 17/12/2022 | 3 | 20/10/2022 | 58 | SAL-039 | Cough | 10011224 | No | 1 | No Relationship | Yes |
| 14/02/2023 | 3 | 23/11/2022 | 83 | SAL-062 | Upper respiratory tract infection | 10046306 | No | 1 | No Relationship | Yes |
| 23/10/2022 | 3 | 20/10/2022 | 3 | SAL-066 | Upper respiratory tract infection | 10046306 | No | 1 | No Relationship | Yes |
| 18/11/2022 | 3 | 20/10/2022 | 29 | SAL-066 | Blood bilirubin increased | 10005364 | No | 1 | No Relationship | Yes |
| 13/01/2023 | 3 | 20/10/2022 | 85 | SAL-066 | Upper respiratory tract infection | 10046306 | No | 2 | No Relationship | Yes |
| 13/12/2022 | 3 | 20/10/2022 | 54 | SAL-067 | COVID-19 | 10084268 | No | 1 | No Relationship | Yes |
| 03/11/2022 | 3 | 20/10/2022 | 14 | SAL-068 | Muscular weakness | 10028372 | No | 1 | No Relationship | Yes |
| 18/11/2022 | 3 | 20/10/2022 | 29 | SAL-068 | Hypertransaminasaemia | 10068237 | No | 1 | No Relationship | Ongoing |
| 27/10/2022 | 3 | 20/10/2022 | 7 | SAL-068 | Flatulence | 10016766 | No | 1 | No Relationship | Yes |
| 15/12/2022 | 3 | 20/10/2022 | 56 | SAL-068 | Upper respiratory tract infection | 10046306 | No | 1 | No Relationship | Yes |
| 13/02/2023 | 3 | 21/11/2022 | 84 | SAL-070 | Muscular weakness | 10028372 | No | 1 | Definite | Ongoing |
| 21/01/2023 | 3 | 21/11/2022 | 61 | SAL-071 | Concussion | 10010254 | No | 3 | No Relationship | Ongoing |
| 23/01/2023 | 3 | 21/11/2022 | 63 | SAL-071 | Upper respiratory tract infection | 10046306 | Yes | 1 | No Relationship | Yes |
| 13/02/2023 | 3 | 21/11/2022 | 84 | SAL-071 | Syncope | 10042772 | No | 1 | No Relationship | Yes |
| 28/11/2022 | 3 | 21/11/2022 | 7 | SAL-076 | Erythema | 10015150 | No | 2 | Definite | Yes |
| 15/02/2023 | 3 | 21/11/2022 | 86 | SAL-076 | Neutropenia | 10029354 | No | 1 | Possible | Ongoing |
| 27/11/2022 | 3 | 23/11/2022 | 4 | SAL-077 | Erythema | 10015150 | No | 2 | Definite | Yes |
| 06/12/2022 | 3 | 23/11/2022 | 13 | SAL-077 | Upper respiratory tract infection | 10046306 | No | 2 | No Relationship | Yes |
| 17/01/2023 | 3 | 23/11/2022 | 55 | SAL-077 | Erythema | 10015150 | No | 3 | Definite | Yes |
| 25/01/2023 | 3 | 23/11/2022 | 63 | SAL-077 | Anaemia | 10002034 | No | 1 | No Relationship | Yes |
| 29/11/2022 | 3 | 22/11/2022 | 7 | SAL-089 | Hypertransaminasaemia | 10068237 | No | 1 | Probable | Yes |
| 19/01/2023 | 3 | 22/11/2022 | 58 | SAL-089 | Ligament sprain | 10024453 | No | 2 | No Relationship | Yes |
| 16/02/2023 | 3 | 22/11/2022 | 86 | SAL-089 | Upper respiratory tract infection | 10046306 | Yes | 1 | No Relationship | Yes |

*Table 26 List of unsolicited adverse from D0-D84 Group 2 and 3 (full dose iNTS-GMMA vaccine versus placebo)*

## 3.4 Discussion

### 3.4.1 Summary

1. The design, set-up, initiation and initial management of the iNTS-GMMA vaccine trial (SALVO study) up to day 28 after second vaccination.

The SALVO study opened for enrolment in May 2022. Groups 1 to 3 completed enrolment in June, September and November 2022, respectively. Day 28 following the last participant to receive the second vaccine was in March 2023.

2. The evaluation of the safety and tolerability of the iNTS-GMMA vaccine at two dose levels: lower dose and full dose iNTS-GMMA vaccine; up to day 28 after second vaccination.

Safety data at two iNTS-GMMA vaccine dose levels (lower and full) up to day 28 after the second vaccination, included solicited, unsolicited, laboratory, and observational data. Solicited adverse events collected up to 7 days following each vaccination revealed mild to moderate transient symptoms for both the lower and full dose vaccine. The most common solicited event were local arm reactions. There were few grade 3 (severe) symptoms across the dose levels, and all were transient. Unsolicited adverse events included respiratory infections, and most were unrelated to the vaccines. No significant laboratory adverse events were noted. During this period, no SAEs or holding rules were met. One participant withdrew unrelated to the vaccine but agreed to telephone safety

follow-up. The iNTS-GMMA vaccine at both lower and full dose appeared safe and well tolerated up to the timepoint investigated (day 28 following second vaccination), limited by an assessment of blinded safety data. To determine overall safety of the iNTS-GMMA vaccine in this phase I study further analysis following third vaccine dose and up to day 350 of unblinded safety data are pending assessment.

### 3.4.2 Demographics

The participants in the trial were skewed towards the younger age group reflecting the local demographics in a university-based town. There was a skew to the male sex, which was likely the result of the recent change (at the time of regulatory review) in guidance regarding the use of contraception in female participants in a first-human trial with barrier methods no longer acceptable[167].

### 3.4.3 Study Progression

The SALVO study was originally due to open for recruitment in early 2020. COVID-19 pandemic affected this study similarly to many clinical trials during 2020-2021, with study progression understandably delayed. Clinical trials could not be conducted during this period due to the safety of participants and staff and altered national urgent public health research priorities. Further delays occurred with the manufacture of the vaccine and regulatory approvals.

Once regulatory approval was in place, additional trial measures were required to ensure the study could be conducted in the background of a dynamic COVID-19 pandemic.

Considerations were made for a further surge in COVID-19 during the trial, the accessibility of COVID-19 vaccines to our participants, the scientific validity should adverse events be complicated by COVID-19 symptoms and most importantly, the safety of each of our participants. Measures included risk assessment for the study start and progression in the context of increased cases of COVID-19, provision of SARS-CoV-2 PCR testing, telephone visits and working with participants to ensure they received any offered COVID-19 vaccines if they became eligible during the trial. Overall COVID-19 had a minimal impact on the study conduct and safety data. One participant confirmed COVID-19 seven days after vaccination, with ten other upper respiratory tract infection cases.

During the study period presented in this chapter, the DSMC reviewed safety data at three scheduled timepoints (seven day following first vaccinations in group 1, sevens day following first vaccinations in group 2 and seven days following second vaccinations in group 3). DSMC review of safety data at all three timepoints allowed progression from group 1 lower dose iNTS-GMMA vaccination to group 2 full dose iNTS-GMMA vaccination, from group 2 to group 3 full dose vaccinations and finally to continue to the third vaccine administration across groups.

### 3.4.4 Safety and Tolerability of the Lower and Full Dose Vaccine

The lower and full dose iNTS-GMMA vaccines appeared safe and well tolerated up to the time points (day 0 to 84) of blinded safety data presented. Further analysis of unblinded safety data following third vaccine and up to day 350 remains pending for confirmation. Local injection site reactions were mild to moderate and transient. As expected, there were more symptoms in the full-dose iNTS-GMMA vaccine group than in the lower-dose

vaccination group with more injection-site inflammation. In addition, there were several upper respiratory infections unrelated to the vaccine coinciding with the winter peak in viral infections. Formal respiratory viral PCR testing may have been helpful in distinguishing upper respiratory tract infections from vaccine-related symptoms.

The sample size in this study is consistent with a first-in-human vaccine trial whose aim is to determine the initial safety of the iNTS-GMMA vaccine before proceeding to a larger cohort of participants: 3-4 participants receiving the lower dose, and 18 participants receiving the full dose iNTS-GMMA vaccine. As a result, the sensitivity of the trial to identify uncommon or rare side effects is expectedly low. In addition, the data presented is blinded, with no certainty that the adverse events seen are unrelated to the placebo or Alhydrogel, particularly with injection site reactions[168 169]. Adverse events following the second vaccination are presented however, further long-term follow-up after the third vaccine is required to determine adverse events with a delayed onset, such as neurological disorders[170]. Given these have been rarely reported in meningococcal OMV vaccines[170], further safety studies would also be required to have the sensitivity required to determine if these are adverse events of concern. No neurological disorders were identified in the study and presented data thus far. Furthermore, there was a trend towards moderate to severe injection site inflammation in the full-dose vaccine group, which was self-limiting. The increase in adverse events following dose escalation from lower to full dose was consistent with the *Shigella* GMMA vaccine trial[129]. However, a larger sample receiving the lower-dose vaccine would be required for confirmation.

There were no significant laboratory adverse events seen in the interim analysis. Whilst 8 participants experienced neutropenia between grade 2 and 3 in the initial phase 1 *Shigella* GMMA vaccine trials in Europe[129], only one participant in the iNTS-GMMA study experienced grade 1 neutropenia. Whilst the discrepancy in numbers may be related to sample size, the fewer neutropenia seen highlight the safety of the GMMA vaccine up to time point analysed.

Few fevers were related to the vaccine, and those who did experience this were likely related to an alternative cause, including a viral infection or SARS-CoV 2 infection. The absence of vaccine-induced fevers is in keeping with the *Shigella* GMMA vaccine studies but not the meningococcal OMV vaccines[171]. The resultant discrepancy between the GMMA and meningococcal OMV studies may be due to the lipid A modification in the GMMA reducing reactogenicity[7] or the presence of more reactogenic endotoxins in the meningococcal OMV vaccines. A reduction in toxicity to OMVs produced from lipid A genetically modified meningococcal strains was less reactogenic in mice studies and *in vitro* human monocyte activation assays[172], suggesting the lipid A may be a significant contributor to the reduction in reactogenicity in the GMMAs.

Reactogenicity profiles vary depending on a number of factors, including host, such as age and number of doses received. The change in the side effect profile between the first and subsequent vaccination is vaccine-dependent, with an increase in reactogenicity believed to be associated with T cell and innate memory activation[77]. A similar reactogenicity profile was seen following the first and second vaccination in the full-dose groups.

Although there was a marked reduction in local injection site erythema following the second vaccination, further sample numbers would be required to determine significance.

Whilst the cause of systemic reactogenicity has been associated with the circulation of cytokines and chemokines from the local vaccination site[77], the association of reactogenicity with low immunogenicity is unclear[173]. The next immunogenicity chapters will determine the immunogenicity of the iNTS-GMMA vaccine despite low reactogenicity seen here.

### 3.4.5 Limitations and further considerations

Studies of meningococcal OMV vaccines demonstrate a variation in adverse events with age, with fever a more common feature in children and infants[170 171]. The blinded limited safety data presented here suggest an adequate safety profile thus far. However, analysis of the unblinded safety profile following third vaccination, further longitudinal follow-up and phase II and III studies will be required, in addition to studies in younger age groups to determine the safety of the vaccine in the target demographic.

The placebo arm provides further accuracy in the estimation of the iNTS-GMMA vaccine safety and is balanced with a pragmatic sample size based on the primary and secondary objectives. In order to achieve the maximum benefit from the inclusion of a placebo, the study is participant-observer blind. As a result, this interim analysis is limited by the uncertainty of vaccine allocation. A full open safety analysis can only be performed once the trial is unblinded.

The second phase I study in an endemic population is planned to follow the SALVO trial. To date an increase in reactogenicity has not been observed in populations previously exposed to the infection targeted by the vaccine[77] although immunogenicity has been shown to vary between different ethnic populations[174]. However, the possibility of differences in reactogenicity and immunogenicity remains possible and will be determined by this follow-on trial.

## 3.5 Conclusions

The iNTS-GMMA vaccine appeared safe and well tolerated up to 28 days following the second vaccination. This was a limited safety assessment of blinded data with further time points after the third vaccination and up to day 350 yet to be assessed in the final study analysis for overall confirmation of safety in this phase I study. Most side effects, if they occurred, were local injection site reactions which were mild to moderate and self-resolved. The observed reactogenicity was similar to *Shigella* GMMA vaccine trials[129 130].

# Immunogenicity of the iNTS-GMMA Vaccine

## 4.1 Introduction

### 4.1.1 GMMAs and Immunogenicity

There are no immunological correlates of protection for invasive non-typhoid *Salmonella* (iNTS). Field seroepidemiology studies in sub-Saharan Africa are underway, with recent developments establishing the role of antibody-associated B cell responses[46]. As an intracellular organism, *Salmonella* sp. must transit the extracellular space to infect adjacent cells. Antibodies are required to arrest this extracellular phase, whilst cell-mediated immunity remains essential in clearing infected cells[68 175]. In Nyirenda et al. (2014)[46], the investigators recruited 449 children under five in Malawi diagnosed with iNTS (STm bacteraemia) following natural infection. The highest incidence in children aged one to three was associated with low titres of bactericidal antibodies. However, increased STm-specific CD4 response with increasing age was followed by a subsequent rise in bactericidal antibodies and fewer cases of iNTS. Whilst this study provides circumstantial evidence for the components of immunity to iNTS, direct evidence is yet to be established. Further indirect evidence of the role of O-antigen antibodies in the prevention of iNTS is discussed in section *1.5 Salmonella Antigens*.

The T cell-dependent B cell response is often insufficient in the under-five age group contributing to a weakened immune response of shorter duration and susceptibility to infection. The *Shigella* GMMA phase I and IIb vaccine trials preceded the *Salmonella*

GMMA first-in-human trial[129 136 132]. The *Shigella* GMMAs are similarly based on O-antigen with linked recognition achieved though proteins contained within the active GMMA vaccine component. Human T-cell immunogenicity data are not available from the *Shigella* GMMA human trials. However, wild-type mice vaccinated with the *Shigella* GMMA demonstrated a more robust antibody response compared with T cell-deficient mice, suggesting that T cells are required for optimal antibody responses[136]. Conjugate-based vaccines exploit the concept of linked recognition to stimulate a T cell-dependent B cell response to polysaccharide-based antigens such as O-antigen[99]. As previously described, outer membrane vesicles such as GMMAs contain multiple protein components. Thus, GMMA vaccines may generate robust T cell-dependent responses to O-antigen by linked recognition with these associated proteins. Given there are multiple protein and polysaccharide antigens in GMMA particles, a polyclonal antibody response is likely, particularly to the outer membrane proteins. *Chapter 5* establishes the distribution of potential immunogenic proteins within SEn and STm GMMA, whilst *Chapter 6* and *Chapter 7* show the immunogenicity of the GMMA protein components to both antibody and T cells as well as the immunodominant and primary cross-reactive antigen.

Although the *Shigella* GMMA vaccine generated elevated antibodies to O-antigen[129 136] in clinical trial volunteers, the controlled human infection model did not show efficacy in preventing gastrointestinal disease[132]. Further work is underway to establish the reasons for this[136]. The dose and vaccine schedule used in the SALVO trial described in this thesis differs from that of the *Shigella* GMMA vaccine[129 136]. In addition, future iNTS-GMMA vaccine trials investigating efficacy using a controlled human infection model would likely be based on a systemic rather than a gastroenteric endpoint as in the *Shigella* trials[132].

This study describes the immunogenicity of the iNTS-GMMA vaccine by comparing response after vaccination with baseline, placebo recipients and, where available, against immune responses following natural infection. As part of this thesis, several immunoassays have been developed using the GMMA as an antigen to determine the response to the active vaccine components. These assays are outlined in the following sections.

### 4.1.2 Mucosal Immune Responses

Identifying the protective antigen and establishing correlates of protection is important for future vaccine studies. GMMA-based ELISAs have been developed to measure the antibody response to the entire GMMA particle for both SEn and STm GMMA (See *Chapter 2 Methods and Materials*). This approach quantifies the total antibody response to the GMMA. Further ELISAs and pre-adsorption assays have been used to identify the immunodominant and cross-reactive antibodies (*See Chapter 6 Non-Typhoid Salmonella GMMAs Demonstrate Functional Cross-Reactivity with Antibodies Generated Across Salmonella Serovars)*.

IgG is the most abundant immunoglobulin in serum and the most important marker of vaccine-induced immunity. IgA is the second most abundant and has a significant role in the mucosal immune response[71]. IgA is found in a secretory dimeric or polymeric form at the mucosal surface, where it neutralises and tethers bacteria[71]. However, it remains predominantly monomeric in serum, where its role is less clear[73]. Exposure to enteric bacteria at the mucosal surface leads to phagocytosis and antigen presentation in the

specialised gut-associated lymphoid tissue (GALT). IgA-specific B cells are selected and release secretory IgA into the intestinal lumen. These B cells predominantly reside in gut-associated lymphoid tissue, forming memory B cells—however, some B cells transit to local lymph nodes and the spleen[176]. Establishing a vaccine-induced mucosal immune response would augment the response against iNTS, preventing *Salmonella* invasion and demonstrating a benefit in preventing or ameliorating NTS gastroenteritis[177]. Vaccine-induced mucosal immune responses may be established by measuring antigen-specific secretory IgA level in stool. However, sampling and assay performance make stool sampling impractical. In this thesis, oral fluid IgA responses are used as surrogate of the gut mucosal immune response which has been shown to correlate between salivary gland and intestinal IgA secretion[178].

### 4.1.3 Functional Antibody Responses to GMMAs

The role of antibodies in the adaptive immune system is well established. The Fragment Antigen Binding (Fab) region binds to antigen-specific epitopes, and should the epitope location and function be crucial for pathogen invasion can lead to neutralisation. The Fc region of the antibody stimulates further downstream immune effector function dependent on several factors, including immunoglobulin subclass, modification of Fc region (e.g., glycosylation), immune effector cell and finally, the Fc receptor. An assessment of all these factors and interactions is called systems serology[79]. The downstream immune effector components which can be influenced or activated include monocyte phagocytosis, neutrophil phagocytosis, complement mediated-killing and NK cell activation. The systems serology approach evaluates these immune effector functions with corresponding assays, including antibody-dependent monocyte phagocytosis

(ADMP), antibody-dependent neutrophil phagocytosis (ADNP), antibody-dependent complement deposition (ADCD), serum bactericidal assay (SBA) and antibody-dependent NK cell activation (ADNKA)[79 179]. An assessment of the functional capacity of an antibody allows the determination of antibody contribution to immune protection[180].

The functional assays chosen for assessment of cross-reactivity and iNTS-GMMA vaccine human immune response are the ADMP and ADNKA. These allow the assessment of two independent immune effector functions. Monocyte phagocytosis has multiple functions, including immune recognition, phagocytosis and pathogen clearance, downstream immune stimulation and antigen presentation[181 182]. The NK cell has a particular role in cytotoxicity and the stimulation of apoptosis in infected host cells[183].

Until this thesis, no functional assays have been established using outer membrane vesicles or GMMAs as antigens. However, SBAs using whole *Salmonella* bacteria (SEn and STm) have been well established demonstrating higher SBA activity in mice antibodies generated from immunisation with GMMA compared with an O-antigen glyconjugate[99]. Further SBA analysis in humans using a similar assay is planned in the SALVO trial as well as a seroepidemiology analysis in endemic countries called the SAiNTS study[138].

### 4.1.4 T Cell Responses

There are few human T-cell studies investigating the response to OMV-based vaccines, with most studies to date based on meningococcal OMV[184 185]. A study in human participants showed an increase in T cell proliferation peaking at two weeks post the initial vaccine, with boosting observed post-second vaccine at six weeks and following the

third vaccine at ten months[184]. Direct T cell responses to proteins within the OMV were identified[184]. Another study showed the development of T cell memory responses to an alternative meningococcal OMV vaccine[185]. CD3+CD4+ and CD3+CD8+ T cell populations are essential for stimulating T cell-dependent B cell response and cell-mediated immunity, respectively. The T cell proliferation assay quantifies antigen-specific clonal expansion, including CD4 and CD8 subsets.

### 4.1.5 Aims and Objectives

This chapter aims to identify whether the iNTS-GMMA vaccine components stimulate GMMA-specific immune responses following the primary vaccination course (first and second vaccine) in human participants.

The objectives include:

1. Evaluation of the serum IgG and IgA antibody response following first and second vaccination up to day 28 after second vaccination using a validated SEnGMMA and STmGMMA IgG and IgA ELISA.
2. Evaluation of the functional antibody response following first and second vaccination up to day 28 after second vaccination using antibody dependent monocyte phagocytosis (ADMP) and NK activation (ADNKA) assays.
3. Evaluation of the mucosal immune response following first and second vaccination by measuring absolute oral fluid IgA using a SEnGMMA and STmGMMA IgA ELISA.
4. Evaluation of the T cell memory response following first and second vaccination using a GMMA-based T Cell proliferation assay.

## 4.2 Methods

### 4.2.1 Sample Collection and Timepoints

SALVO sera, oral fluid and PBMC at baseline and up to D28 post-second iNTS-GMMA vaccine or placebo were collected and analysed in a series of immunoassays described in *Chapter 2 Materials and Methods* (individual sections listed below, where relevant). Immunogenicity assessment including immunoassay and time points tested are listed in table 27.

| **Assay** | **Sample and Timepoint** |
|---|---|
| Serum IgG / IgA, SEn / STm GMMA ELISA | SALVO Sera: *D0, D7, D14, D28, D56, D63, D84* |
| Oral Fluid IgG / IgA, SEn / STm GMMA ELISA | SALVO Oral Fluid: *D0, D14, D28, D56, D84* |
| Functional Antibody assays (ADMP, ADNKA) | SALVO Sera: *D0, D7, D14, D28, D56, D63, D84* |
| GMMA T Cell Proliferation Assay | SALVO PBMCs: *D0, D28, D56, D84* |

*Table 27 SALVO samples (and timepoints) tested across immunoassays*

### 4.2.2 GMMA-Based Enzyme-Linked Immunosorbent Assay (ELISA)

The SEn and STm GMMA-based ELISAs were performed as detailed in *Chapter 2.5 and 2.6*. Positive, negative, quality controls were used as detailed in *Chapter 2.5.3*.

### 4.2.3 Antibody-Dependent Monocyte Phagocytosis (ADMP) Assay

SEn and STm O-antigen bead conjugation, GMMA staining, and THP-1 Cell (*ATCC, Virginia, USA;* TIB-202) preparation were performed as detailed in *Chapter 2.8.*

The SEn and STm GMMA-based ADMP were performed as detailed in *Chapter 2.8*.

The SEn and STm O-antigen bead-based ADMP were performed as detailed in *Chapter 2.8.*

Positive, negative, quality controls were used as detailed in *Chapter 2.5.3*.

### 4.2.4 Antibody-Dependent NK Activation (ADNKA) Assay

NK cell (*ATCC, Virginia, USA;* PTA-8836 NK cells expressing CD16 with a transfected green fluorescent protein) preparation was performed as detailed in *Chapter 2.9*.

The SEn and STm GMMA-based ADMP were performed as detailed in *Chapter 2.9*.

Positive, negative, quality controls were used as detailed in *Chapter 2.5.3*.

### 4.2.5 GMMA-based T Cell Proliferation Assay

The GMMA-based T cell proliferation assay (PBMC CFSE staining, stimulation, fixation, surface marker staining, flow cytometric and statistical analysis) was performed as detailed in *Chapter 2.11*.

### 4.2.6 Statistical Analyses

Statistical analysis for the GMMA-based ELISAs (*Chapter 2.6.4*), ADMP (*Chapter 2.8.5*), ADNKA (*Chapter 2.9.5*), and GMMA-based T cell proliferation (*Chapter 2.11.5*) assay are detailed in Chapter 2. P-values $<0.05$ were considered significant. P-values $>0.01$ are reported to two decimal places; p-values between 0.01 – 0.001 are reported to 3 decimal places; and p-values less than 0.001 are reported as $p<0.001$.

Additional statistical analysis including a mixed-effects analysis with Geisser-Greenhouse correction and Dunnett's multiple comparison tests, paired T-test, ANOVA, Spearman Correlation and Linear Regression were performed using Prism Graphpad™ Software. Further Spearman correlation were performed using R[149] / R Studio[150]. Graphs were produced using Prism Graphpad™ Software and R[149] / R Studio[150].

## 4.3 Results

### 4.3.1 Antibody Response to the iNTS-GMMA Vaccine using IgG and IgA GMMA ELISA

#### Elevated Serum GMMA IgG Responses Following iNTS-GMMA Vaccination

No significant overall difference was seen at baseline (D0) between interventions and placebo for both SEnGMMA (full dose: geometric mean concentration [GMC] 1,283, 95% CI [925-1,780]; lower dose: GMC 1,696, 95% CI [565-5089]; placebo: GMC 2,642, 95% CI [1,650-4,229]; p=0.05) and STmGMMA IgG (full dose: GMC 2840 [2021-3990 ]; lower dose: GMC 3728 [897-15491]; placebo GMC 4325 [2803-6673]; p=0.26) antibodies (figures 24-29).

When compared with baseline, a significant increase in GMMA-specific IgG GMC was observed at 14 days after full dose vaccination (SEnGMMA GMC 11,418 [7,251-17,979], p=0.001; STmGMMA GMC 10,378 [7,848-13,725], p<0.001), but not after lower dose vaccination (SEnGMMA GMC 8,090 [1,555-42,079], p= NA; STmGMMA GMC 17,977 [839-385,087], p=0.50) or placebo (SEnGMMA GMC 1,880 [1,030-3,431], p=0.13; STmGMMA GMC 3,661 [2,373-3,661], p=0.42) for both serovars. The same was seen at 28 days after full dose vaccination (SEnGMMA GMC 8,729 [5,960-12,784], p=0.002; STmGMMA GMC 10462 [6983-15672], p=0.009), lower dose vaccination (SEnGMMA GMC 4,704 [598-36,956], p=0.43; STmGMMA GMC 9,691 [803-116,994], p=0.55) and placebo (SEnGMMA

GMC 2,423 [1,562-3,758], p=0.52; STmGMMA GMC 3,942 [2,633-5,904], p=0.39); and 84 days after full dose vaccination (SEnGMMA GMC 16,991 [11,798-24,469], p<0.001; STmGMMA GMC 11,331 [8,075-15,899], p=0.007), lower dose vaccination (SEnGMMA GMC 6615 [868-50384], p=0.25; STmGMMA GMC 13633 [2.2293-8,336,541], p=0.50) and placebo (SEnGMMA GMC 2,540 [1,448-4,457], p>0.99; STmGMMA GMC 4,946 [3,242-7,546], p>0.99)[figures 27-29].

The peak SEnGMMA and STmGMMA-specific IgG response time varied between D14 and D28, depending on the participant (figure 24-26).

Limited NTS convalescent sera from children under five were tested in parallel [SEn = 2, STm = 5, NTS (Serovar undetermined) = 2] with sera from full dose, lower dose iNTS-GMMA vaccine and placebo recipients. No statistical differences were seen between all D14 convalescent NTS sera and iNTS-GMMA vaccine full dose (SEnGMMA, p=0.42; STmGMMA, p=0.14), lower dose (SEnGMMA, p=0.74; STmGMMA, p=0.94), and placebo recipients (SEnGMMA, p=0.57; STmGMMA, p=0.08). However, the highest NTS serum was greater than the highest D14 SEnGMMA and STmGMMA IgG antibody response to the full dose, lower dose vaccination and placebo (figures 25 and 26).

Finally, a significantly higher fold change from D0 to D14 was seen in the SEnGMMA (GM 9.053 [5.026-16.31]) compared with STmGMMA (GM 3.910 [2.914-5.245]) IgG-specific antibodies in the full-dose vaccine recipients (p=0.02).

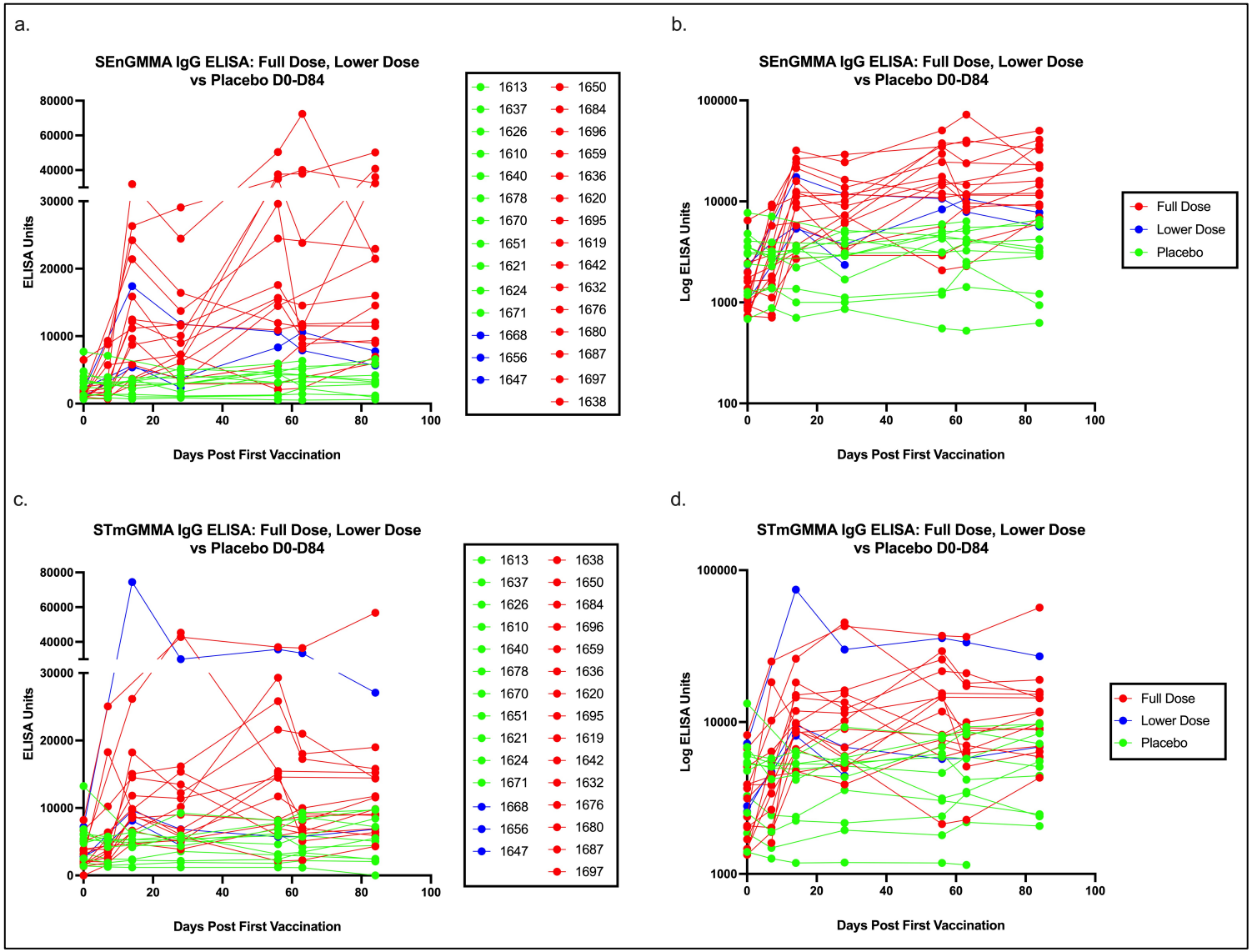



*Figure 24 SEn (a, b) and STm GMMA (c, d) IgG response in healthy adult volunteers following iNTS-GMMA vaccination (full dose, red, n=15; lower dose, blue, n=3) and placebo (green, n=11); D0-D84 following First Vaccination (D0) and Second Vaccination (D56) in ELISA Units (a, c) and Log ELISA Units (b, d)*

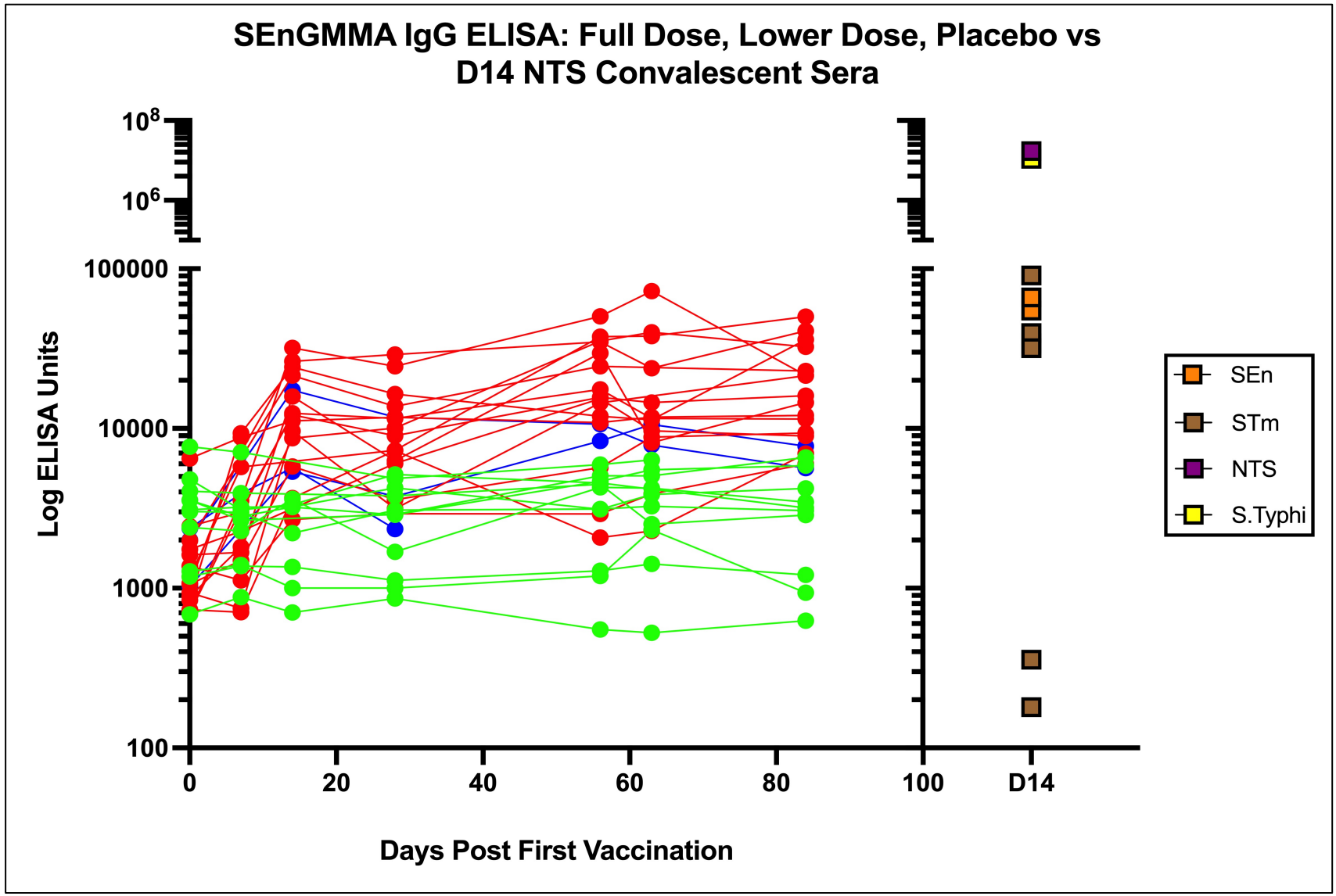



*Figure 25 Comparison of SEnGMMA IgG response following full dose (red, n=15), lower dose (blue, n=3) iNTS-GMMA vaccination and placebo (green, n=11) in healthy UK adult volunteers [D0-D84 (Left)] with D14 convalescent NTS / S.Typhi sera (SEn = 2, STm = 5, Unknown NTS = 2, S.Typhi =1) in children (aged 12 and under) from sub-Saharan Africa (Right)*

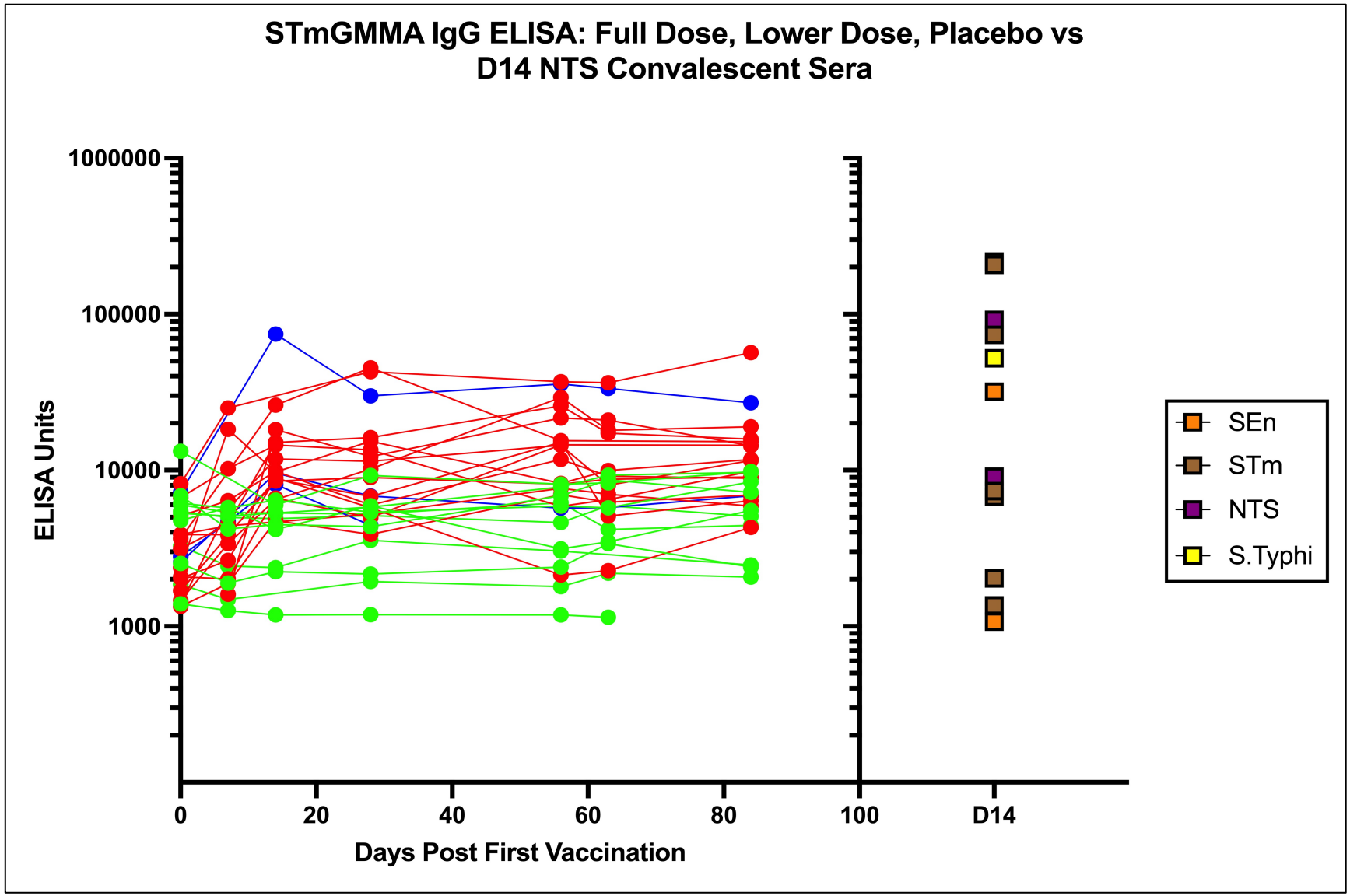



*Figure 26 Comparison of STmGMMA IgG response following full dose (red, n=15), lower dose (blue, n=3) iNTS-GMMA vaccination and placebo (green, n=11) in healthy UK adult volunteers [D0-D84 (Left)] with D14 convalescent NTS / S.Typhi sera (SEn = 2, STm = 5, Unknown NTS = 2, S.Typhi =1) in children (aged 12 and under) from sub-Saharan Africa (Right)*

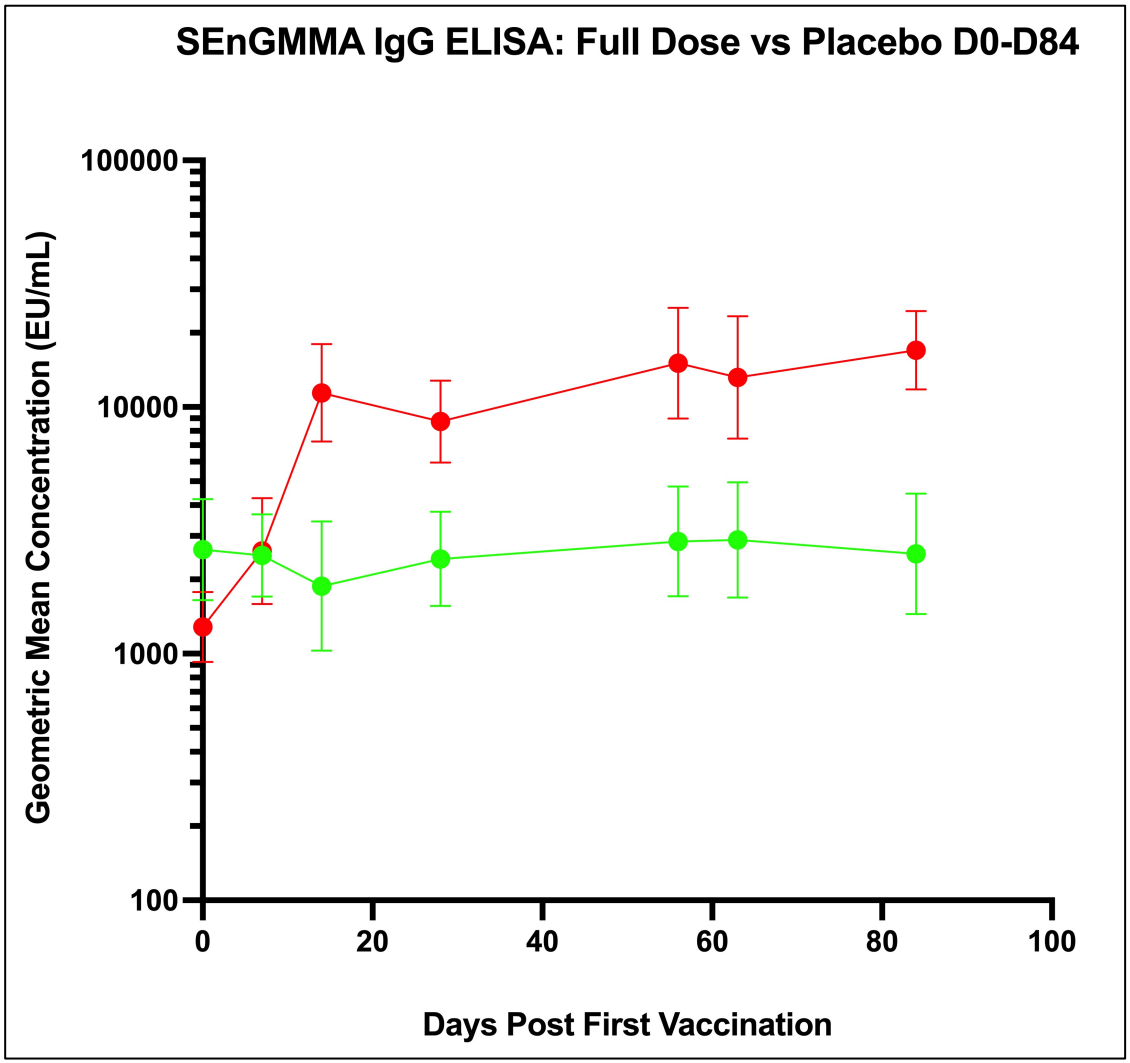



*Figure 27 Comparison of geometric mean concentration (GMC) with 95% confidence interval (CI) SEnGMMA IgG response in healthy adult volunteers following full dose (red, n=15) iNTS-GMMA vaccination vs placebo (green, n=11) D0-D84; full dose iNTS-GMMA vaccine vs placebo demonstrates a significant increase above placebo in GMC from D14 to D84; NB: Lower dose has not been shown due to the wide confidence interval (n=3)*

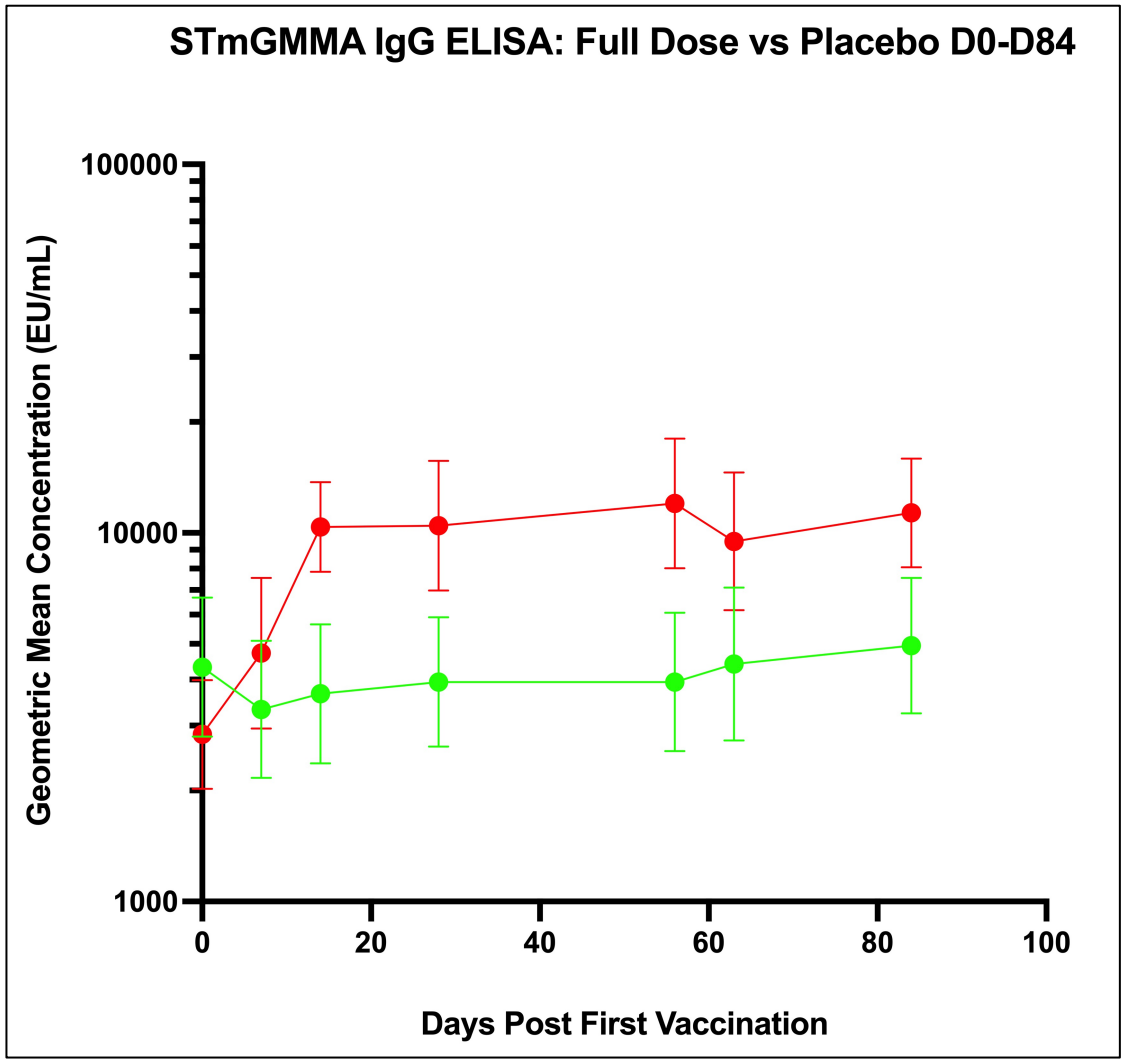



*Figure 28 Comparison of geometric mean concentration (GMC) with 95% confidence interval (CI) STmGMMA IgG response in healthy adult volunteers following full dose (red, n=15) iNTS-GMMA vaccination vs Placebo (green, n=11) D0-D84; full dose iNTS-GMMA vaccine vs placebo demonstrates a significant increase above placebo in GMC from D14 to D84; NB: Lower dose has not been shown due to the wide confidence interval (n=3)*

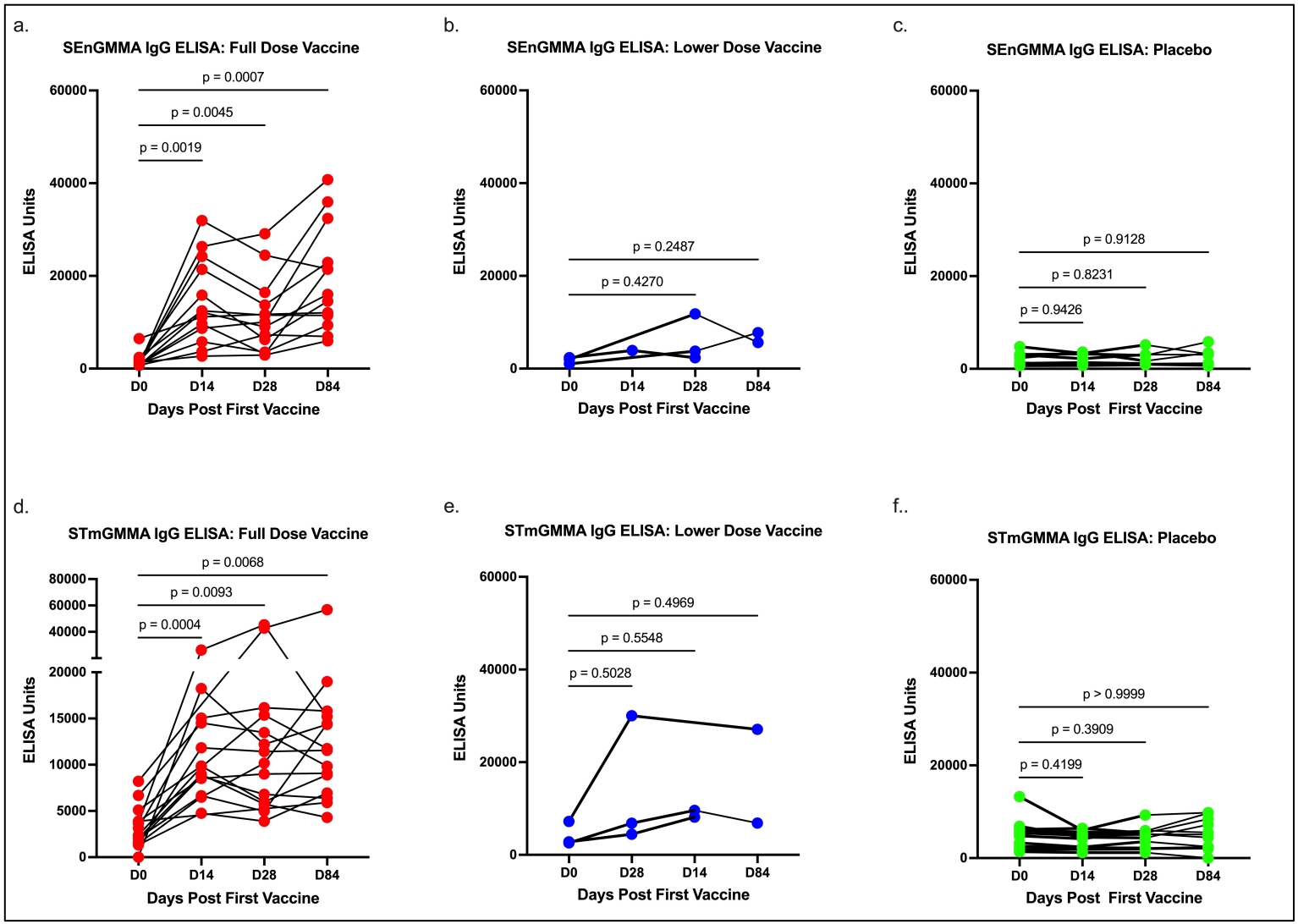



*Figure 29 Full Dose iNTS-GMMA vaccine (red, n=15) demonstrates a significant increase in SEn GMMA (a) and STm GMMA (d) IgG levels at D14, D28 and D84 in healthy adult volunteers; No significant difference in IgG levels for both lower dose iNTS-GMMA vaccine (blue, n=3)[SEnGMMA, b; STmGMMA, e] and placebo (green, n=11)[SEnGMMA, c; STmGMMA, f]. Statistical Test: Mixed-effects analysis with Geisser-Greenhouse correction and Dunnett's multiple comparison tests were used to compare the mean differences between each timepoint within each intervention or placebo.*

## Elevated Serum GMMA IgA Response Following iNTS-GMMA Vaccination

The samples in the GMMA IgG ELISA were also tested in the IgA ELISA. Baseline SEnGMMA IgA-specific antibodies in the placebo (GMC 14,494 95% CI [8,162-25,739]) recipients were significantly higher than the full dose (GMC 5,691 [3,733-8,677], p=0.03) but not significantly different from lower dose (GMC 4,029 [717-22,636], p=0.13) vaccine recipients. In contrast, there was no significant overall difference seen at baseline (D0) between full dose (GMC 3,759 [2,114-6,684], p= 0.89), lower dose vaccine (4,546 [255-81,170], p= 0.98) and placebo (GMC 4,953 [3,239-7,576]) for STmGMMA IgA antibodies (figure 30-32).

When compared with baseline, a significant increase in GMMA-specific IgA GMC was observed at 14 days after full dose vaccination (SEnGMMA GMC 11,418 [7,251-17,979], p=0.046; STmGMMA GMC 10,378 [7,848-13,725], p=0.03), but not after lower dose vaccination (SEnGMMA GMC 8,090 [1,555-42,079], p=0.40; STmGMMA GMC 17,977 [839-385,087], p=0.63) or placebo (SEnGMMA GMC 1,880 [1,030-3,431], p=0.87; STmGMMA GMC 3,661 [2,373-3,661], p=0.79) for both serovars. A similar pattern was seen at 28 days after full dose vaccination (SEnGMMA GMC 8,729 [5,960-12,784], p<0.001; STmGMMA GMC 10462 [6983-15672], p=0.009), lower dose vaccination (SEnGMMA GMC 4,704 [598-36,956], p=0.41; STmGMMA GMC 9,691 [803-116,994], p=0.48) and placebo (SEnGMMA GMC 2,423 [1,562-3,758], p=0.98; STmGMMA GMC

3,942 [2,633-5,904], p=0.94). At 84 days after full dose vaccination, a significant increase in GMMA-specific IgA GMC above baseline was seen in SEnGMMA (SEnGMMA GMC 16,991 [11,798-24,469], p=0.006) but not in STmGMMA (STmGMMA GMC 11,331 [8,075-15,899], p=0.05). Again, there was no significant increase seen in either the lower dose (SEnGMMA GMC 6615 [868-50384], p=0.41; STmGMMA GMC 13633 [2.2293-8,336,541], p=0.35) vaccine recipients or placebo (SEnGMMA GMC 2,540 [1,448-4,457], p=0.14; STmGMMA GMC 4,946 [3,242-7,546], p=0.34)[figure 33-35].

The highest IgA response among the NTS sera was similar to the highest vaccine response. No overall significant difference in IgA antibodies was seen between all D14 convalescent NTS sera IgA and iNTS-GMMA vaccine full dose (SEnGMMA, p=0.84; STmGMMA, p=0.96), lower dose (SEnGMMA, p>0.99; STmGMMA, p=0.12), and placebo recipients (SEnGMMA, p=0.66; STmGMMA, p=0.59)[figure 31-32].

There was no significant difference in increased fold change from D0 to D14 between SEnGMMA (GM 16.50 [6.947-39.19]) and STmGMMA (GM 10.41 [3.891-27.84]) IgA-specific antibodies in the full-dose vaccine recipients (p=0.36).

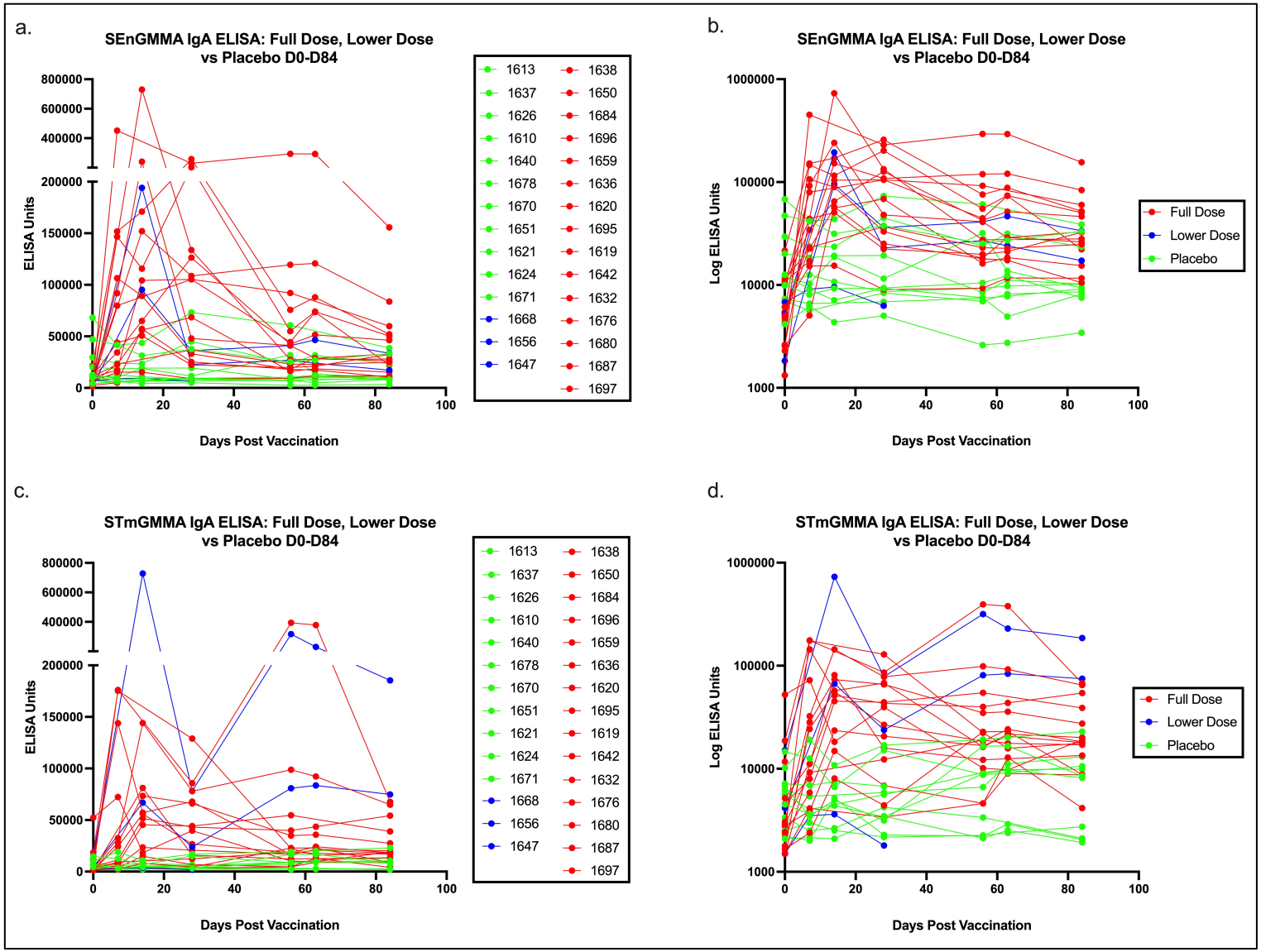



*Figure 30 Generation of SEn (a, b) and STm GMMA (c, d) IgA response in healthy adult volunteers following iNTS-GMMA vaccination (full dose, red, n=15; lower dose, blue, n=3) and placebo (green, n=11); D0-D84 following First Vaccination (D0) and Second Vaccination (D56) in ELISA Units (a, c) and Log ELISA Units (b, d)*

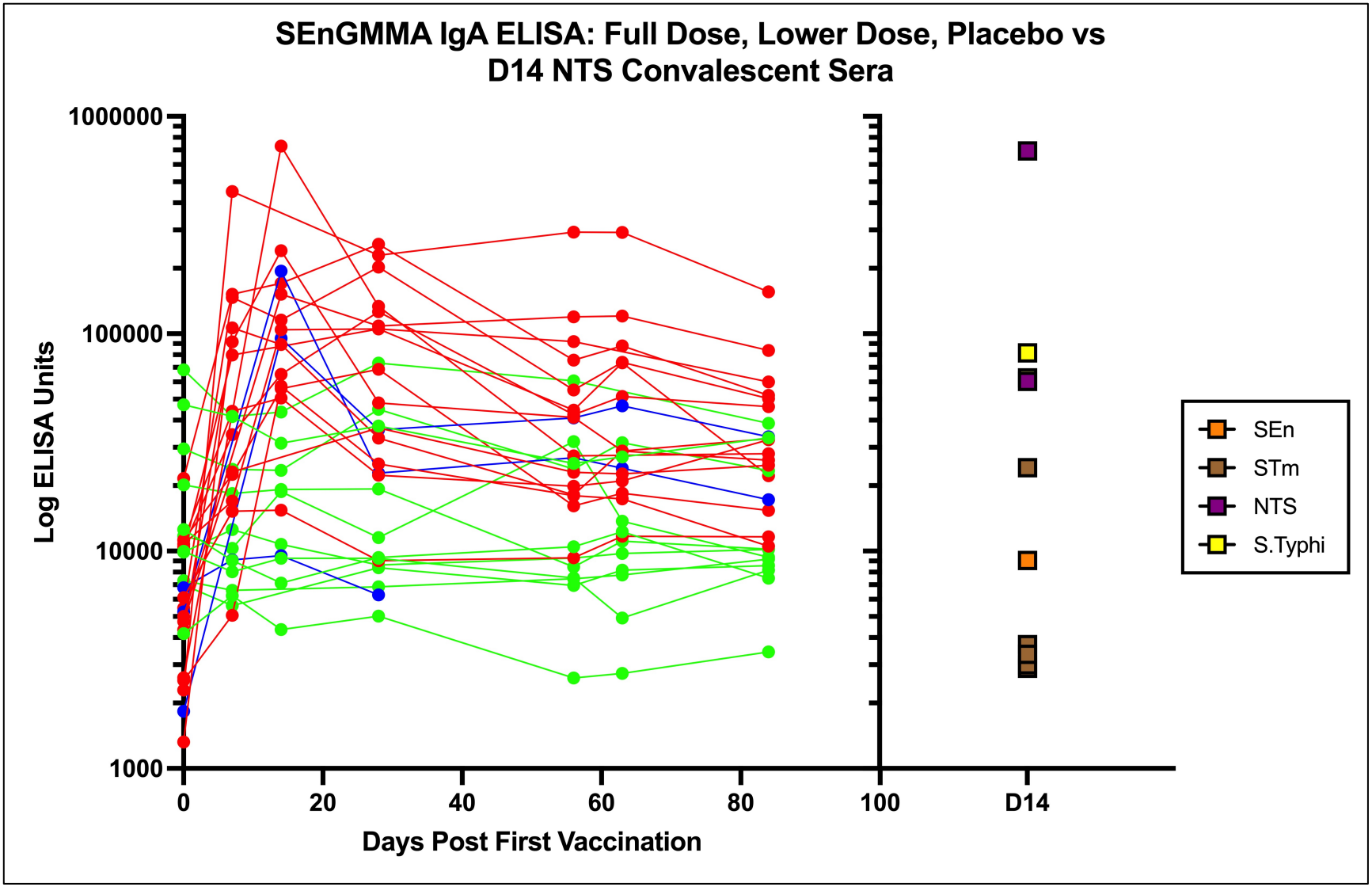



*Figure 31 Comparison of SEnGMMA IgA response following full dose (red, n=15), lower dose (blue, n=3) iNTS-GMMA vaccination and placebo (green, n=11) in healthy UK adult volunteers [D0-D84 (Left)] with D14 convalescent NTS / S.Typhi sera (SEn = 2, STm = 5, Unknown NTS = 2, S.Typhi =1) in children (aged 12 and under) from sub-Saharan Africa (Right)*

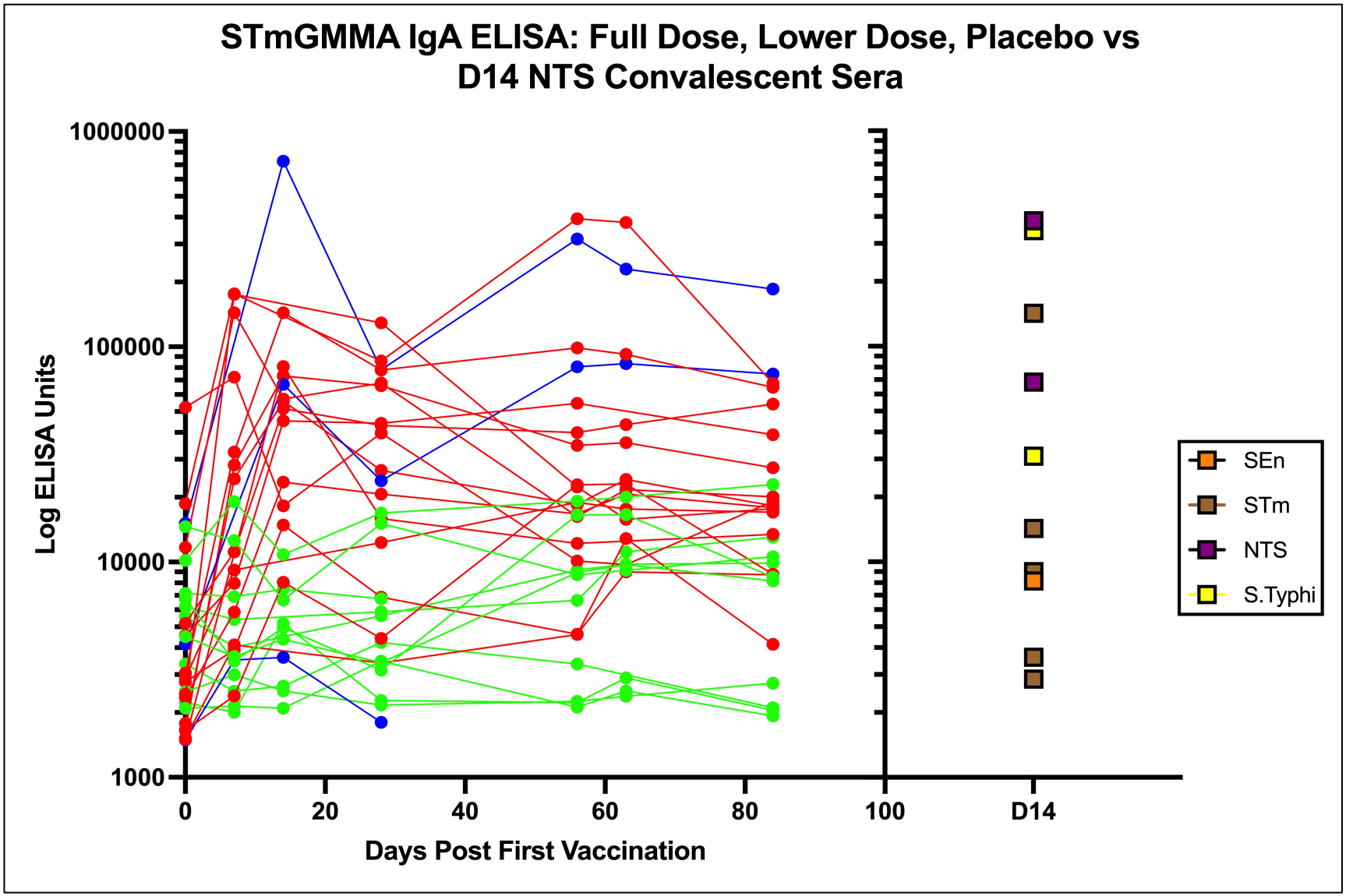



*Figure 32 Comparison of STmGMMA IgA response following full dose (red, n=15), lower dose (blue, n=3) iNTS-GMMA vaccination and placebo (green, n=11) in healthy UK adult volunteers [D0-D84 (Left)] with D14 convalescent NTS / S.Typhi sera (SEn = 2, STm = 5, Unknown NTS = 2, S.Typhi =1) in children (aged 12 and under) from sub-Saharan Africa (Right)*

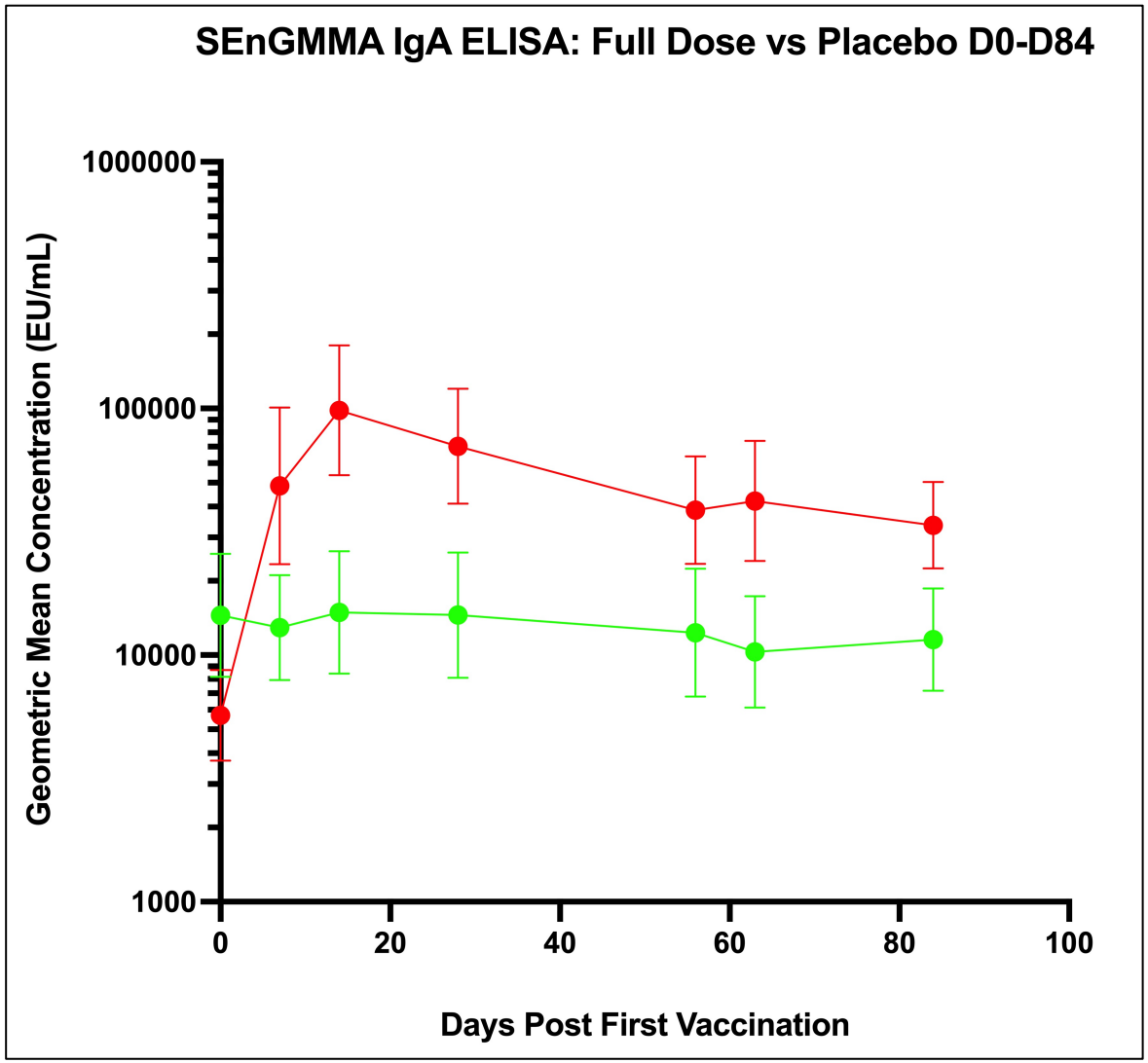



*Figure 33 Comparison of geometric mean concentration (GMC) with 95% Confidence Interval (CI) SEnGMMA IgA response in healthy adult volunteers following Full Dose (Red, n=15) and Lower Dose (Blue, n=3) iNTS-GMMA vaccination vs Placebo (Green, n=11) D0-D84; Full Dose iNTS-GMMA vaccine vs placebo demonstrates significant increase above placebo in GMC from D7 to D84; NB: Lower dose has not been shown due to the wide confidence interval (n=3)*

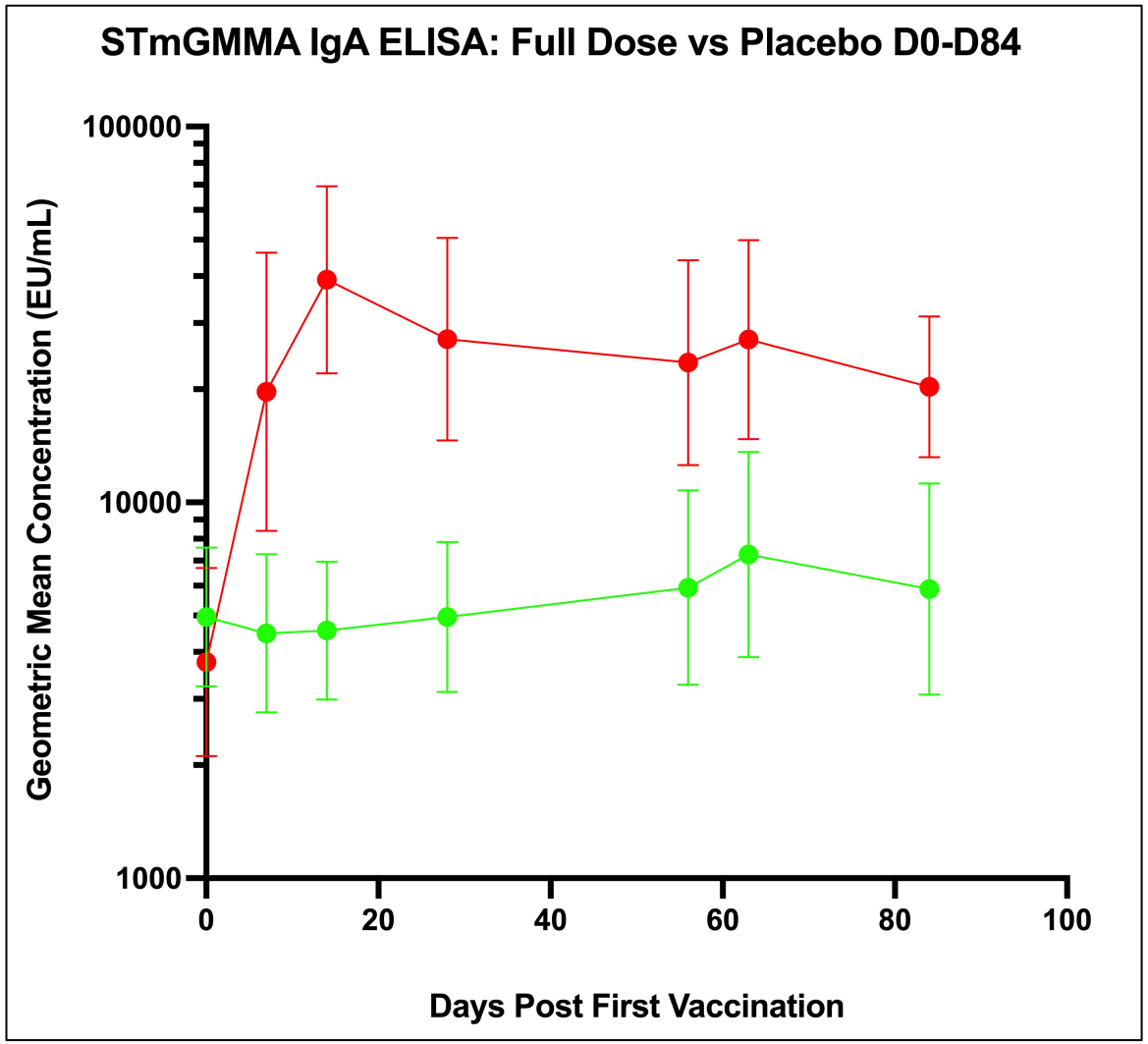



*Figure 34 Comparison of geometric mean concentration (GMC) with 95% Confidence Interval (CI) STmGMMA IgA response in healthy adult volunteers following Full Dose (Red, n=15) and Lower Dose (Blue, n=3) iNTS-GMMA vaccination vs Placebo (Green, n=11) D0-D84; Full Dose iNTS-GMMA vaccine vs placebo demonstrates significant increase above placebo in GMC from D7 to D84; NB: Lower dose has not been shown due to the wide confidence interval (n=3)*

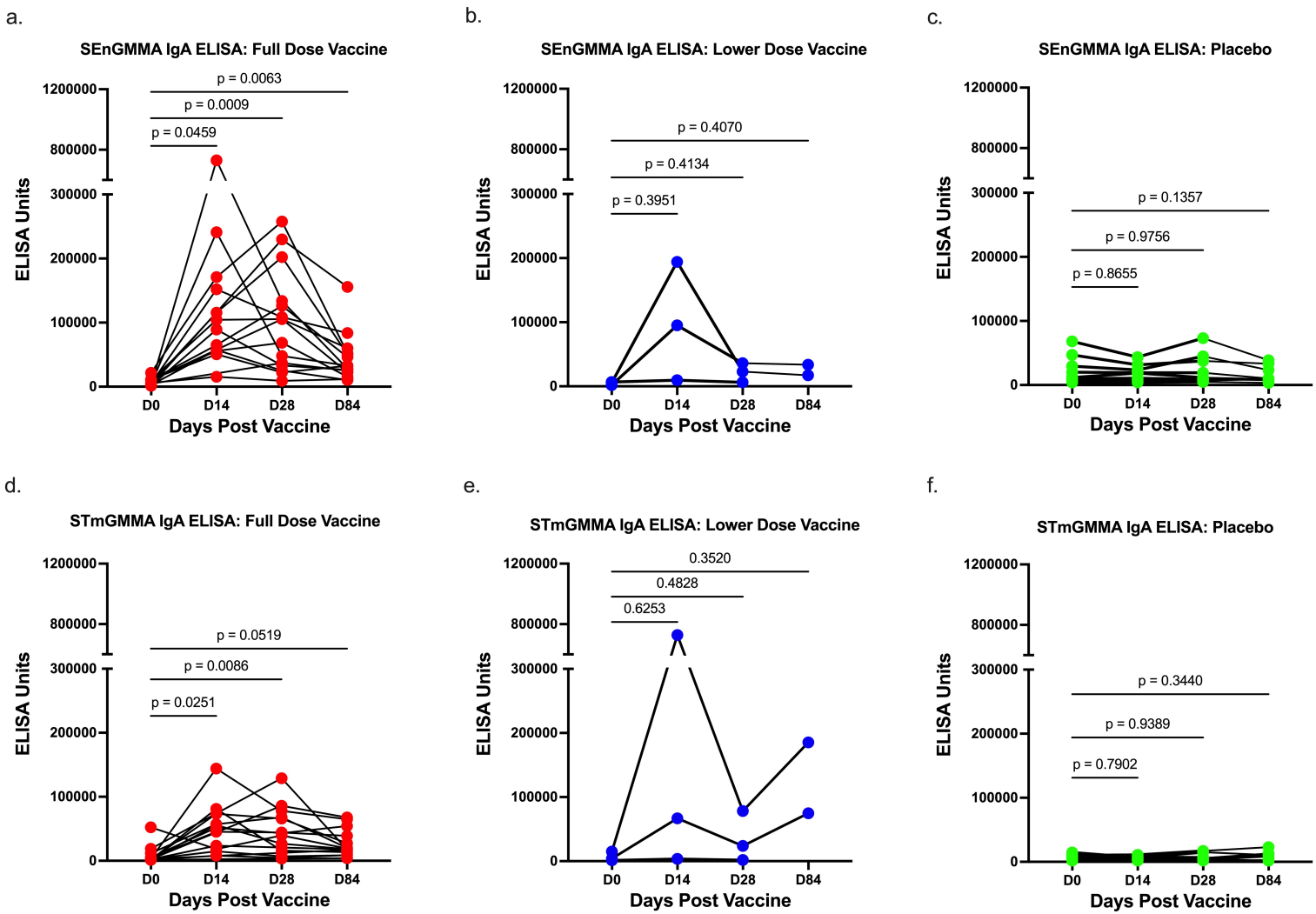



*Figure 35 Full Dose iNTS-GMMA vaccine (red, n=15) demonstrates a significant increase in SEn GMMA (a) and STm GMMA (d) IgA levels at D14, D28 and D84 in healthy adult volunteers; No significant difference in IgA levels for both lower dose iNTS-GMMA vaccine (blue, n=3)[SEnGMMA, b; STmGMMA, e] and placebo (green, n=11)[SEnGMMA, c; STmGMMA, f]. Statistical Test: Mixed-effects analysis with Geisser-Greenhouse correction and Dunnett's multiple comparison tests were used to compare the mean differences between each timepoint within each intervention or placebo.*

## Elevated Oral Fluid GMMA IgA Response on Day 14 Following iNTS-GMMA Vaccine

Oral fluid samples following iNTS-GMMA vaccination or placebo taken at baseline (D0), D14, D28, D56 and D84 were tested in the SEn and STm GMMA IgA ELISA. Full dose, n= 15 (red), lower dose, n= 3 (blue) and placebo, n= 4 (green) samples were tested (figure 36). Peak SEnGMMA IgA antibodies were seen at D14 in some participants following iNTS-GMMA vaccination, rapidly declining to baseline after that. Some participants also demonstrated a similar peak in STmGMMA IgA ELISA at day 14, but the majority of STmGMMA IgA antibody responses were similar to placebo. The oral fluid IgG GMMA antibody levels was not tested as prior studies showed a correlation between serum and oral fluid (crevicular fluid) IgG due to the overspill of IgG from the circulation[98].

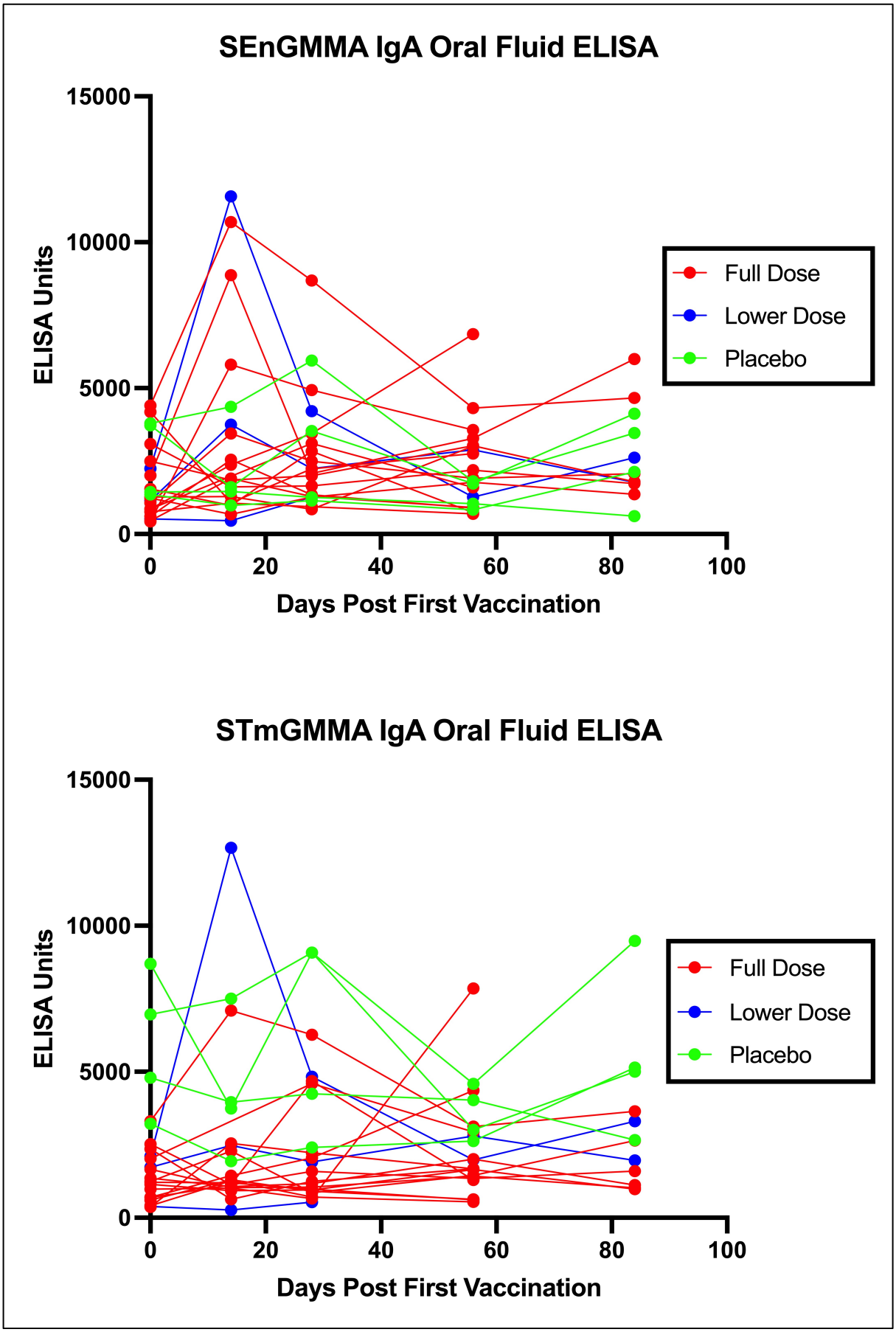



*Figure 36 GMMA-specific oral fluid IgA response in healthy UK adult volunteers following iNTS-GMMA vaccination (full dose, lower dose) and placebo from D0-D84; SEnGMMA (top) and STmGMMA (bottom)*

## Correlation Analysis of IgG and IgA GMMA ELISA

There was a significant positive correlation between antigen specific serum IgG and IgA titres for each of SEnGMMA and STmGMMA as determined by Spearman correlation (Figures 37a and b). However, there was no correlation between antigen specific serum IgA sera and oral fluid IgA for SEnGMMA or STm GMMA (Figures 37c and d).

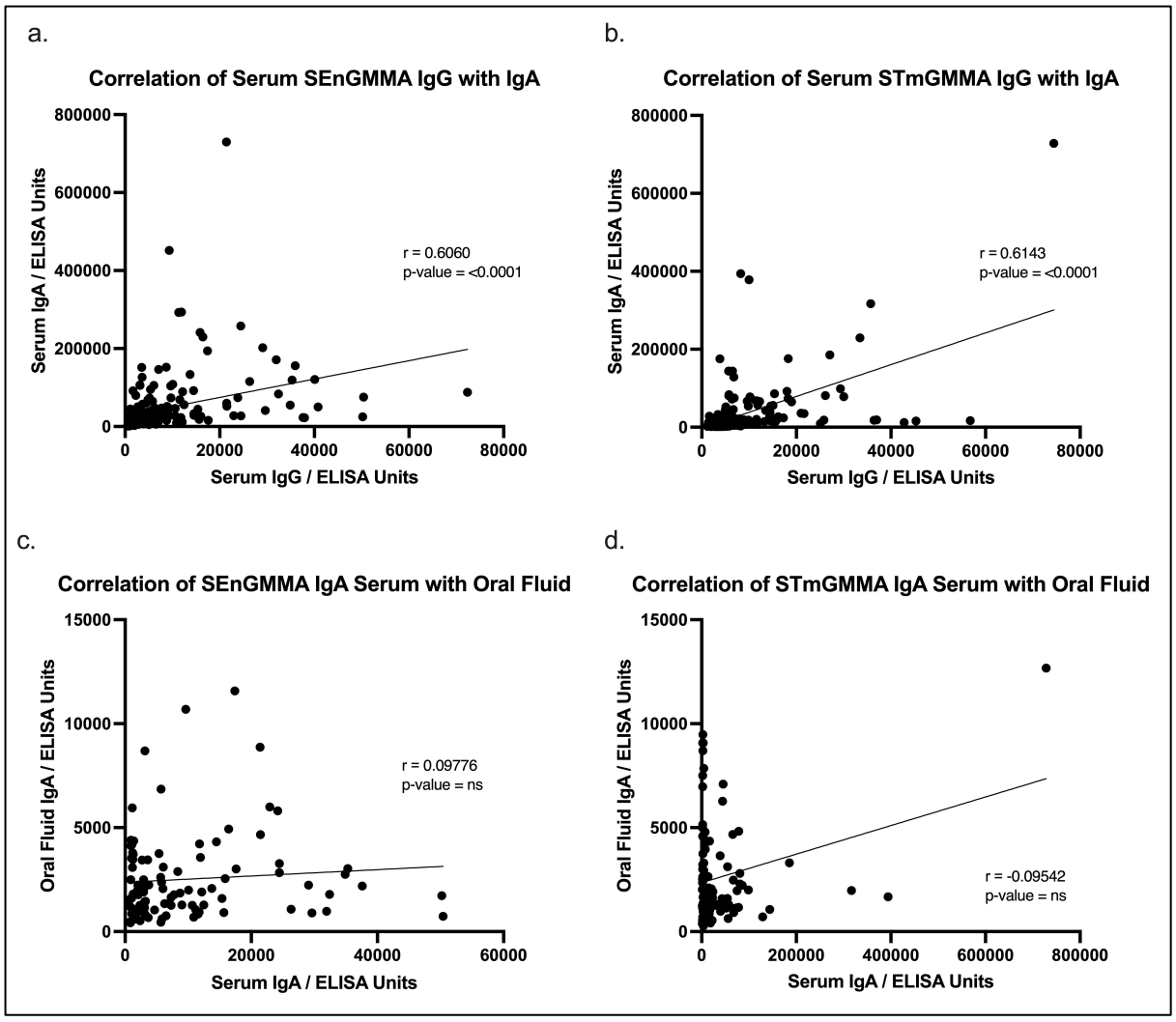



*Figure 37 Correlation Analysis of SEnGMMA (a) and STmGMMA (b) serum IgG vs IgA; and SEnGMMA (c) and STmGMMA (d) serum vs oral IgA; Spearman correlation across available matched time points in healthy adult volunteers between D0-D84 per analysis*

### 4.3.2 Elevated Functional Antibody Response to the iNTS-GMMA Vaccine Using GMMA and OAg-based Antibody-dependent Monocyte Phagocytosis (ADMP) Assays

Available sera from participants vaccinated with either the full dose (n=15) or lower dose iNTS GMMA vaccine (n=3) and placebo (n=7) and following the first and second vaccine administration (Day 0-84) were tested using both GMMA and O-antigen specific ADMP assays (figures 38-43).

No significant difference in ADMP activity at baseline (D0) was seen between full dose, lower dose and placebo recipients in assays using GMMA SEn (full dose, p=0.92; lower dose, p=0.94), GMMA STm (full dose, p=0.62; lower dose, p=0.31), SEn OAg beads (full dose, p=0.95; lower dose, p=0.63), and STm OAg beads (full dose, p=0.93; lower dose, p=0.88).

A significant increase from baseline to 28 days following vaccination in antibody-dependent phagocytosis of both SEn and STm GMMAs was seen in the full dose vaccine recipients (SEnGMMA: GM MedFI 232 [205-262], p<0.001; STmGMMA: GM MedFI 765 [684-855], p<0.001) but not in the lower dose vaccine recipients (SEnGMMA: GM MedFI 184 [92-366], p=0.46; STmGMMA: GM MedFI 915 [352-2358], p=0.39 or placebo recipients (SEnGMMA: GM MedFI 155 [136-176], p=0.90; STmGMMA: GM MedFI 540 [433-672], p=0.15) (figures 38, 40 and 43).

A significant increase above baseline at 28 days in ADMP activity was observed in the full dose vaccine recipients (SEn OAg: GM MedFI 898 [719-1120] p=0.02; STm OAg: GM MedFI 538 [404-717], p=0.045). Lower dose vaccine recipients had a significant increase in ADMP measured using STm OAg beads (GM MedFI 1075 [751-1539], p=0.002) but not SEn OAg beads (GM MedFI 866 [304-2473], p=0.13) . No significant change was seen in placebo recipients (SEn OAg: GM MedFI 682 [566-822], p=0.76; STm OAg: GM MedFI 303 [193-475], p=0.47) (figures 41 and 43). Where samples (full dose, n=3; lower dose, n=2; placebo, n=4) were available for further time points, functional antibodies remained elevated above baseline for both SEn and STm GMMA at D84 (figure 38). However, several individual participants did not appear to mount a functional antibody response against the O-antigen (figure 39, 41 and 43). The sample size at D84 was not sufficient for statistical testing.

Fold change in ADMP activity from day 0 to day 28 in full dose recipients was similar when measured in assays using SEn GMMA (GM 1.414 [1.236-1.619]) and STm GMMA (GM 1.318 [1.180-1.1.473]) (p= 0.34) [figure 42]. This was also true when comparing ADMP fold change using SEn OAg beads (GM 1.274 [1.043-1.555]) and STm OAg beads (GM 1.527 [1.069-2.180]) (p=0.21) at these time points [figure 43].

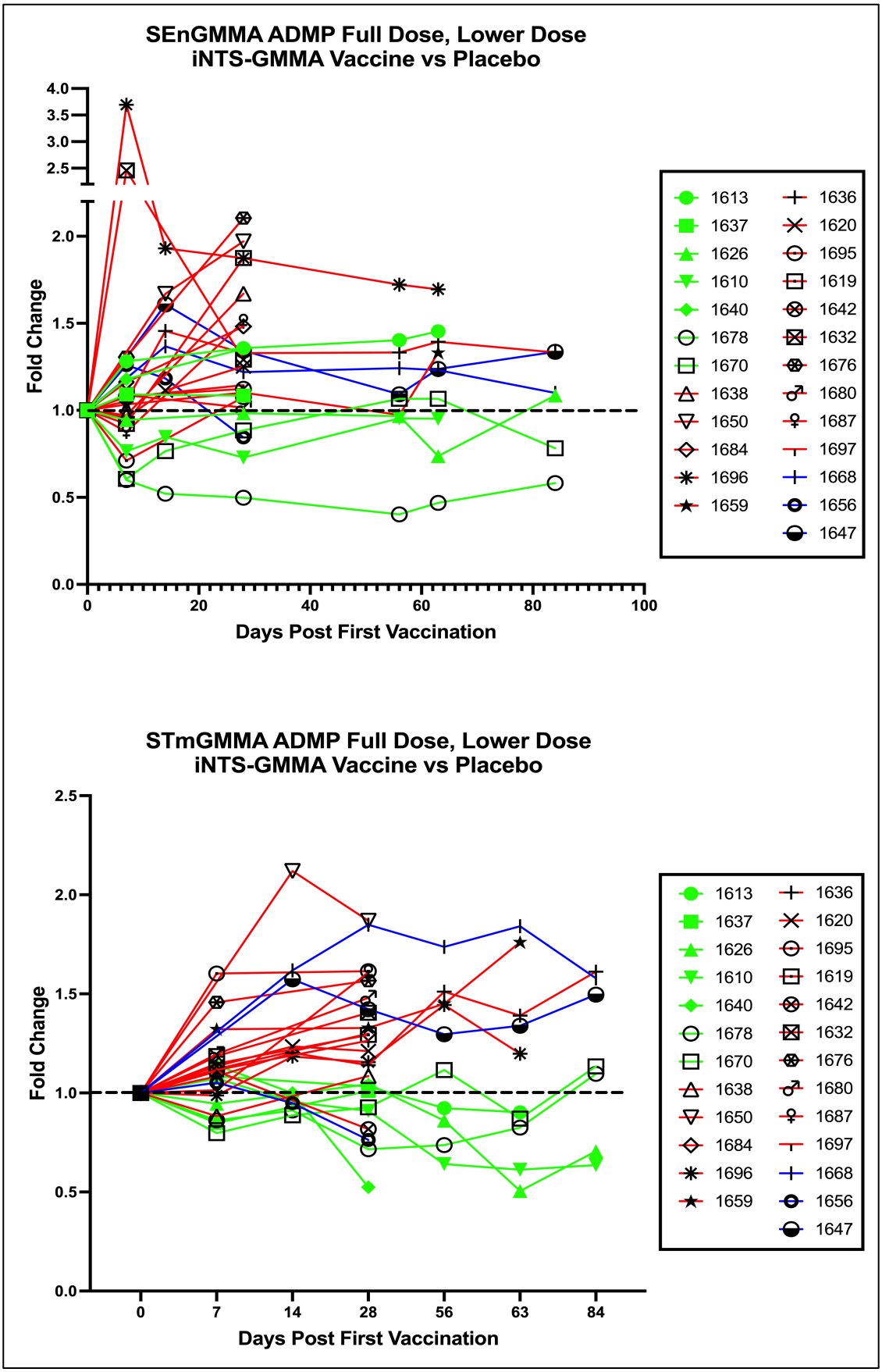



*Figure 38 SEn (Top) and STm (Bottom) GMMA-based ADMP response in healthy adult volunteers following iNTS-GMMA vaccination (full dose, red, n=15; lower dose, blue, n=3) and placebo (green, n=7) ); D0-D84 following First Vaccination (D0) and Second Vaccination (D56), fold change from baseline (D0)*

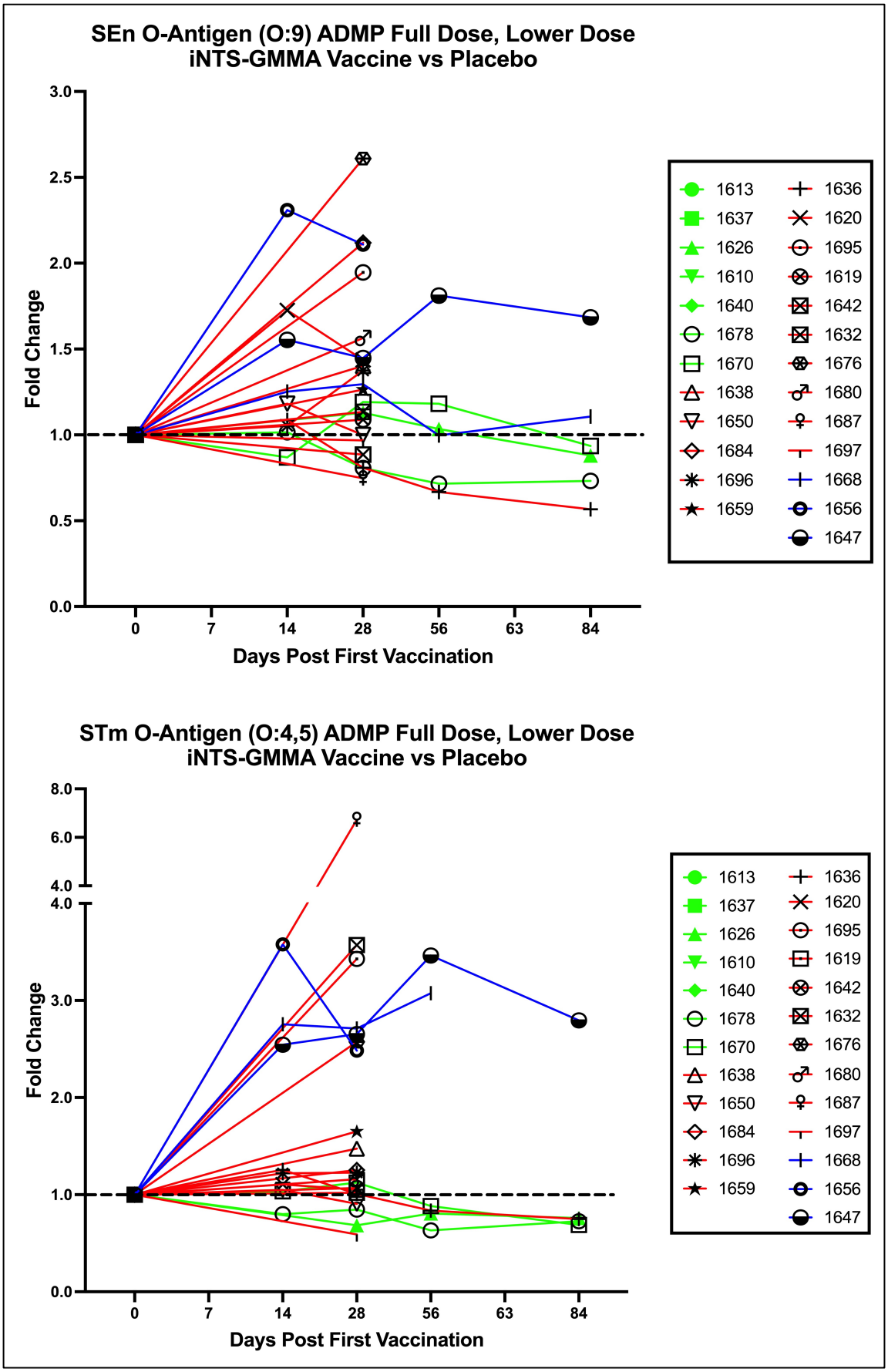



*Figure 39 SEn (Top) and STm (Bottom) O-Antigen based ADMP response in healthy adult volunteers following iNTS-GMMA vaccination (full dose, red, n=15; lower dose, blue, n=3) and placebo (green, n=7) ); D0-D84 following First Vaccination (D0) and Second Vaccination (D56), fold change from baseline (D0)*

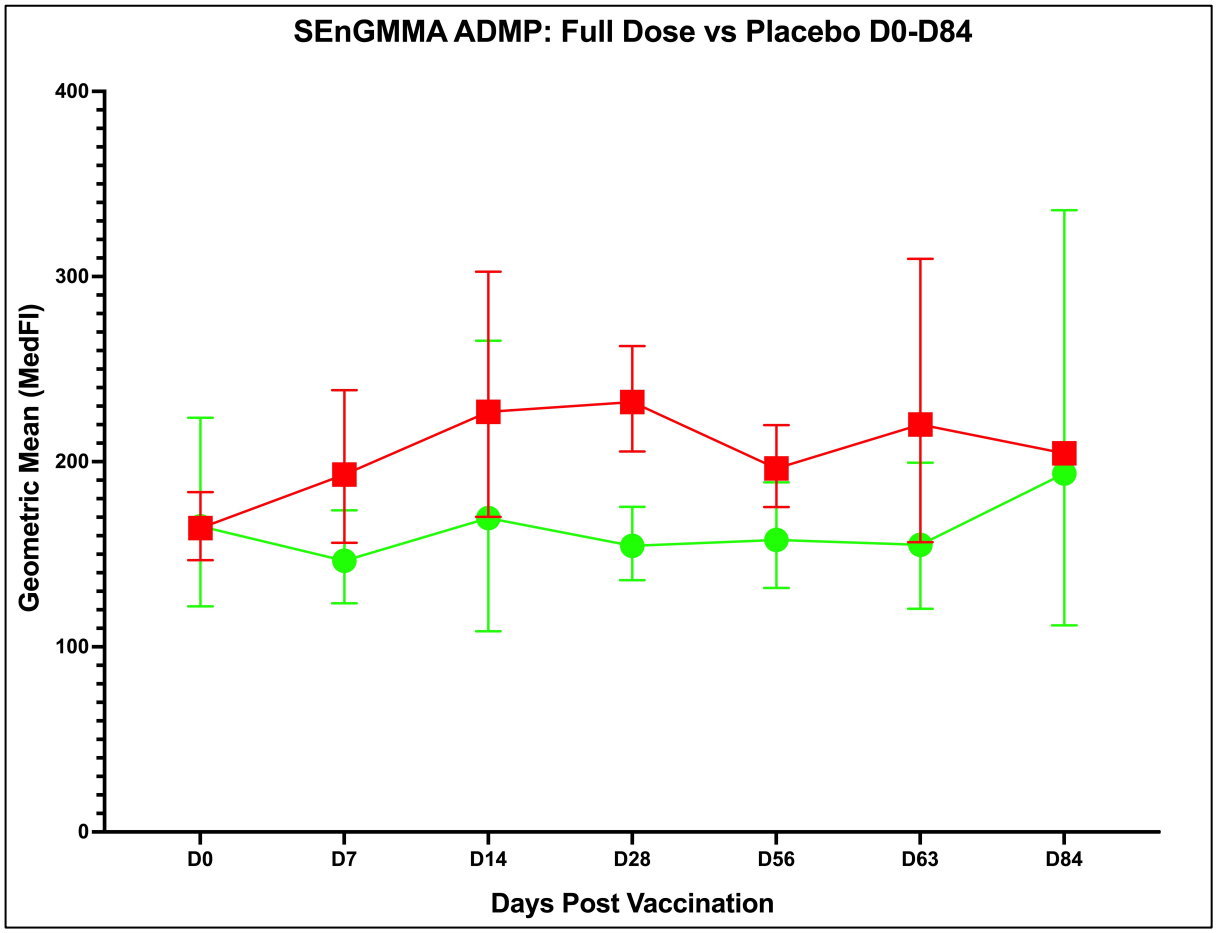



*Figure 40 Comparison of geometric mean (of median Fluorescent Intensity) with 95% Confidence Interval (CI) SEnGMMA ADMP response following full dose (red, n=15) iNTS-GMMA vaccination vs placebo (green, n=11) D0-D84, in healthy adult volunteers; full dose iNTS-GMMA vaccination demonstrates a significant increase in geometric mean above placebo at D28 in the SEnGMMA based assay; NB: lower dose has not been shown due to the wide confidence interval (n=3)*

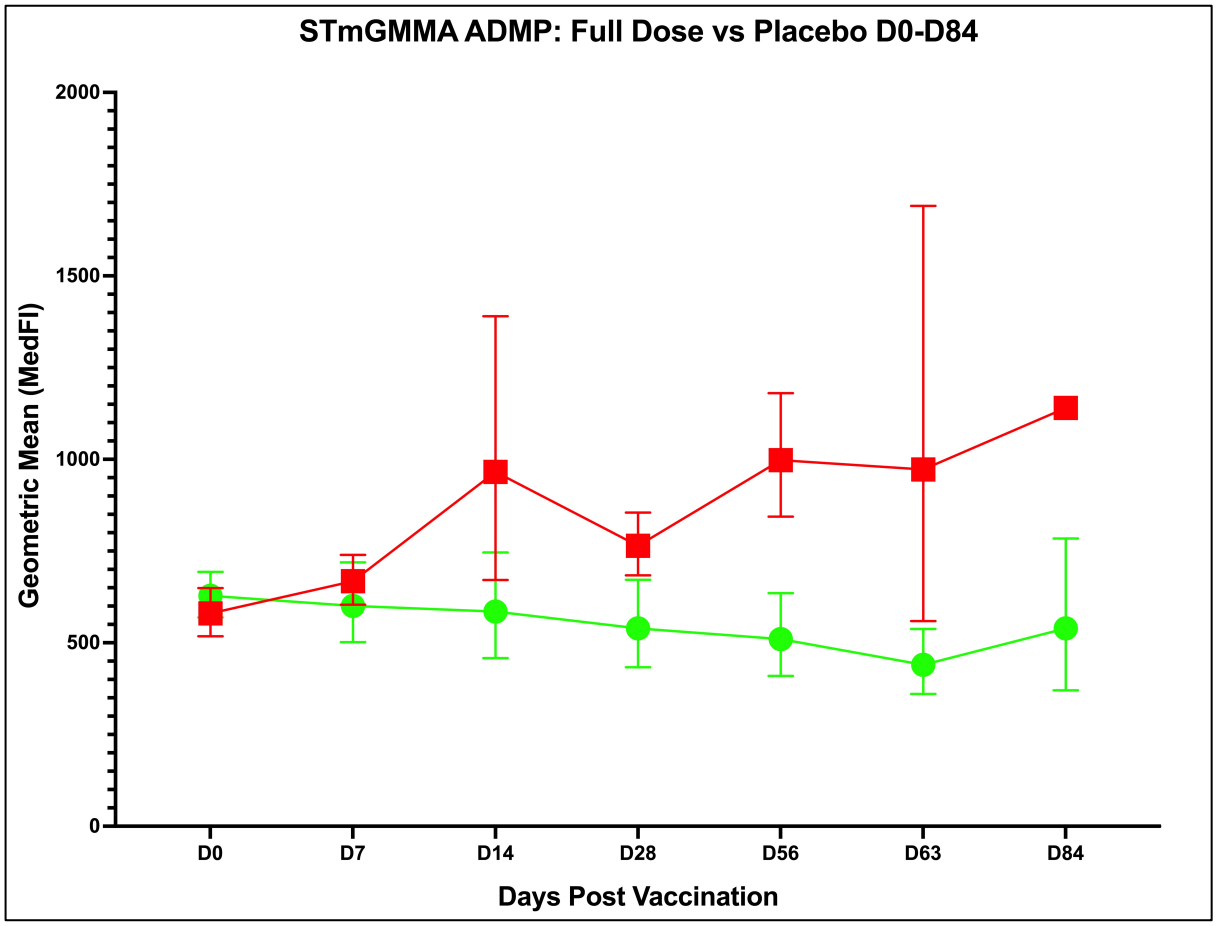



*Figure 41 Comparison of geometric mean (of Median Fluorescent Intensity) with 95% Confidence Interval (CI) STmGMMA ADMP response following full dose (red, n=15) iNTS-GMMA vaccination vs placebo (green, n=11) D0-D84, in healthy adult volunteers; full dose iNTS-GMMA vaccination demonstrates a significant increase in geometric mean above placebo at D28 in the STmGMMA based assay; NB: lower dose has not been shown due to the wide confidence interval (n=3)*

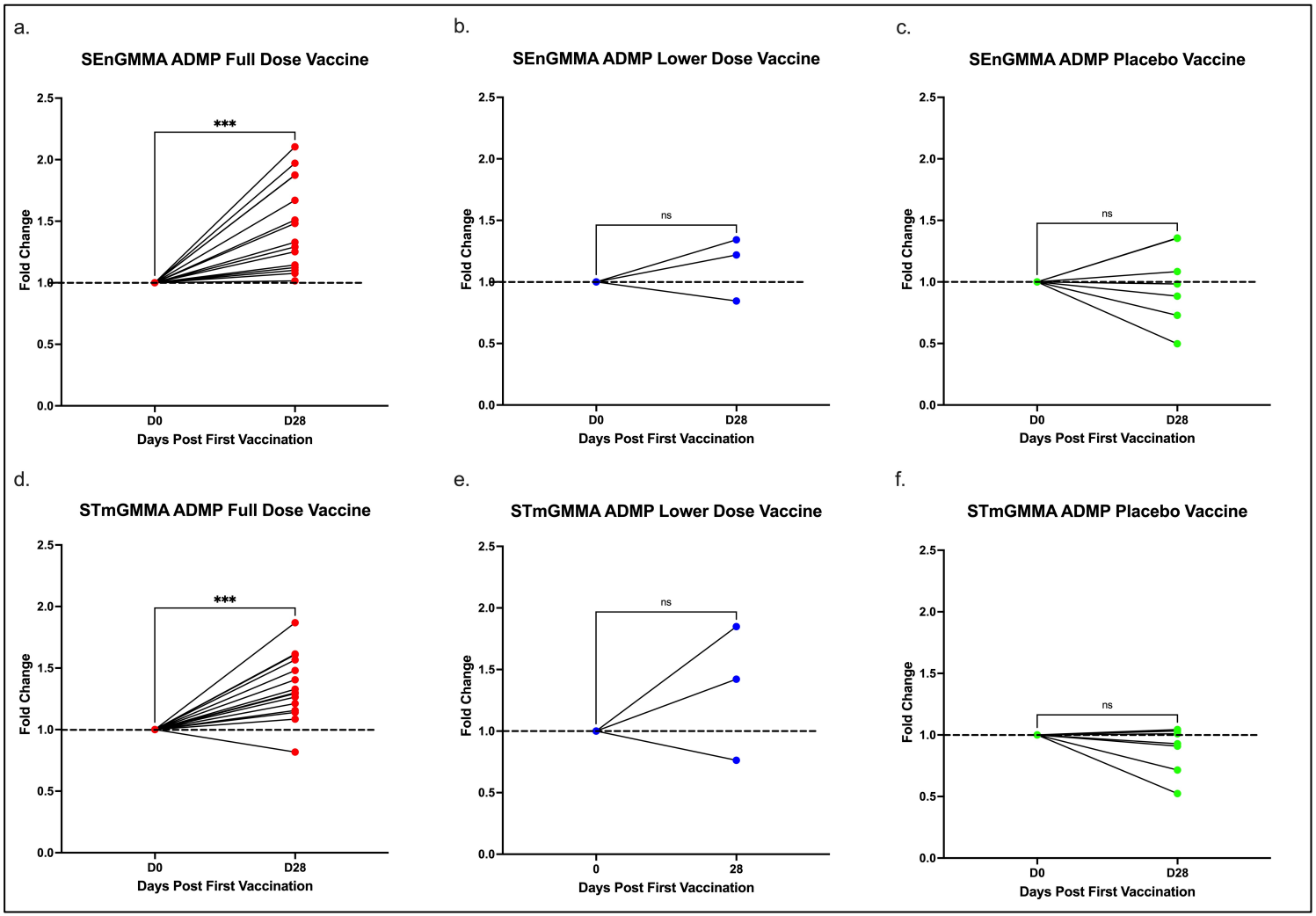



*Figure 42 SEn (Top, a-c) and STm (Bottom, d-f) GMMA-based ADMP response in healthy adult volunteers from D0 - D28, full dose iNTS-GMMA vaccine (red, n=15), lower dose iNTS-GMMA vaccine (blue, n=3) and placebo (green, n=7); Statistical analysis: Paired T-test comparing the overall mean fold change between D0 and D28. SEnGMMA ADMP: (a) full dose vaccine D0 vs D28, p<0.001; (b) lower dose vaccine, D0 vs D28, p=0.46; (c) placebo, D0 vs D28, p=0.90. STmGMMA ADMP: (d) full dose vaccine D0 vs D28, p<0.001; (e) lower dose vaccine, D0 vs D28, p=0.39; (f) placebo, D0 vs D28, p=0.15. *P<0.05, **p<=0.01, ***p<=0.001.*

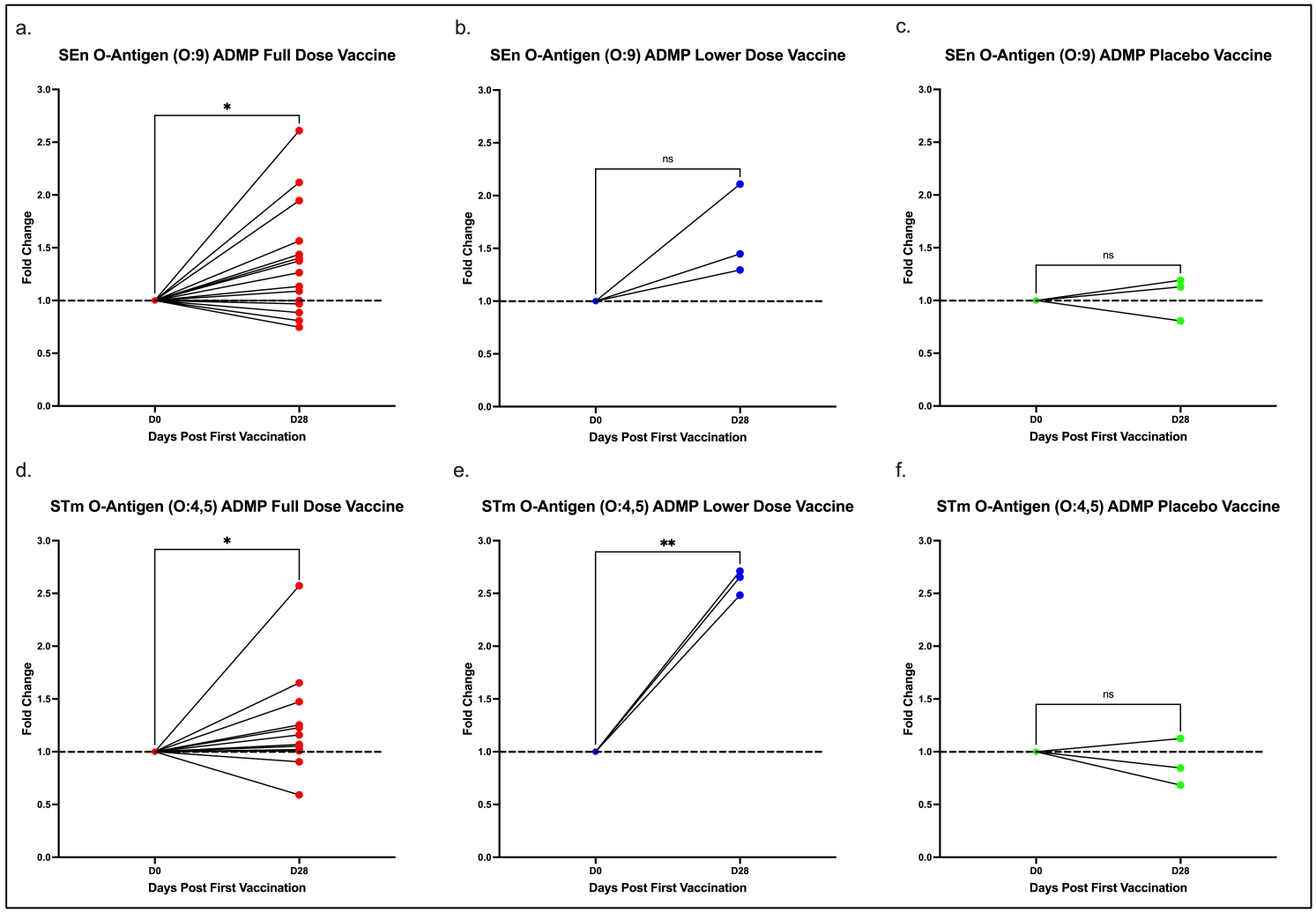



*Figure 43 SEn (Top, a-c) and STm (Bottom, d-f) O-Antigen based ADMP response in healthy adult volunteers from D0 - D28, full dose iNTS-GMMA Vaccine (red, n=15), lower dose iNTS-GMMA Vaccine (blue, n=3) and placebo (green, n=7); Statistical analysis: Paired T-test comparing the overall mean fold change between D0 and D28. SEn O-Antigen ADMP: (a) Full Dose Vaccine D0 vs D28, p=0.02; (b) Lower Dose Vaccine, D0 vs D28, p=0.13; (c) Placebo, D0 vs D28, p=0.76. STm O-Antigen ADMP: (d) Full Dose Vaccine D0 vs D28, p=0.045; (e) Lower Dose Vaccine, D0 vs D28, p=0.002; (f) Placebo, D0 vs D28, p=0.47. *P<0.05, **p<=0.01, ***p<=0.001.*

### 4.3.3 Antibody elicited by iNTS-GMMA Vaccine Stimulated Minimal NK Cell Activation

Antibody dependent NK activation following iNTS-GMMA vaccination demonstrated a minimal a response (as opposed to the ADMP response) with no significant difference in fold change from D0-D28 in either full dose (SEnGMMA, p=0.13; STmGMMA, p=0.93), lower dose (SEnGMMA, p=0.16; STmGMMA, p=0.89) or placebo recipients (figure 44). Of note, one lower dose recipient individual (sample 1668) had the highest overall activation across NK assays, whilst most participants who received the full dose vaccine, with few exceptions, demonstrated minimal NK activation. The minimal activation seen could be assay related, although the controls (positive NTS convalescent and negative control) performed adequately across NK assays.

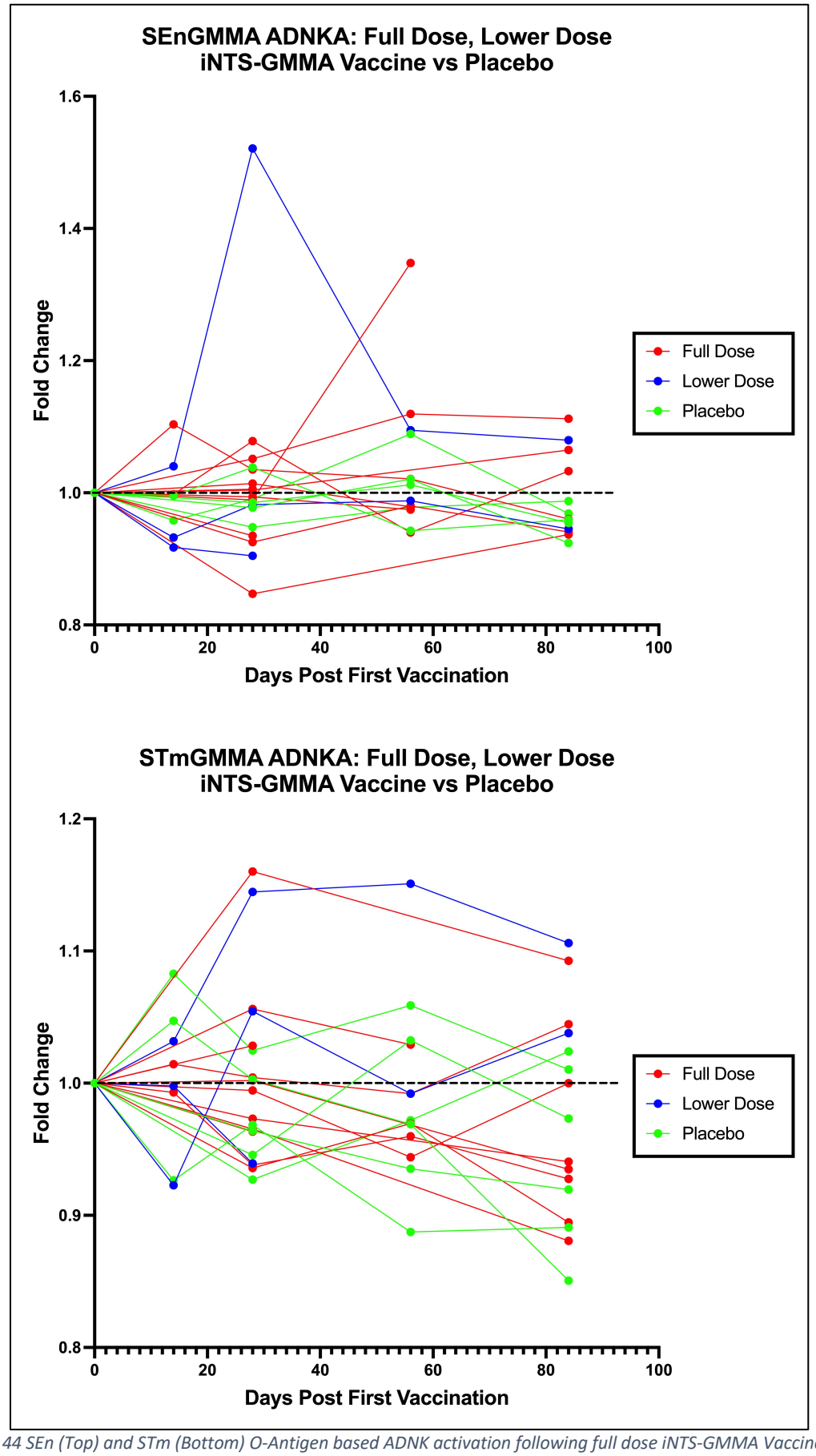



*Figure 44 SEn (Top) and STm (Bottom) O-Antigen based ADNK activation following full dose iNTS-GMMA Vaccine (red), lower dose iNTS-GMMA vaccine (blue) and placebo (green) in healthy adult volunteers*

### 4.3.4 T Cell Response to the iNTS-GMMA Vaccine

#### $CD3^+CD4^+$ Memory Response to the iNTS-GMMA Vaccine

Peripheral blood mononuclear cells (PBMCs) obtained at D28 following full dose iNTS-GMMA vaccination demonstrated significant proliferation in the $CD3^+CD4^+$ T cell subset compared with paired baseline (D0) PBMC when stimulated ex-vivo with 100 microgram SEn and STm GMMAs (SEnGMMA, 1.22% mean proliferation, n=11, p=0.046; STmGMMA, 1.49% mean proliferation, n=11, p=0.04 respectively). A borderline non-significant increase was seen when 10 micrograms of GMMAs were used in the stimulation (SEnGMMA, 3.73%, n=11, p=0.07; STmGMMA, 3.18% n=11, p=0.05). PBMCs at D28 following lower dose iNTS-GMMA vaccination demonstrated increased proliferation on stimulation with high dose GMMA above baseline, but this was not statistically significant (SEnGMMA 100µg, 1.25%, n=3, p=0.26; STmGMMA 100µg, 0.73%, n=3, p=0.24). In addition, PBMCs taken at D28 following placebo did not demonstrate proliferation in response to high dose ex vivo GMMA stimulation (SEnGMMA 100µg, 0.12%, n=3, p=0.26; STmGMMA 100µg, 0.22%, n=3, p=0.24) [figure 45 and 46].

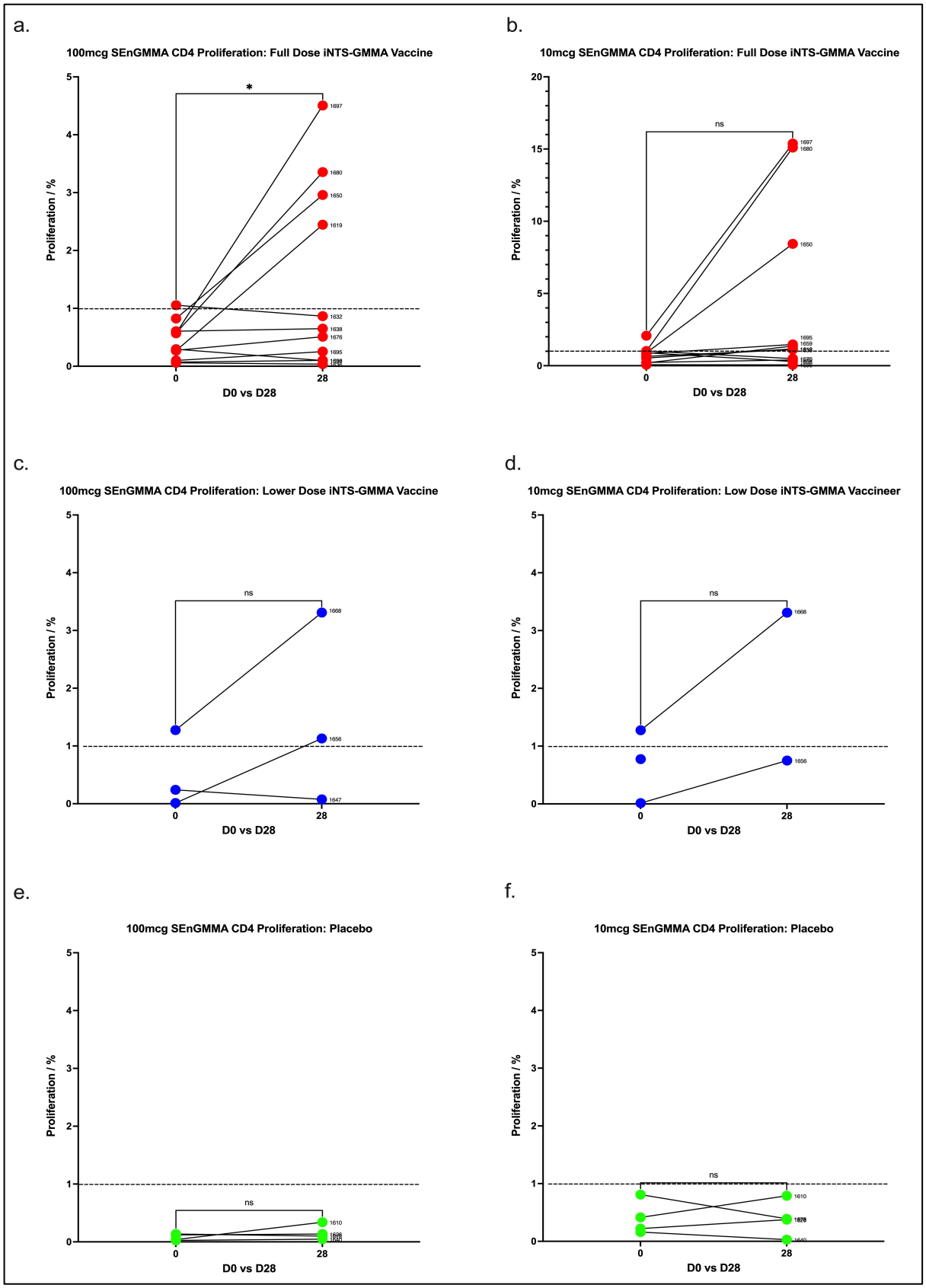



*Figure 45 PBMCs (CD3+CD4+ subset) proliferation in healthy adult volunteers at baseline (D0) vs D28 following iNTS-GMMA vaccine or placebo stimulated ex-vivo with SEnGMMA 100µg and 10µg. T-test comparing sample mean between D0 and D28, CD3+CD4+ subset following: full dose iNTS-GMMA vaccine (RED) with 100µg [n=11, p=0.046] (a) or 10µg [n=11, p=0.07] (b) stimulation; lower dose iNTS-GMMA vaccine (BLUE) with 100µg [n=3, p=0.26] (c) or 10µg[n=2, p=0.28] (d); Placebo (GREEN) with 100µg [n=4, p=0.39] (e) or 10µg[n=4, p=0.98] (f). T cell Proliferation <1% is considered negative. *P<0.05, **p<=0.01, ***p<=0.001.*

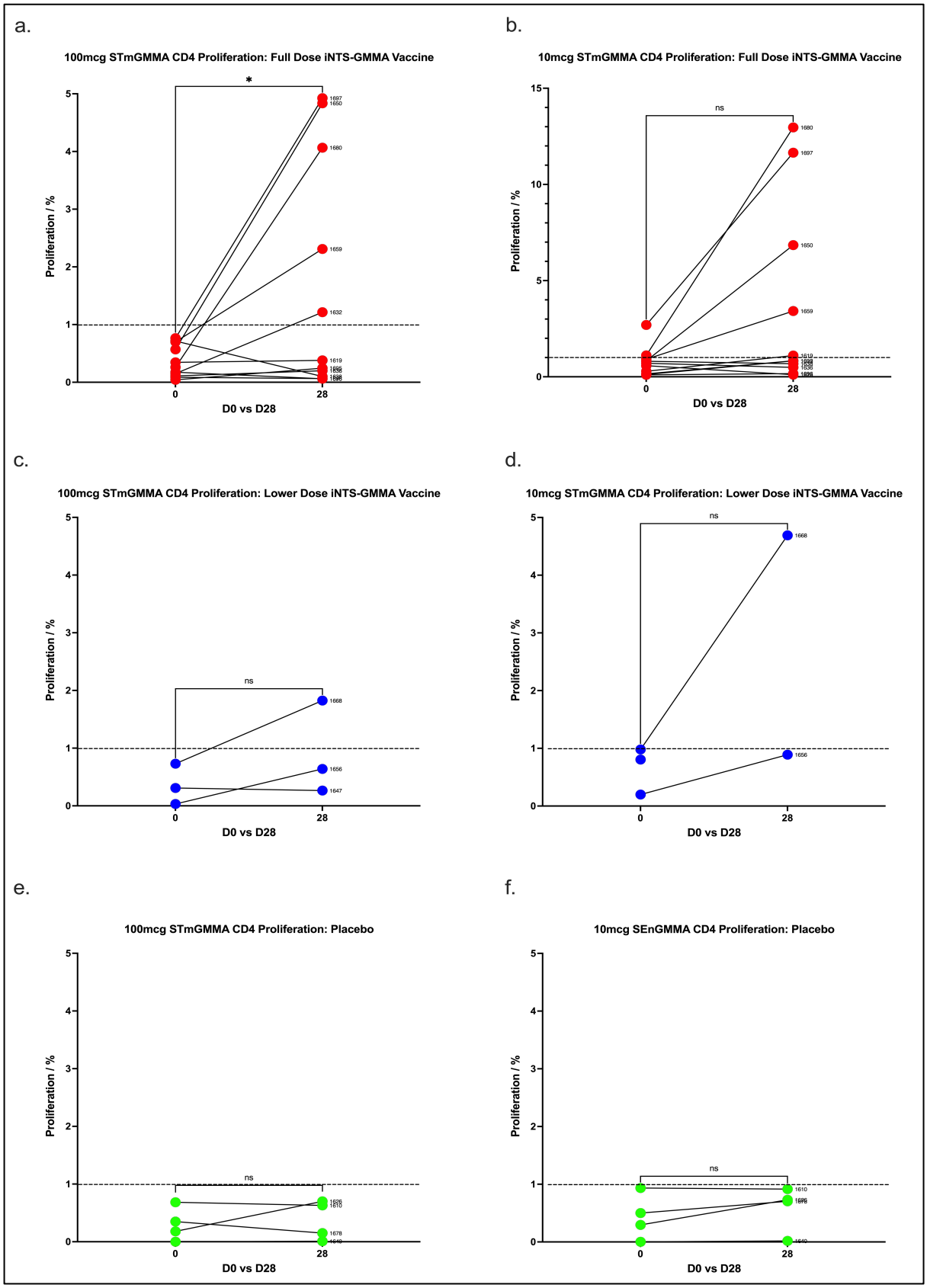



*Figure 46 PBMCs (CD3+CD4+ subset) proliferation in healthy adult volunteers at baseline (D0) vs D28 following iNTS-GMMA vaccine or placebo stimulated ex-vivo with STmGMMA 100µg and 10µg. T-test comparing sample mean between D0 and D28, CD3+CD4+ subset following: full dose iNTS-GMMA vaccine (RED) with 100µg [n=11, p=0.04] (a) or 10µg[n=11, p=0.05] (b) stimulation; lower dose iNTS-GMMA vaccine (BLUE) with 100µg [n=3, p=0.24 ] (c) or 10µg[n=2, p=0.38] (d); Placebo (GREEN) with 100µg [n=4, p=0.69] (e) or 10µg[n=4, p=0.23] (f). T cell Proliferation <1% is considered negative. *P<0.05, **p<=0.01, ***p<=0.001.*

## $CD3^+CD8^+$ Response Memory Response to the iNTS-GMMA Vaccine

Similar to the $CD3^+CD4^+$ cells, the full dose iNTS-GMMA vaccine stimulates a significant SEnGMMA (100µg SEnGMMA, 1.95%, n=11, p=0.03) and STmGMMA (100µg STmGMMA, 2.50%, n=11, p=0.03) $CD3^+CD8^+$ memory T cell response. Specifically, peripheral blood mononuclear cells (PBMCs) at D28 following full dose iNTS-GMMA vaccination demonstrated significant proliferation in the $CD3^+CD8^+$ T cell subset compared with paired baseline (D0) PBMC when stimulated ex-vivo by SEn and STm GMMAs. No significant difference was seen when 10 micrograms of GMMAs were used in the stimulation (10µg SEnGMMA, 2.27%, n=11, p=0.07; 10µg STmGMMA, 3.11%, n=11, p=0.02). PBMCs at D28 following lower dose iNTS-GMMA vaccination demonstrated increased proliferation above baseline, but this was not statistically significant (SEnGMMA 100µg, 0.44%, n=3, p=0.29; STmGMMA 100µg, 1.18%, n=3, p=0.22). In addition, PBMCs at D28 following placebo were not statistically different from baseline (SEnGMMA 100µg, 0.26%, n=3, p=0.29; STmGMMA 100µg, 0.46%, n=3, p=0.22)[figure 47 and 48].

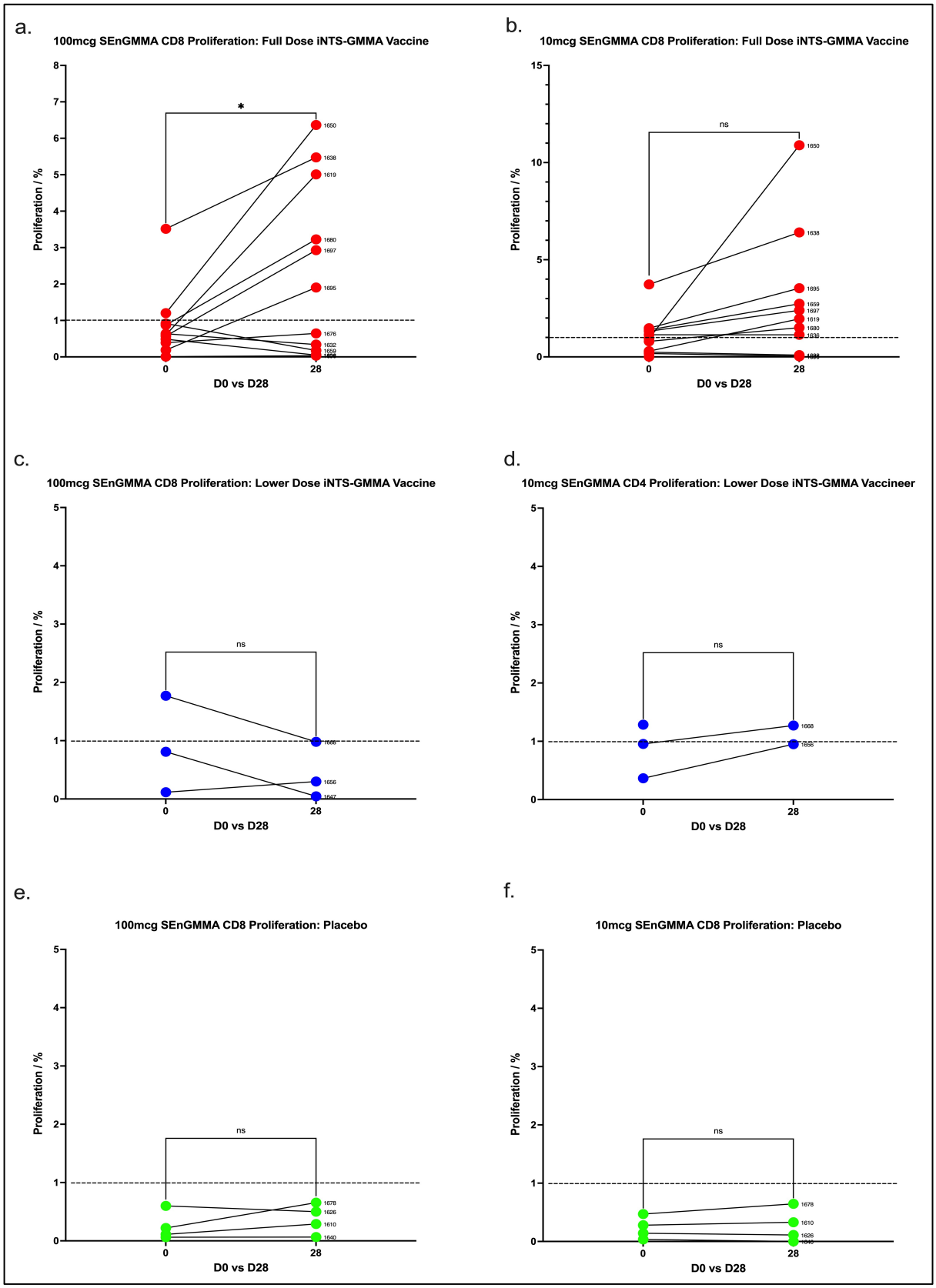



*Figure 47 PBMCs (CD3+CD8+ subset) proliferation in healthy adult volunteers at baseline (D0) vs D28 following iNTS-GMMA vaccine or placebo stimulated ex-vivo with SEnGMMA 100µg and 10µg. T-test comparing sample mean between D0 and D28, CD3+CD8+ subset following: full dose iNTS-GMMA vaccine (RED) with 100µg [n=11, p=0.03 ] (a) or 10µg[n=11, p=0.07] (b) stimulation; lower dose iNTS-GMMA vaccine (BLUE) with 100µg [n=3, p=0.29] (c) or 10µg[n=2, p=0.19 ] (d); Placebo (GREEN) with 100µg [n=4, p=0.35] (e) or 10µg[n=4, p=0.48] (f). T cell Proliferation <1% is considered negative. *P<0.05, **p<=0.01, ***p<=0.001.*

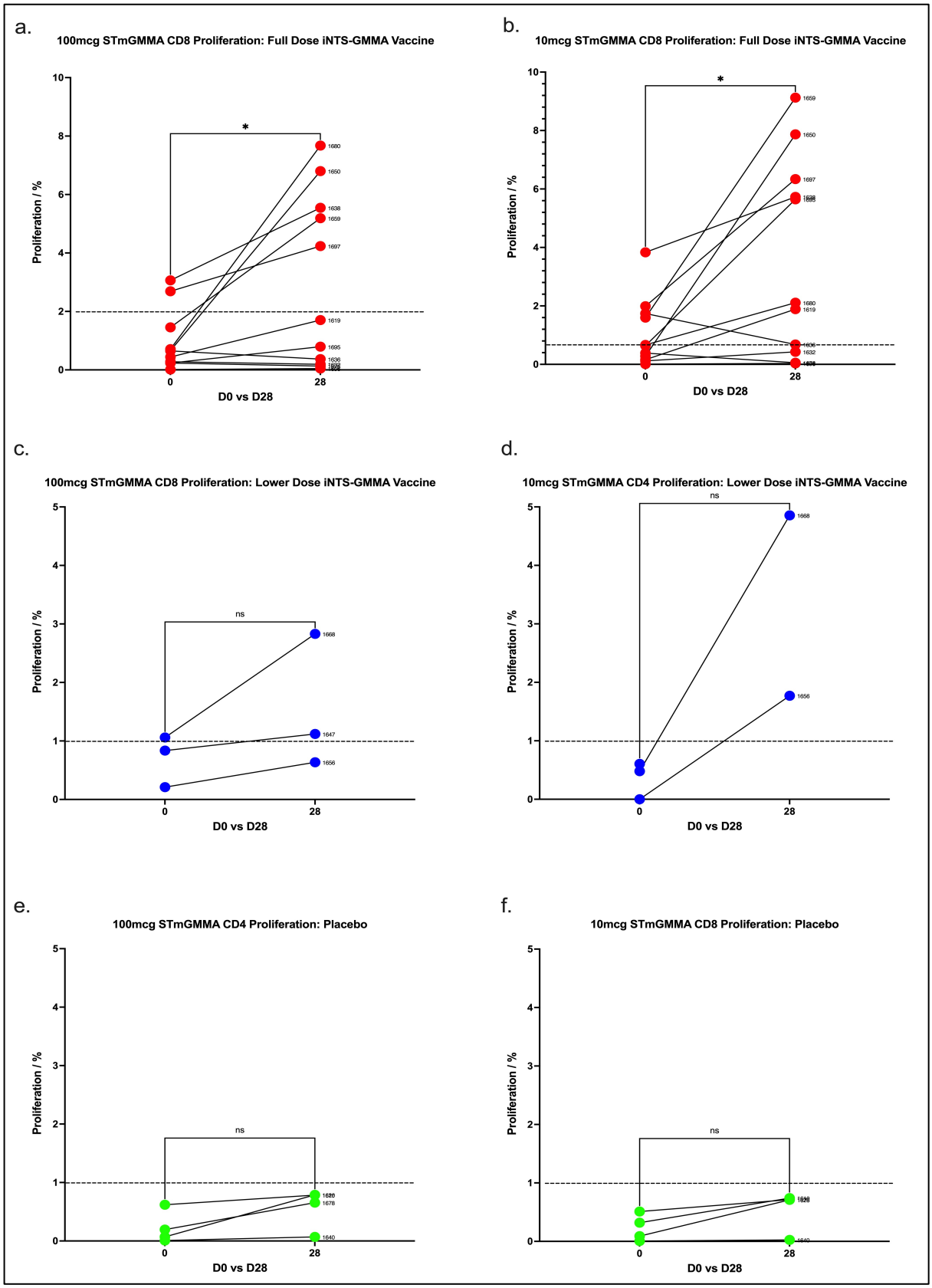



*Figure 48 PBMCs (CD3+CD8+ subset) proliferation in healthy adult volunteers at baseline (D0) vs D28 following iNTS-GMMA vaccine or placebo stimulated ex-vivo with STmGMMA 100µg and 10µg. T-test comparing sample mean between D0 and D28, CD3+CD8+ subset following: full dose iNTS-GMMA vaccine (RED) with 100µg [n=11, p=0.03] (a) or 10µg[n=11, p=0.019] (b) stimulation; lower dose iNTS-GMMA vaccine (BLUE) with 100µg [n=3, p=0.22] (c) or 10µg[n=2, p=0.26] (d); Placebo (GREEN) with 100µg [n=4, p=0.10] (e) or 10µg[n=4, p=0.09] (f). T cell Proliferation <1% is considered negative. *P<0.05, **p<=0.01, ***p<=0.001.*

### 4.3.5 Correlation of Immune Responses following iNTS-GMMA Vaccination

A Spearman correlation analysis was performed across all immunoassay responses [SEn/ STm GMMA Serum IgG / IgA levels (GM); SEn/ STm GMMA Oral Fluid IgA (GM); SEn / STm OAg / GMMA ADMP (GM MedFI); SEn / STm GMMA ADNKA (% NK Activation); 10 / 100µg SEn / STm GMMA CD4+/CD8+ (% Proliferation)] following iNTS-GMMA vaccination and antigens using available time points from Day 0 and Day 28 and matching participants in R[149]/R Studio[150] (figure 49). A further correlation analysis of antibody levels and functional responses [SEn/ STm GMMA Serum IgG / IgA levels (GM); SEn/ STm GMMA Oral Fluid IgA (GM); SEn / STm OAg / GMMA ADMP (GM MedFI); SEn / STm GMMA ADNKA (% NK Activation)] to the iNTS-GMMA vaccination assays was performed with an expanded set of time points. P-values in the correlation analysis are reported <0.05 as significant and >=0.05 non-significant.

Following iNTS-GMMA vaccination serum GMMA IgG strongly correlated with serum IgA responses (SEnGMMA IgG/IgA, r=0.72, p<0.05; STmGMMA IgG/IgA, r=0.77, p<0.05). STmGMMA IgG response positively correlated with the GMMA ADMP response but not OAg ADMP (STmGMMA IgG/GMMA, r=0.62, p<0.05; STmGMMA IgG/OAg, p=>0.05)[figure 50].

SEn and STm CD3+CD4+ positively correlated (r=0.73, p<0.05). SEn and STm CD3+CD4+ response positively correlated with a CD3+CD8+ response, across both SEn and STm GMMA (100µg SEnGMMA CD4+/CD8+, r=0.59, p<0.05; 100µg STmGMMA CD4+/CD8+,

r=0.78, p<0.05). In addition, 10 and 100 microgram GMMA stimulants positively correlated with each other (SEnGMMA CD4 10/100, r=0.94, p<0.05; STmGMMA CD4, r=0.93, p<0.05).

SEn and STm CD3+CD4+ proliferation did not significantly correlate with GMMA-specific IgG (SEnGMMA CD4/IgG, p=>0.05; STmGMMA CD4/IgG, p=>0.05), GMMA-specific IgA (SEnGMMA CD4/IgA, p=>0.05; STmGMMA CD4/IgA, p=>0.05), and GMMA-specific ADMP (SEnGMMA CD4/ADMP, p=>0.05; STmGMMA CD4/ADMP, p=>0.05), or OAg ADMP (SEnGMMA CD4/ADMP, p=>0.05; STmGMMA CD4/ADMP, p=>0.05).

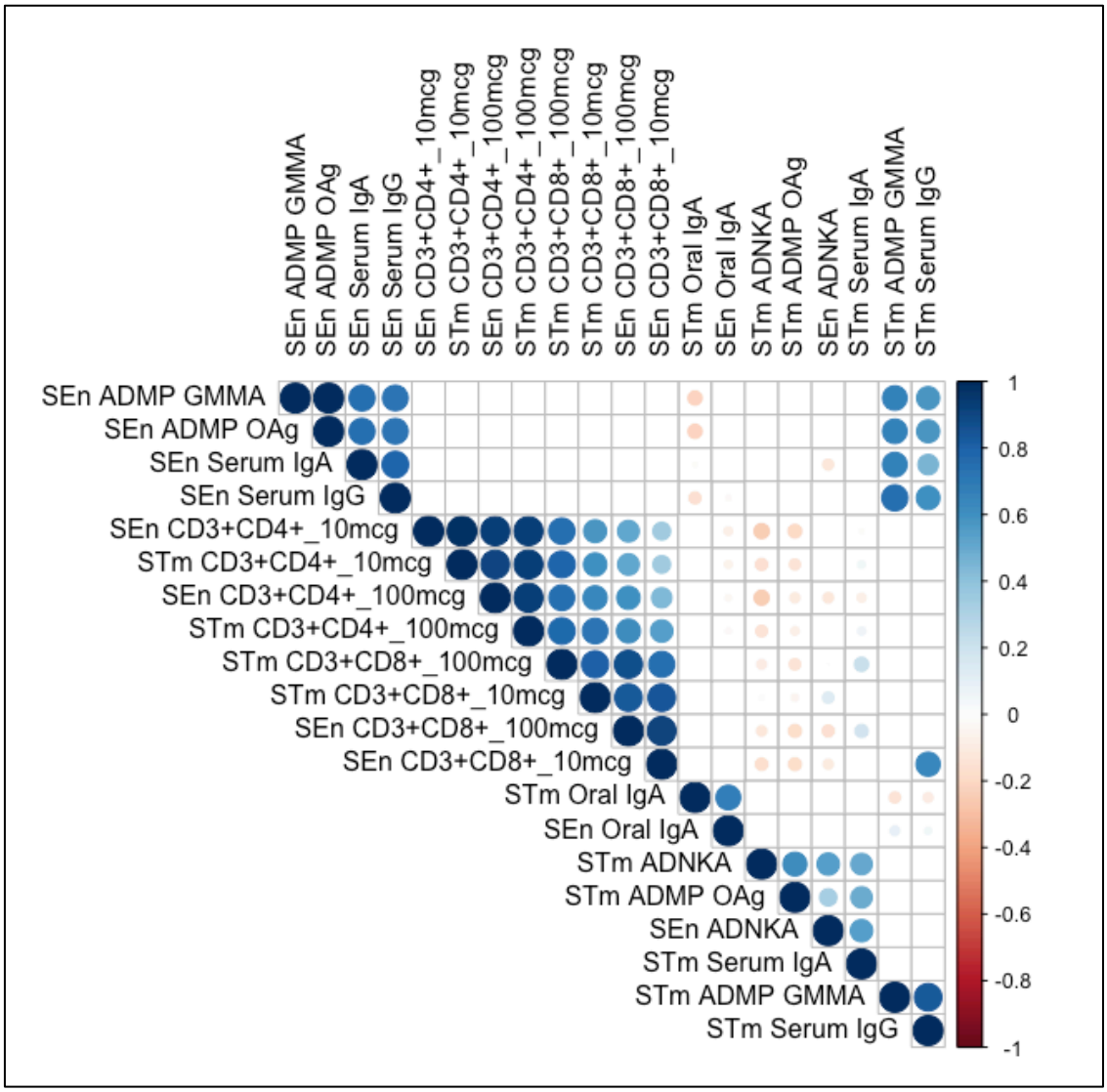



*Figure 49 Spearman correlation analysis between antibody and T cell immunoassays and antigens (GMMA and O-Antigen) across available matched participants (n=12) and time points (D0 and D28). Correlation (r) represented 1 to -1 by colour (blue to red) and size of the circle (1 to 0 or 0 to -1; large to small). Significance (p-value) <0.05 represented by present circles and non-significance (>=0.05) by absent circles.*

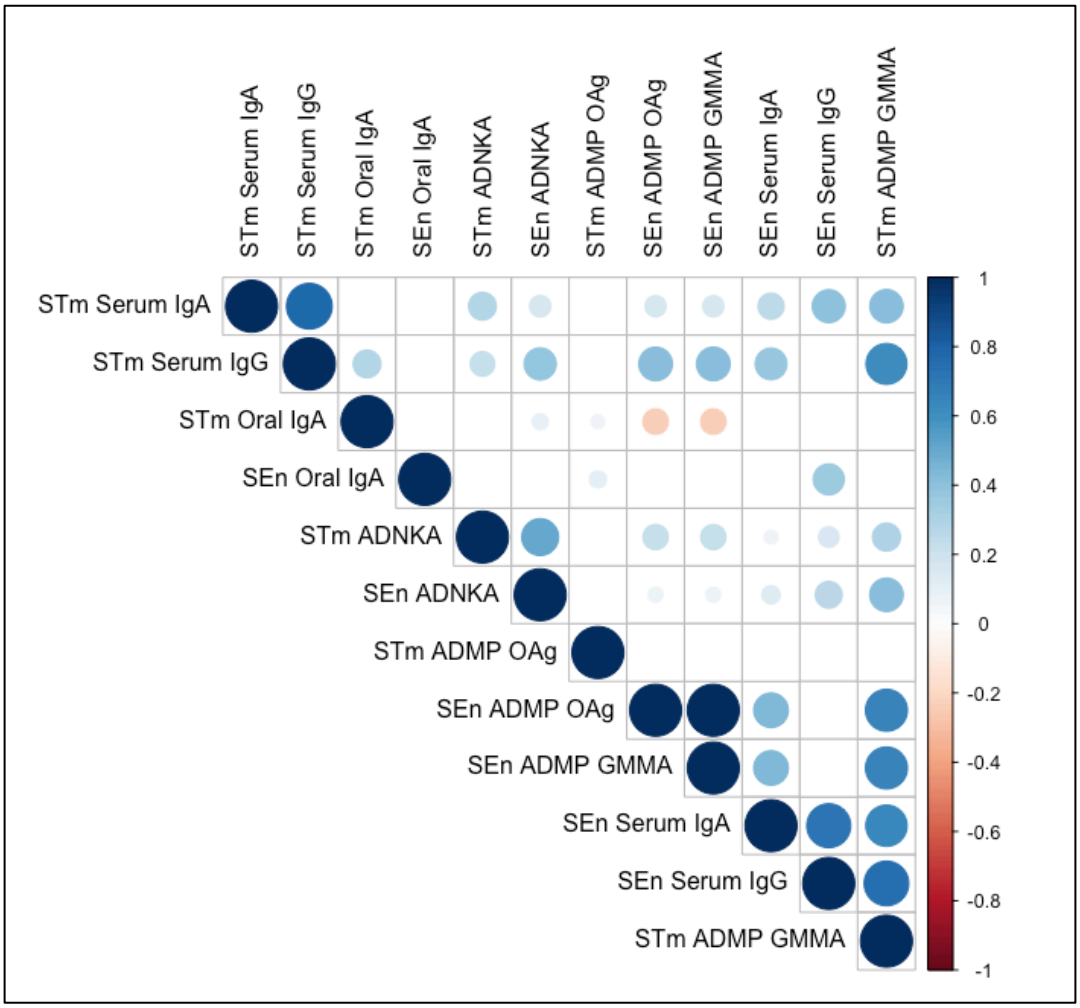



*Figure 50 Spearman correlation analysis across antibody assays and antigens (GMMA and O-Antigen) across available matched participants (n=15) and time points from D0-D84. Correlation (r) represented 1 to -1 by colour (blue to red) and size of the circle (1 to 0 or 0 to -1; large to small). Significance (p-value) <0.05 represented by present circles. Non-significance (>=0.05) by blanks.*

## 4.4 Discussion

### 4.4.1 Summary

1. Evaluation of the serum IgG and IgA antibody response following first and second vaccination up to day 28 after second vaccination using a validated SEnGMMA and STmGMMA IgG and IgA ELISA.

The iNTS-GMMA primary vaccination course stimulates a significant and sustained elevation in serum IgG and IgA antibody response from D14 to D84 above baseline (D0) in participants who received the full dose iNTS vaccine.

2. Evaluation of the mucosal immune response following first and second vaccination by measuring absolute oral fluid IgA using a SEnGMMA and STmGMMA IgA ELISA.

The iNTS-GMMA first vaccination stimulates a rapid peak in mucosal SEnGMMA IgA but not STmGMMA at D14. Mucosal IgA levels returned to baseline thereafter. Whilst a transient mucosal IgA response was seen, this was not sustained beyond day 14 following first vaccination including beyond second vaccination. Oral (mucosal) fluid IgA did not correlate with serum IgA.

3. Evaluation of the functional antibody response following first and second vaccination up to day 28 after second vaccination using antibody dependant monocyte phagocytosis (ADMP) and NK activation (ADNKA) assays.

The full dose iNTS-GMMA vaccine stimulated a significant functional antibody response as measured by monocyte phagocytosis to both SEn and STm GMMA at D28, which was maintained to D84 in the samples tested. However, no significant responses were seen in NK activation following full dose iNTS-GMMA vaccination.

4. Evaluation of the T cell memory response following first vaccination using a GMMA-based T Cell proliferation assay.

SEn and STm GMMA-specific CD3+CD4+ and CD3+CD8+ T cell subset at D28 were observed following first (full dose) vaccination as demonstrated by the significant proliferation of D28 PBMCs (full dose vaccination group) in response to ex-vivo stimulation by 100 micrograms of GMMA.

### 4.4.2 The iNTS-GMMA primary vaccination course stimulates a significant and sustained elevation in serum IgG antibody response from D7 to D84 above baseline (D0)

The full dose iNTS-GMMA vaccine stimulates a significant SEn and STm GMMA-specific IgG rise above baseline and placebo recipients from D14 to D84. This antibody level remained significantly elevated following the second vaccination (D56) to D84. The

geometric mean SEn and STm GMMA-specific IgG level in the lower dose vaccine recipients was higher than the placebo, but significance could not be determined due to the insufficient sample size (n=3). Should the full dose be determined more reactogenic following the 3rd vaccination, further investigation of the lower dose vaccine could be considered. Further testing from the 3rd vaccination (D168) to the final time point (D350) would be required to determine the initial longevity of GMMA IgG response.

Whilst no significant difference was seen between full dose, lower dose and placebo baseline (D0) SEnGMMA and STmGMMA-specific IgG, a small subset of vaccine and placebo samples demonstrated IgG GMMA levels above the assay background (as determined by OD450 in blank wells). Placebo samples with a high baseline antibody level maintained this GMMA-specific IgG level throughout the remaining time points from D7 to D84. High baseline IgG-GMMA levels in some participants may be due to pre-existing GMMA-binding antibodies. This could be explained by pre-existing direct or cross-reactive antibodies generated following prior exposure to *Salmonella* spp. or other Gram-negative Enterobacteriaceae.

Furthermore, participants who received the full dose iNTS-GMMA vaccine mounted one of three responses. Several participants demonstrated a peak GMMA-specific IgG level at D28, while the remaining peaked at D14. These later participants either mounted a second peak antibody level following 2nd vaccination or a sustained GMMA-specific IgG response to the final time point (D84). The typical response following initial exposure of a naïve antigen to the human immune recognition and effector cells is for antibody levels to peak between D21-D28[186]. An earlier rise in antibody level (D14) and a high initial

baseline level in the full dose-iNTS vaccine recipients may suggest a boosted rather than a primary response to the first administration of the iNTS-GMMA vaccine for both SEn and STm.

*Salmonella* exposure has been demonstrated in developed countries from direct sero-incidence studies[187], foodborne disease surveillance[188], and smaller NTS IgG comparative studies[98 189]. In the UK, the number of laboratory reports of non-typhoid *Salmonella* is 15.48 per 100,000 cases[190]; and is likely an underestimate of actual exposure. Results from laboratory cases corroborate findings from a small UK cohort identifying pre-existing antibodies to *Salmonella* O-antigen[129 98] in individuals without confirmed prior *Salmonella* infection. The *Shigella* GMMA trials, which informed the vaccine schedule of the subsequent *Salmonella* GMMA vaccine trial, required a second vaccination to achieve a peak response in those with no pre-existing antibody[130]. However, participants who received the full dose iNTS-GMMA vaccine peaked after the first vaccine, with few demonstrating a further increase in antibody levels after the second. Whilst pre-existing antibodies may be common in the adult population in the UK, this may not be the case in the target demographic. The planned second phase I study in adults in a highly endemic country may show similar results, and it is unlikely until the vaccine is administered to children under twelve months[46] that prior exposure and pre-existing antibody may not contribute to the vaccine-induced response and be reflective of the response in this target demographic.

GMMAs contain multiple antigens predominantly from the bacterial outer membrane and can be considered to contain a subset of the total potential antigen pool of the SEn and

STm bacteria[65 122]. The availability of these antigens may increase the possibility of cross-reactive responses, which may also explain the high baseline response to GMMA seen. Antibody cross-reactivity to outer membrane proteins has been shown in *Neisseria* sp. between the pathogenic *Neisseria meningitidis* and non-pathogenic commensal *Neisseria lactamica*[191]. This has been further investigated in a series of protein ELISAs in *Chapter 6.3.6*. The planned direct O-antigen ELISAs (not included in this thesis) or the O-antigen pre-adsorbed sera GMMA ELISA assay (*Chapter 6.3.5*) may identify the cross-reactive antigen. The phase I *Shigella* GMMA studies identified pre-existing antibodies to lipopolysaccharide in approximately 50% of participants[129].

Convalescent iNTS sera from children under 5 in sub-Saharan Africa demonstrated a wide variation in GMMA-specific IgG response. As a result, no significant difference was seen between the geometric mean concentration of all available convalescent NTS sera (excluding *S*. Typhi sera) and the full dose iNTS-GMMA vaccine or placebo. The variation in antibody levels might be explained by the age of donor and time of bacteraemia, which was conditional on several confounding factors. Further matched sample numbers at controlled time points would be required to determine significance. However, the highest antibody level in a convalescent specimen against both SEn and STm GMMA exceeded the highest level induced by the full-dose iNTS-GMMA vaccine. Whilst, in general, adults mount a higher antibody response to infection than the under-fives (due to immune system maturity [192]), the sustained antigen stimulation following bacteraemia may account for the high responses seen in several convalescent sera samples.

Paradoxically, extremely high antibody responses following natural infection in individuals with HIV have been shown to inhibit antibody-mediated cell killing[64]. One reason for this may be immune complexes sterically preventing the complement membrane attack complex from forming near the bacterial membrane[64]. This may be limited to the immune dysregulation seen with HIV; however, further examination of high antibody responses may be warranted, particularly should the vaccine be deployed in HIV-endemic areas.

### 4.4.3 The iNTS-GMMA primary vaccination course stimulates a significant and sustained elevation in serum GMMA-specific IgA antibody response from D7 to D84 above baseline (D0)

Given that NTS is a gut pathogen, and exposure will be via the mucosal route it is promising that the iNTS-GMMA vaccine stimulates serum IgA responses. SEnGMMA IgA baseline (D0) response was higher than the assay background and was not significantly different between full dose, lower dose and placebo. However, the STmGMMA IgA baseline (D0) response was higher at baseline in placebo recipients than full dose vaccine recipients, suggesting pre-existing cross-reactive antibodies in these individuals.

Following full dose vaccination, SEn and STm GMMA-specific IgA antibodies increased above baseline compared with placebo recipients with a peak at day 14. Lower dose recipients showed a trend towards a rise in GMMA-specific IgA when compared with placebo recipients. The evidence of pre-existing antibodies and an early peak in antibody level again suggests a boosted rather primary response in some individuals.

This boosted IgA response might be because of either cross-reactive or prior *Salmonella* sp. exposure, and via a mucosal route, given that an IgA antibody response is typically primed following mucosal exposure[71 193]. The exact mechanism of serum IgA boosting by an intramuscular vaccine is unclear[73]. However, the immunological picture seen in this study is not without precedence[194]. Sequential intramuscular inactivated polio vaccine (IPV-IPV) combination produces a strong serum IgG but minimal serum IgA response[195]. When an oral polio vaccine known to stimulate a mucosal IgA response is followed by an intramuscular inactivated polio vaccine (OPV-IPV), the IM IPV vaccine produces a rapid boost in serum IgA as soon as day 7 following vaccination[195]. However, a significant primary serum IgA response to an intramuscular typhoid vaccine which is associated with protection from infection was seen in participants challenged in the UK with *Salmonella* Typhi with presumed no prior exposure[196], suggesting that the GMMA IgA kinetics observed here may reflect a primary response. The discrepancy between these two different intramuscular vaccines administered to human participants is unclear. This may be due to either a primary IgA response elicited by one vaccine and not the other, a boosted cross-reactive IgA response to exposed prior enteric antigens or a primary IgA response skewed toward an enteric pathogen with Gram-negative pattern associated molecular patterns (PAMPs)[197].

Whilst the role of serum IgG in the immune response has been extensively investigated, the role of IgA in serum needs to be clarified, with much of the literature focusing on IgA-mediated neutralisation of toxins and pathogenic antigens at mucosal surfaces. Recent work has highlighted the role of monomeric serum IgA Fc-mediated effector cell function, including NK activation and opsonophagocytosis, except for complement-mediated killing

in contrast to IgG[73]. The Ty21a vaccine, a live attenuated *S*. Typhi, was shown to stimulate NK activation *in vivo* via an IgA-mediated response[198]. As such, whilst not directly tested, the ADMP and ADNKA response may include IgA as a mediator of effector function considering the high IgA antibody levels stimulated. This is particularly the case with the ADMP with a positive correlation seen between SEn and STm GMMA IgA responses and SEn and STm GMMA ADMP responses following vaccination.

Furthermore, IgA-producing B cells primed in the gut-associated lymphatic tissue have been shown to translocate to the spleen, where IgA is released into the bloodstream. The benefit of this mechanism has been postulated as a second line of IgA-mediated defence should the gut pathogen penetrate beyond the mucosal response into the systemic circulation[176]. The rapid increase in IgA seen from D7 following full dose iNTS-GMMA vaccination may thus be a non-mucosal IgA-secreting plasma cell booster response to a perceived breach of mucosal immunity.

Whilst there was no significant difference between SEn and STm convalescent sera and STmGMMA-specific IgA antibody levels following full dose, lower dose vaccination or placebo, the convalescent NTS sera sample sizes were too small to account for the heterogeneity and variation of the immune response in children. However, the highest GMMA-specific IgA levels following full dose vaccination were comparable with the highest convalescent NTS sera, suggesting that the full dose vaccination stimulated IgA responses consistent with natural infection.

Despite being a parenteral vaccination, the iNTS-GMMA stimulates a strong IgA-mediated response. This increase in serum IgA antibody was not seen in mice vaccinated with STm GMMA, with only IgG and IgM demonstrating a significant rise in antibody levels[199] which may reflect the differences in immunoglobulin responses between mice and humans.

### 4.4.4 The iNTS-GMMA first vaccination stimulates a rapid peak in mucosal SEnGMMA IgA but not STmGMMA at D14

The oral or salivary fluid contains both IgG and IgA. The source of IgG is extravasation from the bloodstream into the crevicular fluid. Oral fluid IgG has been shown to correlate with serum IgG following invasive non-typhoid *Salmonella* infection[98]; therefore, IgG was not tested in oral fluid in this thesis. In contrast, IgA in oral fluid consists of monomer and dimeric forms. The monomeric IgA predominantly arises from extravasation into the crevicular fluid. Dimeric IgA comprises the majority of IgA in oral fluid and is produced by IgA-secreting B cells in the salivary glands, representing the IgA-specific mucosal response. The lack of correlation between SEn and STm GMMA-specific IgA in serum and oral fluid may be consistent with stimulating local secretory IgA plasma cells independent of the monomeric IgA released into the bloodstream. Absolute Oral fluid IgA titres were lower than serum titres for both SEn and STm GMMA, similar to past studies investigating serum and oral fluid[98].

The iNTS-GMMA vaccine stimulates an increase in oral fluid SEnGMMA IgA in several participants at D14 compared with baseline (D0). A rise at D14 in STmGMMA IgA was seen

in fewer participants. The lack of correlation between oral fluid and serum IgA and rapid decline to baseline after day 14 in both SEn and STmGMMA IgA suggests that this peak is likely secretory IgA produced by local lymphoid tissue. As with serum IgG and IgA, the rise in oral fluid IgA response at D14 following iNTS-GMMA vaccination suggests prior mucosal exposure to *Salmonella* sp. Similar to the serum IgA response, the higher peak at D14 seen in the SEnGMMA IgA may be consistent with the greater incidence of SEn in the UK[190].

In participants with an oral fluid IgA response at D14, levels declined substantially to near baseline, in contrast to serum. Furthermore, there was no response after administering the second iNTS-GMMA vaccine at two months. A similar decline with a muted response to a second vaccination was seen following intramuscular COVID-19 mRNA vaccination in previously unvaccinated long-term care workers, where a mild peak in anti-spike IgA was seen in the majority at day 14-28 post-initial vaccination, which then typically declined to baseline at month 3 with no boosting of mucosal IgA response following second vaccination at the same interval. Mucosal IgA levels were sustained in some, preventing further infection over the short period following the second dose[200]. An intramuscular vaccination may have a role in boosting an IgA mucosal response. Still, this boosting is short-lived, and antibody levels reached are below those achieved in natural infection or further oral vaccination[195 200].

Across both serum IgG and IgA assays, SEnGMMA antibodies were consistently higher than STmGMMA antibodies following full-dose vaccination. A significantly higher D0-D14 fold change between SEn and STm was seen in the GMMA IgG assays. Whilst fold change

between SEn and STm was not significant in the GMMA-specific IgA assays, SEnGMMA IgA geometric mean was higher than STmGMMA IgA with statistical significance compared with baseline sustained to D84 in SEnGMMA IgA but not STm GMMA IgA. This trend may be more evident in the observed difference in oral fluid SEn and STm GMMA-specific IgA response with more samples demonstrating a peak in SEnGMMA-specific IgA antibodies at D14.

In many instances, STmGMMA-specific oral fluid IgA levels at baseline were higher in placebo than full dose vaccination group. This is consistent with the significantly higher IgA response at baseline in placebo compared with full dose vaccination in serum and the lower overall oral fluid IgA response stimulated by the active vaccines.

Lower absolute oral fluid IgA titres compared with serum may be due to several factors: including transience of memory IgA-producing B cells in lymphoid and gut-associated lymphoid tissue[176]; limitation of antigen concentration and presentation at lymphoid tissue; small oral mucosal memory responses due to primary invasion of *Salmonella* in the intestinal mucosa[201]; dose of intramuscular vaccination; or sensitivity of the oral fluid antibody assay itself. The mechanism of this intramuscular boosting remains unclear, with direct antigen stimulation at the local oral or gut-associated lymphoid tissue translocating antigen-specific B cells to oral lymphoid tissue[74]. Indeed, it remains to be seen why there was no response after the second vaccination when one might be expected. Rapid antigen clearance at the point of intramuscular vaccination and secondary response might have prevented antigen presentation to the mucosal lymphoid system, which may or may not

be affected by the interval of response. The forthcoming 3rd vaccination and response following waning immunity may provide further information.

Overall serum IgA antibody levels are sustained following the second vaccination augmenting the immune response against systemic iNTS infection, which may meet the requirements for immunity to invasive disease. The peak at D14 of oral mucosal IgA may provide additional protection against invasive disease and could contribute to attenuation and protection against NTS gastroenteritis. Should there be a protective effect against NTS gastroenteritis, the cost-effectiveness and deployability of the vaccine might be significantly enhanced. However, the oral mucosal immune response may not be directly equivalent to the intestinal mucosal response[202]; the most important site of *Salmonella* infection and invasion. GMMA-specific IgA testing in stool samples would be beneficial to examine this further; however, stool IgA testing requires considerable sample optimisation compared with oral fluid testing[193]. Whilst a high NTS endemic country might produce more sustained immune boosting, the target demographic for vaccine deployment may be *Salmonella* naïve; therefore, it may rely on a primary rather than boosted response, which in respect of mucosal IgA as a component of the immune response, may not be sufficient in the target demographic.

IgA testing in the oral fluid has multiple limitations. Age and periodontal disease may influence variation in oral fluid IgA[193]. When testing oral fluid, IgA normalisation to salivary proteins or total IgA is often included in analysis. However, these normalisation methods are also subject to variation in the reference measure chosen[193]. As discussed, the overall reduced mucosal GMMA IgA antibody levels may represent a true response to

the vaccine; however, total IgA should be tested to account for poor sampling and variations in overall IgA in the oral fluid, which could have affected the result. In addition, the lowest dilution (1:50) was tested to preserve limited samples. A lower dilution (1:2) of samples might have shown a greater response. Other factors in the oral fluid or storage media may have contributed to an overall reduced response compared with serum.

### 4.4.5 Functional antibody response following iNTS-GMMA primary vaccination

#### Antibody-Dependent Monocyte Phagocytosis

Whilst antibody-dependent monocyte phagocytosis is presumed IgG mediated, more evidence is emerging for the role of IgA[73]; which may be particularly important considering the serum IgA antibody level generated by the vaccine. The full dose iNTS-GMMA vaccine stimulated a significant functional antibody response as measured by monocyte phagocytosis to both SEn and STm GMMA at D28, which was maintained to D84 in the samples tested. The O-Antigen bead assay demonstrated a significant functional response in the full dose group at D28, suggesting that the OAg contributes significantly to the ADMP response. This is the first instance of OMVs being used as direct antigens in an ADMP or opsonophagocytic assay. Using SEn and STm GMMA allows an assessment of the polyclonal functional response and the contribution of OAg to antibody dependant phagocytosis.

In addition to antibody phagocytosis, GMMA phagocytosis by monocytes was seen in the absence of sera. This may indicate that the GMMAs alone stimulate innate effector cells and phagocytosis, a concept further explored in the monocyte activation assays in *Chapter 7.3.1.*

### Antibody-Dependent NK Activation

The NK activation stimulated by both SEnGMMA and STmGMMA was equivocal. In some samples, the GMMAs appeared to inhibit NK responses, even at the highest antibody levels. Whilst high serum concentrations well above that of the antibody-dependent monocyte phagocytosis and the GMMA ELISA antibody assays, and a wide range of dilution tested in assay optimisation were used, it is possible but unlikely that even higher concentrations might have increased the sensitivity of the assay. Another possibility may be that the NK cell Fcγ receptors do not recognise the class of antibodies[203] bound to the GMMAs. An IgG subclass analysis would be required to elucidate this further. However, overall further samples, including following vaccine and convalescent sera could be tested to confirm the response to GMMAs in the first instance.

## 4.4.6 The iNTS-GMMA vaccine stimulates T memory cells to GMMAs

The full dose iNTS-GMMA vaccine stimulates memory $CD3^+CD4^+$ and $CD3^+CD8^+$ for SEn and STm GMMAs, as confirmed by statistically significant T cell responses at D28. Having established that both GMMAs elicit antibody responses, the addition of CD4 and CD8

responses provides evidence for the increase in T cell-dependent B cell response and stimulation of a CD8+ population which may contribute to clearance of infected cells in the event of subsequent exposure to iNTS. In addition, Day 28 T cell responses are more reflective of memory T cell responses and thus the ability to form secondary responses following repeat stimulation[204].

Outer membrane vesicles have been previously shown to stimulate a human T cell response, particularly with meningococcal Group B vaccines, given that they are the most advanced and only licenced group of OMV vaccines[184]. However, T cell analysis in this chapter represents the first evidence for a human T cell response to a GMMA-based vaccine.

### 4.4.7 Immunoassay correlation analysis

GMMA-specific CD3+CD4+ and CD3+CD8+ cells positively correlated with each other, whilst neither CD4+ nor CD8+ reached significance on correlation testing with the antibody assays. This may have been due to the sample size for correlation analysis or the variation in peak response between immune system components. In addition, both GMMA-specific CD4+ and CD8+ positively correlated between each serovar, suggesting similar or T cell cross-reactive proteins in both SEn and STmGMMAs. However, the degree of cross-reactive responses and relative contribution of SEn and STmGMMA must be clarified. A direct comparison is problematic due to variation caused by antigen and assay conditions. However, the trend across assays was for a greater response to SEnGMMA than STm GMMA, possibly due to a higher incidence of SEn in the UK[190], as previously discussed. A similar trend may not be seen in sub-Saharan Africa which has more STm

(particularly ST313) cases[24]. STmGMMA IgG demonstrated a significant positive correlation with STm GMMA ADMP, but although the SEnGMMA IgG and ADMP trended towards a positive correlation, this was not significant. Further sample numbers may have been required to confirm the correlation.

### 4.4.8 Limitations

As expected, the sample size was a limitation in several assays, including ADNKA and T cell proliferation. This was a particular issue for immunogenicity testing of the lower dose iNTS-GMMA vaccines (n=3). A larger sample size might have revealed a statistical significance increase with the lower dose vaccine in several assays.

GMMAs were used in multiple assays as an antigen. Whilst OMVs have been used in ELISAs, the use of GMMAs in the ADMP and ADNKA assays is novel. Using GMMAs as antigens allows an investigation of the polyclonal response to the iNTS-GMMA vaccine. In this thesis, it is clear that GMMAs can be used as an antigen when whole bacteria as antigens are problematic in other vaccine-preventable diseases. However, the use of GMMAs also led to the identification of high-baseline potentially cross-reactive antibodies, which by itself was not a significant limitation.

### 4.4.9 Future plans and considerations

The next steps include the evaluation of immunogenicity at 4 months post the second vaccine and following the booster vaccine (3rd vaccine) to determine the longevity of response at 4 months following the primary course and boosted response up 6 months after the 3rd vaccine. Further data on the longevity of response is essential to determine

the temporal coverage in the demographic with the highest burden. In addition, the planned second phase I study will evaluate the response to the iNTS-GMMA vaccine in adults in a country with high endemic non-typhoid *Salmonella*. The response to the primary course will likely be a boosted response with the possibility of higher baseline responses than in the UK cohort. This could be tested with a direct comparison with UK post-vaccination samples or independently with avidity testing. The UK cohort in this study suggests that the vaccine is immunogenic. However, the responses in children under 5 with an immature immune system are yet to be determined.

Further immunological assessment of the iNTS-GMMA vaccine will be undertaken to determine the immunodominant antigen. O-antigen specific ELISAs may reveal a reduction in background antibody response compared with the GMMA ELISA. The use of OAg-negative GMMAs will allow a further assessment of the dominance of the O-antigen. O-antigen pre-adsorbed iNTS-GMMA vaccine sera tested on the GMMA-specific ELISAs will enable evaluation of the O-antigen contribution to both immunodominance and cross-reactivity and is further investigated in *Chapter 6*. Serum bactericidal assays to determine complement-mediated bacterial killing are planned to assess stimulation by the vaccine of a significant functional antibody component of the immune system.

## 4.5 Conclusion

The full dose iNTS-GMMA vaccine stimulates a significant functional antibody, CD3+CD4+ and CD3+CD8+ response to both SEnGMMA and STmGMMA. This response is consistent with a boosted rather than a primary response in many participants who were likely previously exposed to *Salmonella*. The antibody response consists of both IgG and IgA

systemic responses with some evidence of an initial mucosal IgA response following an intramuscular iNTS-GMMA full dose vaccination. The IgG and IgA antibody levels peaked at greater than 4-fold the baseline suggesting a convincing immune response following full dose iNTS-GMMA vaccination, whilst the monocyte phagocytosis-induced antibody response peaked 2-2.5-fold above baseline. The CD3+CD4+ and CD3+CD8+ although significant did not elicit a response in some participants following full dose iNTS-GMMA vaccine. Immunogenicity in this small sample size suggests a vaccine that stimulates significant components of the immune system, with the results in this chapter suggesting a promising iNTS vaccine candidate.

# Proteomic Analysis of the Active GMMA Vaccine Components

## 5.1 Introduction

This chapter describes the proteomic analysis of the active GMMA vaccine components and includes a computational analysis of conserved proteins across GMMAs and *Salmonella* sp. and their predicted immunogenicity. Selected immunogenic proteins are identified to be produced and tested in enzyme-linked immunosorbent assays (ELISA) and T cell proliferations assays with samples from the iNTS-GMMA vaccine trial to confirm immunogenicity. Furthermore, given the dual burden of iNTS and *Salmonella* Typhi in sub-Saharan Africa, establishing proteins which may stimulate a cross-reactive response may be of particular benefit to any future polyvalent vaccine involving OMVs.

### 5.1.1 Proteins in Vaccinology

Pathogen-derived proteins are essential targets of protective immune responses, recognised by the adaptive immune system's humoral (antibodies and B cells) and cellular (T cell) arms. Whilst antibodies can be developed to other molecules, including polysaccharide antigens (e.g., O-antigen), T cells commonly recognise peptide (although not exclusively[205]) epitopes presented by MHC I or II[206]. CD3+CD4+ T helper cells are essential to promote T cell-dependant B cell activation of linked T and B cell epitopes on a given antigen[207]. T cell help facilitates affinity maturation and antibody class switching, improving antibody-antigen binding quality and subsequent downstream effector response[206]. Protein conjugate vaccines (e.g., pneumococcal conjugate PCV13) exploit

linked recognition to enhance immune responses over plain polysaccharide vaccines (e.g., PPV23)[208 209]. Stimulating T cell-dependent B cell responses in children under five years is particularly challenging due to an immature immune system, as illustrated by the requirement for repeated vaccine boosts in this age group[80 210]. Similar challenges would be faced by any O-antigen-based iNTS vaccine deployed in the group with the highest disease burden (the under-five age group).

### 5.1.2 Proteins in Outer Membrane Vesicles (OMVs) and GMMAs

Gram-negative bacteria naturally release outer membrane vesicles (OMVs) that contain multiple proteins localised to the outer membrane, inner/cytoplasmic membrane, periplasm and cytoplasm. However, initial proteomic characterisation demonstrates a disproportionate skew towards membrane proteins[125 211-214]. GMMAs are OMVs produced by genetic modification of the Gram-negative organism to increase production and reduce the reactogenicity of the OMV[122]. Proteomic analysis of the *Shigella* GMMA vaccine revealed 95-99% of identified proteins were either outer membrane or periplasmic, with the most abundant proteins being OmpA, OmpC, and OmpX[122]. The proteomics distribution has proved similar across multiple analyses of outer membrane vesicles from Gram-negative bacteria, including Acinetobacter[215] and *Pseudomonas*[216]. However, the exact distribution of proteins can be modified by the culture conditions of the bacteria at the time of OMV shedding[125]. OMVs derived from *Salmonella* Typhimurium cultured in acidic, low magnesium conditions comparable to within macrophage vacuoles had a higher proportion of type 3 secretion system proteins than when culture in Luria-Bertani (LB) media under typical laboratory conditions[125].

Whilst the iNTS-GMMA vaccine has been developed with the O-antigen as the likely immunodominant antigen (See *Chapter 6.3.6, 6.4*), protein components might either stimulate an antigen-specific immune response in themselves or act similarly to protein carriers in a polysaccharide-protein conjugate vaccine demonstrating linked recognition to the O-antigen or other antigens present[210]. Mice studies have revealed that *Salmonella* outer membrane vesicles administered intramuscularly promote a significant CD4 T cell and B cell response which contributed to a reduction in bacterial burden when these mice were subsequently challenged with *Salmonella* bacteria[217].

In addition, OMVs have been found to deliver effector proteins, including type 3 secretion system proteins, into host cells to allow bacterial invasion and manipulation of a favourable environment for bacterial growth and immune escape[123 218 55 216 219]. An immune response against these proteins might therefore reduce bacterial burden within cells and prevent infection[220].

Unlike polysaccharide-protein conjugate vaccines, OMVs do not require complex conjugation chemistry, making them cheaper to manufacture and easier to produce in large quantities[122]. Additionally, outer membrane vesicles have the potential to present multiple antigens, making them an attractive vaccine platform. At present they have been employed in the licensed vaccine for Meningococcal B, Bexero[128], with multiple vaccine candidates currently in development, including vaccines against *Acinetobacter*[221], *E.Coli*[222], *Pseudomonas*[216], and *Shigella*[122].

## 5.1.3 Proteomic Analysis in Vaccinology

The ability to identify the proteomic composition within pathogenic organisms has facilitated the antigen discovery and selection[223]. Proteins within a target organism can now be accurately determined, with analysis pipelines developed to predict the immunogenicity of selected proteins[224 225]. Immunogenicity can be verified by pre-clinical *in vitro* and animal testing before being identified as future vaccine candidates and progressing to first-in-human phase I clinical trials. Numerous databases and vaccinology software have been developed, some of which have been used in the current analysis[226-228]. This software includes identifying protein localisation within cells[226], MHC I and MHC II epitope binding capacity[229] and similarity to human proteins[227] to avoid molecular mimicry[230].

## 5.1.4 Identification of potentially Immunogenic Proteins Within GMMA Particles

Protein candidate selection criteria (modified from Esmailnia et al. (2020)[224], Soltan et al. (2021)[231], and Enayatkhhani et al. (2021)[223]:

| | | |
|---|---|---|
| i. | *Production – Identify proteins that can be readily produced for immunoassay testing.* | The production of structurally accurate proteins requires the correct amino acid sequence and the ability to fold into secondary, tertiary, and quaternary structures. Whilst this is less relevant for linear T cell epitopes, this is of greater importance for the 3-dimensional structure of B cell epitopes[232]. Furthermore, some protein structures are increasingly challenging to produce and will be excluded[233]. |
| ii. | *Adhesin Probability – Identify proteins which interact with host cell receptors.* | Proteins which bind to host cell receptors have been preferentially selected based on a higher probability of antibody binding and |

| | | |
|---|---|---|
| | | neutralisation[234 235] and the possibility of acting as T-cell antigens[236]. |
| iii. | *Non-Human Proteins – Identify and exclude proteins with similar sequences to human proteins.* | Proteins with similar amino acid sequences to human proteins were excluded to reduce the risk of molecular mimicry and autoimmunity or suppression of immune response to the invading organism[230]. |
| iv. | *Abundance - Identification of the most abundant shared proteins across SEn and STm GMMA and Bacteria.* | For abundance, log10 label-free quantification was used as the primary measure calculated by MaxQuant (*Max-Planck-Institute of Biochemistry, Germany*)[148]. To facilitate an appropriate immune response, proteins must be sufficiently abundant in the GMMAs to prime the immune system. Whilst there is a degree of complexity regarding antigen quantity and relation to B cell differentiation, memory B cell formation and Th1/Th2 stimulation, the most abundant proteins were identified for protein selection[236]. Additionally, during any secondary antigen exposure, the abundance of the antigen must reach a threshold such that the immune system recognises it. Whilst I have preferentially selected abundant proteins, there is some evidence that abundance (up to a point) is less critical, and that subcellular location might be of greater importance for B and T Cell stimulation[105]. |
| v. | *Subcellular Location – Identification of the localising site of the protein: Outer Membrane, Cytoplasm (Inner) Membrane, Periplasmic, Cytoplasmic, Unknown.* | The availability of antigens on the surface of invading organisms (outer membrane) is accepted as a clear advantage for both antibody access and B-cell stimulation[105]. However, this is yet to be clarified for T cell stimulation which requires antigen presentation from specialised antigen-presenting cells via MHC II or other non-specialised cells via MHC I complexes[206]. Given the abundance of cytoplasmic proteins (figure 8) in bacteria (and GMMAs), these proteins could play a role in T cell antigen stimulation. However, proteins currently identified to have stimulated *Salmonella*-exposed CD3+ T cells are located either in the periplasm or extracellular space, including PhoN (periplasm), HlyE |

| | | |
|---|---|---|
| | | (extracellular/periplasm), and CdtB (extracellular)[106 237]. In addition, it has been hypothesised that outer membrane proteins may be of greater importance due to the higher percentage of intact bacteria that remain live within antigen-presenting cells, thus only allowing the presentation of outer membrane proteins[105]. |
| vi. | *Immunogenicity – Identify potentially immunogenic proteins as defined by the predicted antigenic epitopes.* | |
| vii. | *Current Evidence – Identify proteins with established evidence as possible antigens.* | Once selected proteins were filtered using selections 1-6, a literature search was conducted using PubMed (*NIH, National Library of Medicine, USA*) to identify any current evidence of antigenicity. |
| Further criteria for discovering possible antigens that may stimulate a cross-reactive antibody response across *Salmonella* species were then completed. | | |
| viii. | *Homologous Salmonella Typhi and Paratyphi Proteins – Identify proteins with > 95% sequence similarity to Salmonella Typhi Proteins.* | *Salmonella* Typhi homologous proteins were identified using BLAST[237] against NTS proteins based on accession numbers identified through MaxQuant[148] using *Salmonella* Typhimurium and Enteritidis reference organisms. |
| ix. | *Microarray-identified Antigens – Identify potential cross-reactive or non-cross-reactive antigens from protein microarrays exposed to convalescent Salmonella species serum.* | |

### 5.1.5 Current Evidence for Immunogenic and Cross-Reactive Proteins in *Salmonella* sp.

Humoral responses to *Salmonella* sp. have largely investigated the polysaccharide Vi capsule (*S*.Typhi)[238], flagellin and LPS O antigen[65 238]. Absolute IgM and IgG antibody titres to OAg were higher than antibody titres to porins, including OmpC, OmpF and OmpD, in

mice injected with STmGMMA, suggesting that a combination of OAg abundance to porin ratio (predicted at 10:1) and the distance from the cell membrane might provide a more significant advantage to OAg in the development of a robust antibody response[65]. However, mice immunised with flagellin and SSeB developed antibody and CD4 responses with longer survival times following *Salmonella* challenge, suggesting protein responses may play a role in protection[93]. Several *Salmonella* proteins, including PhoN, HlyE, and CdtB, have been found to stimulate human T cell-specific responses to *Salmonella* Typhi and other *Salmonella* sp., including *Salmonella* Paratyphi and non-typhoid *Salmonella*[106]. Liang et al. (2013)[239] and Darton et al. (2017)[240] investigated multiple proteins as antigens in microarray studies using serum from convalescent NTS, Typhoid and Paratyphoid infection, identifying multiple further potential *Salmonella* antigens for investigation.

### 5.1.6 Aims

This chapter describes the proteomic analysis of the iNTS-GMMA Vaccine active components: SEnGMMA and STmGMMA. It includes a comparison with SEn and STm origin bacterial strains and identification of proteins which may be immunogenic across non-typhoid and typhoid *Salmonella* species.

The aims of this chapter are to:

1. Evaluate of the proteomic components of the iNTS-GMMA vaccine.
2. Identify of immunogenic homologous *Salmonella* proteins conserved across *Salmonella* serovars for protein production and immunoassay confirmatory testing.

## 5.2 Method

### 5.2.1 Culture, Digestion and Quantification of Bacteria and GMMAs

SEn (Strain CMCC4314; GVGH1835) and STm Bacteria (Strain D23580; GVGH1842) were cultured and, together with SEnGMMA and STmGMMA, digested as per *Chapter 2 Material Methods, section 2.3 and 2.4.*

### 5.2.2 Mass Spectrometry Protein Identification and Quantification

Three biological replicates were processed at 200ng of peptide mix per sample. These peptides were separated on a 1h nanoLC (*SCIEX, Washington USA*) gradient and analysed on the Q Exactive mass spectrometer (*Thermo Scientific, USA*). Samples were run together to reduce the batch effect. Results were then analysed in MaxQuant (*Max-Planck-Institute of Biochemistry, Germany*)[148] using *S*. Typhimurium (strains D23580, SL1344) and *S*. Enteritidis (strain P125109) reference databases to identify and quantify unique proteins present by Label-Free Quantification (LFQ) Intensity. Abundance was assigned to each protein, as measured by log10 Label-Free Quantification (LFQ) intensity using MaxQuant[148] for raw LFQ values and R[149] / R Studio[150] for downstream data analysis. Collaborators (R.Fischer and S.Flannery) performed Mass Spectroscopy and MaxQuant analysis at Target Discovery Institute.

### 5.2.3 Quality Control and Variation Between Replicates and Sample Types

Following protein identification and quantification assignment in MaxQuant[148], further data analysis was conducted using R[149]/RStudio[150], where a principal component analysis (PCA), an analysis of missing proteins per sample and replicate, and median normalisation was performed.

### 5.2.4 Grouping by Higher Order Protein Function

Higher-order function protein grouping was conducted using STRING (*STRING Consortium*)[241]. The 118 proteins shared between sample types were imputed against a *Salmonella* Typhimurium reference strain, classifying individual proteins according to STRING term, Gene Ontology (GO) and KEGG Pathway. Cluster analysis of the 118 shared proteins was performed in STRING using Markov Clustering (MCL) with the inflation parameter set to two.

To further identify differences in the pattern of protein composition between GMMA and bacteria, groups of proteins were organised by higher-order function utilising STRING[242] to assign proteins to Gene Ontology (GO) terms[243] and KEGG Pathways[244]. GO classifies proteins non-exclusively into three functional groups: molecular function, biological process, and cellular component. The molecular function includes any singular enzymatic and transport protein activity (e.g. GO:0003824_Catalytic activity)[243] . Biological processes include a sequence of molecular functions constituting an entire process (e.g. GO:1901564_Organonitrogen compound metabolic process)[243]. The cellular component

includes structural components of the cell (e.g. GO:0110165_Cellular anatomical entity)[243]. KEGG was used to identify metabolic pathways to establish differences in functional themes[244]. The percentage contribution of protein to each term was calculated against the total number of proteins identified in each process using this classification.

### 5.2.5 Assigning Subcellular Location

Individual protein subcellular location was identified using PSortb (*Simon Fraser University, Canada*)[226] version 3.0 with FASTA files inputted with bacteria and Gram-negative stain filtering. The output file was analysed using R[149] / R Studio[150].

### 5.2.6 Differential Abundance Analysis

Differential abundance analysis was performed in R[149] / R Studio[150] using the r packages ProDA[245] and limma[246].

### 5.2.7 Filtering for Transmembrane Helices, Adhesin Probability and Self-Antigens

Transmembrane helices, adhesin probability and self-antigens were identified using Vaxign (*University of Michigan, USA*)[227]. Transmembrane helices >1 and adhesin probability <0.5 were excluded.

### 5.2.8 Identifying Homologous *Salmonella* Typhi and Paratyphi Proteins

Homologous S.Typhi and S.Paratyphi proteins were identified via Basic Local Alignment Search Tool (BLAST) via the UniProt Database[237] from the non-typhoid *Salmonella* reference database identified accession numbers. Only homologous proteins with >95% sequence alignment were included.

### 5.2.9 Assigning B and T Cell Epitopes

Accession number FASTA files were imputed into CD4 T Cell Immunogenicity Prediction (*IEDB, USA*)[229] and Bepipred Linear Epitope Prediction 2.0 (*IEDB, USA*)[228] tools to identify T cell and B cell epitopes, respectively. The output file was analysed using R[149] / R Studio[150].

## 5.3 Results

### 5.3.1 Proteomic Composition of GMMA and Equivalent Bacterial Strains

#### Laboratory Total Protein Quantification

Following culture and digestion, a Protein BCA assay (*Thermo Scientific, USA*) quantified proteins per sample type and biological replicate (table 28). A higher protein concentration was seen across all three STmGMMA replicates than SEnGMMA despite initial selection by identical O-antigen content.

| | Protein Concentration in mcg/ml | | |
|---|---|---|---|
| | *1* | *2* | *3* |
| SEn GMMA | 554.39 | 609.51 | 654.04 |
| STm GMMA | 766.22 | 684.79 | 675.62 |
| SEn | 801.19 | 1444.98 | 1082.29 |
| STm | 1137.20 | 732.77 | 1091.75 |

*Table 28 : Protein concentration (µg/mL) across sample type and biological replicate*

#### Mass Spectrometry Unique Protein Quantification

The number of unique proteins identified per sample and biological replicate indicates a higher number of individual proteins in origin bacteria compared with serovar specific GMMA and also in STmGMMA compared with SEnGMMA (table 29).

| | Unique Proteins Per Sample Type | | |
|---|---|---|---|
| | *1* | *2* | *3* |
| SEnGMMA | 165 | 266 | 327 |
| STmGMMA | 435 | 486 | 515 |
| SEn | 1096 | 1239 | 1185 |
| STm | *174* | 1211 | 1210 |

*Table 29 Unique proteins across sample type and biological replicate per 200ng of sample tested; NB: STm Replicate 1 removed from the analysis.*

## Quality and Variation Between Sample Types and Replicates

To assess the quality and variation between sample type and replicates by both unique proteins and the abundance of each protein, a principal component analysis (PCA) was performed using R[149] / R Studio[150]. The PCA showed clustering of all replicates of each bacterial origin strain and GMMA along variables 1 and 2, with one exception for a single STm bacterial replicate (figure 51, red arrow). Both bacterial origin strains clustered together whilst the SEn and STm GMMA formed distinct clusters along variable two whilst separated from the bacterial strains along variable 1 (figure 51 and 52), demonstrating a higher protein variation between bacteria and GMMA than across GMMA sample types.

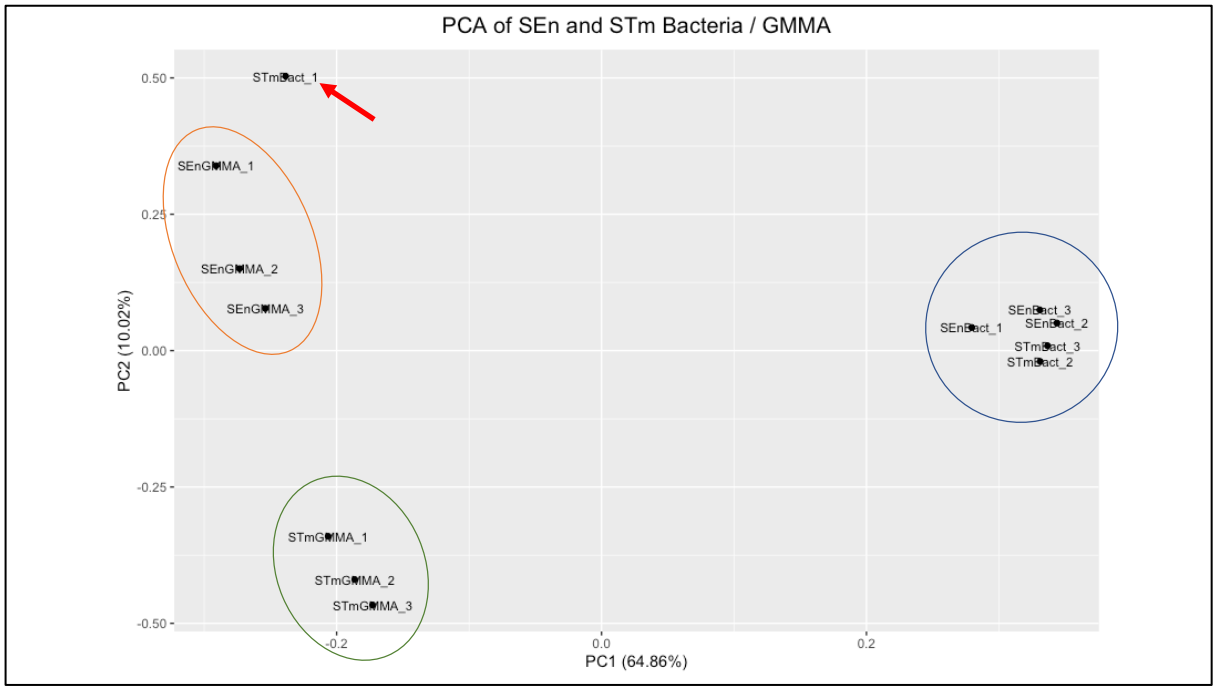



*Figure 51 Principal Component Analysis (PCA) across sample type and replicates, including each protein's unique protein composition and abundance. Three clusters: 1. SEnBact and STmBact (blue ellipse); 2. SEnGMMA (green ellipse); 3. STmGMMA (red ellipse). Outlier: STmBact_1 replicate (red arrow)*

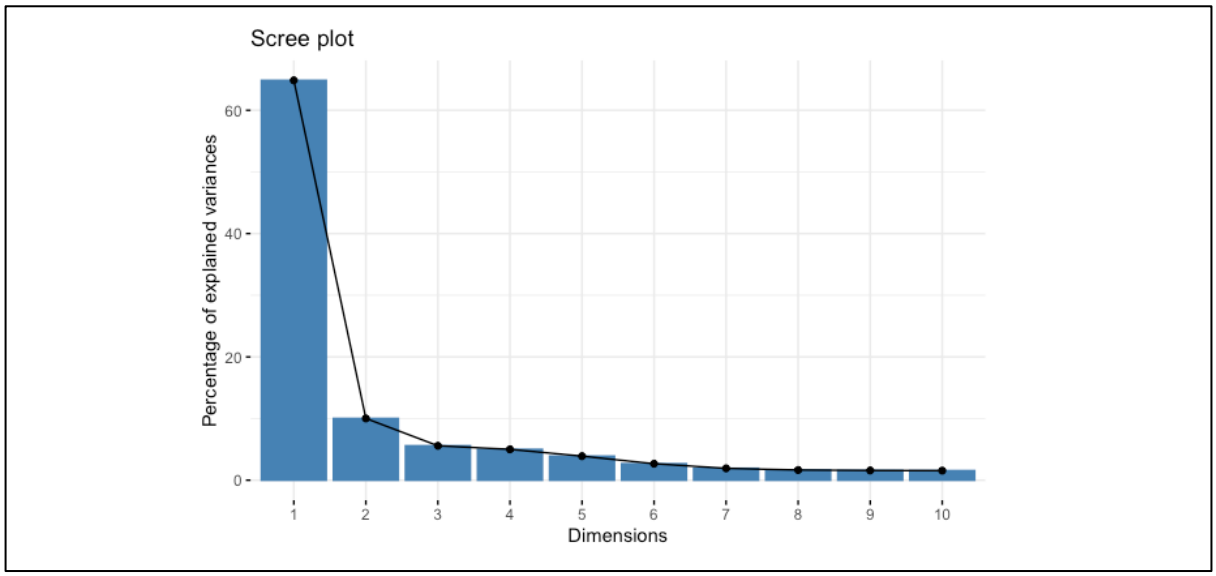



*Figure 52 Scree plot: Corresponding to PCA (figure 51). Showing variance principally related to variable 1, with variable two the next (albeit lower) principal component*

STm Bacterial replicate one was removed from further analysis following quality control assessment. In addition to the identification of an outlier on principal component analysis (figure 51, red arrow), further investigation revealed a high level of missing values commensurate with GMMAs (figure 53, red box) and higher than average median

intensity value distribution following medium normalisation compared with other bacterial samples (figure 54, red arrow).

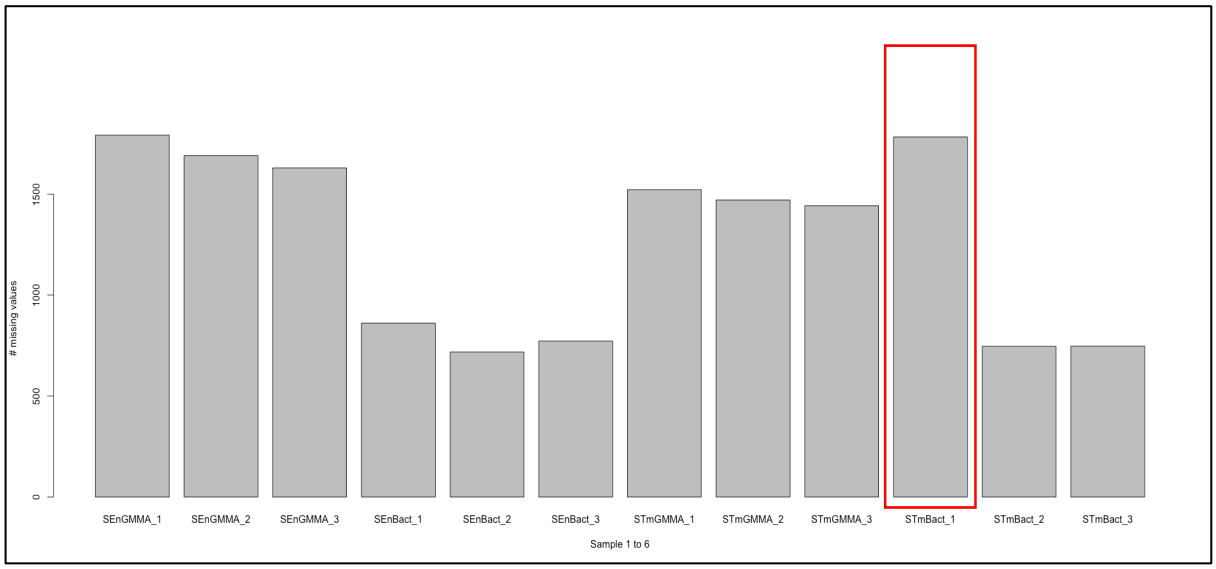



*Figure 53 Bar chart of missing proteins (NAs) between sample types showing STmBact_1 to have a higher number of missing proteins compared with other STmBact replicates*

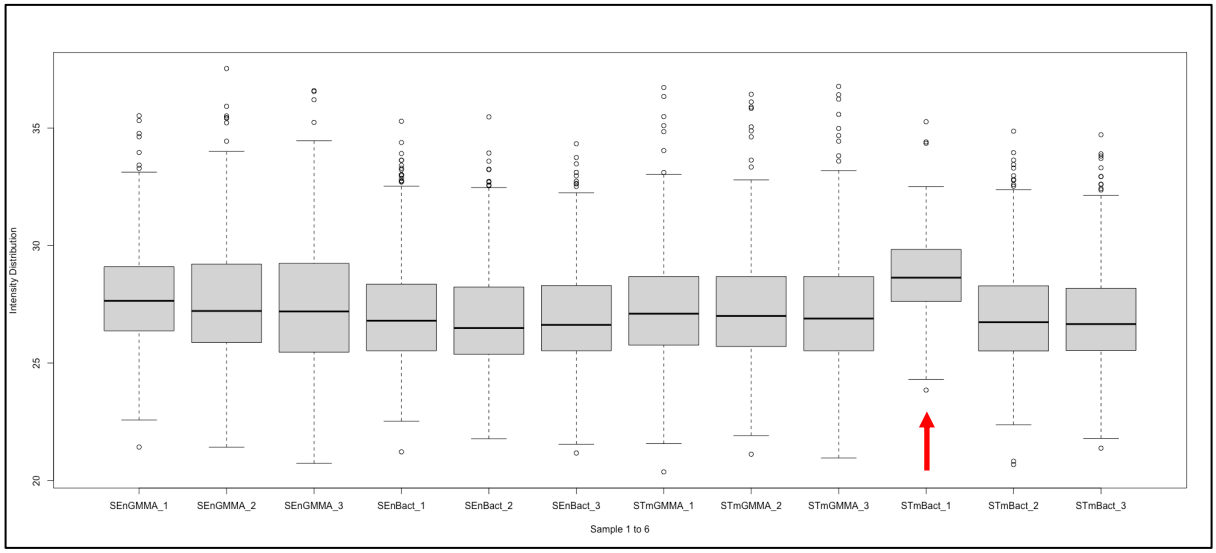



*Figure 54 Median normalised log10 Label-Free Quantification (LFQ) intensity distribution between all sample types and replicates. Outlier: STmBact_1 replicate (red arrow)*

## Comparison of Shared Unique Proteins Between Replicates

Of the three replicates of each sample type, SEnGMMA shared 143 unique proteins (figure 56), STmGMMA 369, SEn bacteria 998 and STm Bacteria (with replicate 1 excluded) 976. The shared proteins across replicates were used for further analysis unless otherwise stated.

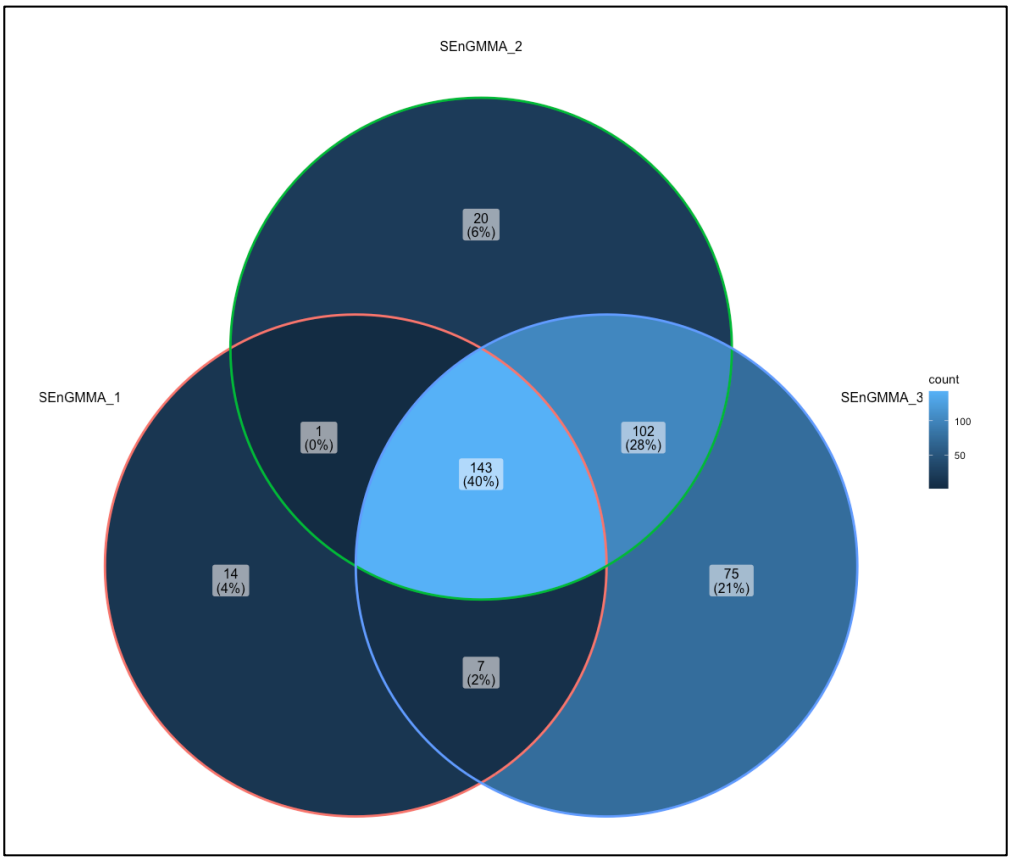



*Figure 55 Venn diagram of unique proteins shared between SEnGMMA replicates*

## Comparison of Shared Unique Proteins Between GMMA and Bacteria

A comparison of GMMA and bacteria by replicate with the most abundant unique proteins revealed that 90% and 80% of the proteins found in the SEnGMMA and STmGMMA were identified in the corresponding bacterial origin strain (figure 56).

Furthermore, 81% of unique proteins were shared between SEn and STm bacteria, which declined to 51% between SEn and STmGMMA (figure 57). In addition, STmGMMA (515 unique proteins) contained more proteins compared with SEnGMMA (327 unique proteins).

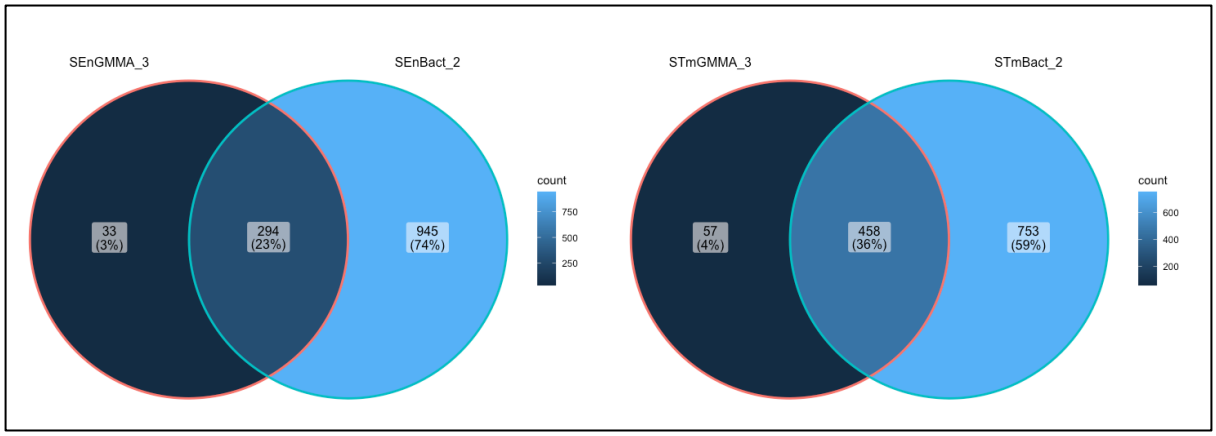



*Figure 56 Venn diagram of unique proteins shared between SEnGMMA and Bacteria (left) and STmGMMA and Bacteria (right) by most abundant replicate*

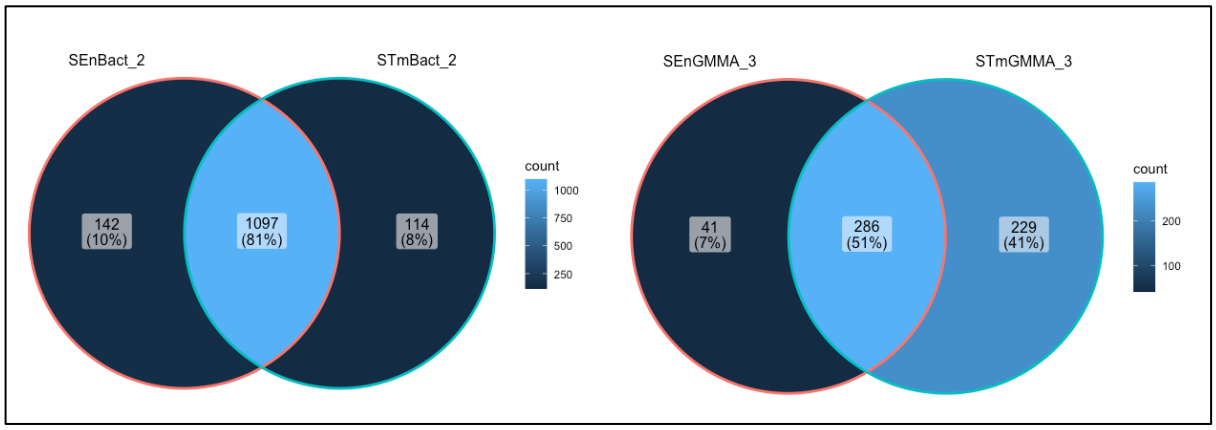



*Figure 57 Venn diagram of unique proteins shared between GMMAs (left) and Bacteria (right) by most abundant replicate*

## Identification and Comparison of Subcellular Location Across GMMA and Bacteria

The subcellular location of all proteins was assigned using PSortB[226] (Methods section 5.2.5). Cytoplasmic proteins were the most frequent unique proteins in all sample types, whilst bacteria had a disproportionately higher number of unique cytoplasmic proteins between all sample types: SEnGMMA 29%, STmGMMA 51%, SEn bacteria 68% and STm Bacteria 67%. STmGMMA possessed a higher cytoplasmic component than SEnGMMA which could be contributing to differences noted in the PCA (figure 51) and unique proteins between GMMAs (figure 57). In addition, a similar distribution was seen once abundance as measured by log10 LFQ intensity was assigned to each protein (Methods section 5.2.5) and factored in to determine the total protein assigned to each subcellular location (figure 58). However, once the outer membrane components (outer membrane, cytoplasmic membrane, periplasmic space) were summated, these proteins constituted the single largest protein group for SEnGMMA but not for STmGMMA.

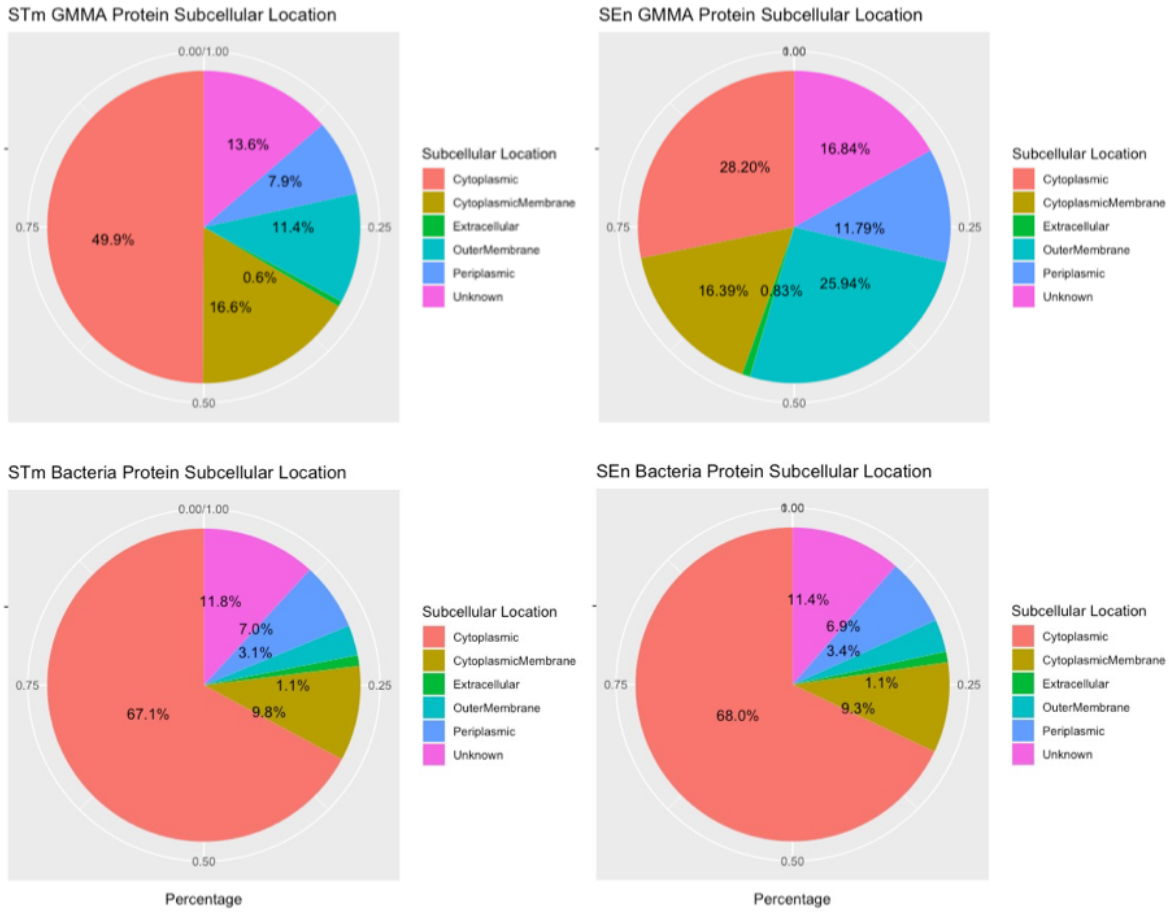



*Figure 58 Subcellular distribution by unique protein and abundance across all four sample types*

## Identification of Most Abundant Proteins and Their Subcellular Location Across GMMA and Bacteria

The 20 most abundant proteins per sample type (table 30) in GMMAs were either membrane-bound or periplasmic, whereas, in bacteria these were cytoplasmic proteins. Whilst OmpA was the most abundant protein found in the GMMAs, it remained among the 20 most abundant proteins found in bacteria, highlighting a possible role as an antigenic target with abundance in both the active vaccine components and in infecting bacteria. Another abundant protein identified across GMMA and bacteria was cypD or cyclophilin D, known for regulating mitochondrial function[247]. LysS or Lysine tRNA ligase,

an enzyme involved in tRNA and lysine formation[248], was the most abundant protein found in both bacterial serovars.

| | Gene Names | | | | Localisation | | | |
|---|---|---|---|---|---|---|---|---|
| | SEnGMMA | STmGMMA | SEn Bacteria | STm Bacteria | SEnGMMA | STmGMMA | SEn Bacteria | STm Bacteria |
| 1 | ompA | ompA | lysS | lysS | Outer Membrane | Outer Membrane | Cytoplasmic | Cytoplasmic |
| 2 | ompX | lpp | tuf | groES | Outer Membrane | Outer Membrane | Cytoplasmic | Cytoplasmic |
| 3 | ompC | ompX | groES | tuf | Outer Membrane | Outer Membrane | Cytoplasmic | Cytoplasmic |
| 4 | lpp | ompC | osmY | osmY | Outer Membrane | Outer Membrane | Periplasmic | Periplasmic |
| 5 | fljB | cypD | cypD | cypD | Extracellular | Cytoplasmic Membrane | Cytoplasmic Membrane | Cytoplasmic Membrane |
| 6 | cypD | groEL | hupA | rplD | Cytoplasmic Membrane | Cytoplasmic | Cytoplasmic | Cytoplasmic |
| 7 | ompD | pal | ompA | hupA | Outer Membrane | Outer Membrane | Outer Membrane | Cytoplasmic |
| 8 | slyB | PagC | rplD | ompA | Outer Membrane | Outer Membrane | Cytoplasmic | Outer Membrane |
| 9 | osmE | traT | mdh | ybiB | Unknown | Outer Membrane | Unknown (multiple) | Cytoplasmic |
| 10 | mipA | osmE | hns | rplL | Outer Membrane | Unknown | Cytoplasmic | Unknown |
| 11 | pal | ompF | rplL | STM0402 | Outer Membrane | Outer Membrane | Unknown (multiple) | Cytoplasmic |
| 12 | pagN | degP | STM0402 | hupB | Unknown | Periplasmic | Cytoplasmic | Cytoplasmic |
| 13 | ygdI | lysS | ompW | rpsH | Unknown | Cytoplasmic | Outer Membrane | Cytoplasmic |
| 14 | sucB | fliC | eno | mdh | Cytoplasmic | Extracellular | Cytoplasmic | Unknown |
| 15 | degP | ompD | ybiB | fusA | Periplasmic | Outer Membrane | Cytoplasmic | Cytoplasmic |
| 16 | tolB | bamC | fljB | hns | Periplasmic | Outer Membrane | Extracellular | Cytoplasmic |
| 17 | yiaD | aceF | sodB | eno | Outer Membrane | Cytoplasmic | Periplasmic | Cytoplasmic |
| 18 | yraP | lptA | crr | acpP | Periplasmic | Unknown | Cytoplasmic | Cytoplasmic |
| 19 | bamC | yiaD | yciF | sodB | Outer Membrane | Outer Membrane | Cytoplasmic | Cytoplasmic |
| 20 | STM1254 | bamB | fusA | rpsD | Unknown | Outer Membrane | Cytoplasmic | Cytoplasmic |

*Table 30 Comparison of all sample types, the 20 most abundant proteins, and localisation by mean log10 label-free quantification (LFQ)*

### Higher Order Function of Proteins Across GMMA and Bacteria

All sample types contained proteins which contributed to metabolic processes. However, as expected, more metabolic process proteins were found in the bacteria than GMMAs. In addition, STmGMMA had more proteins covering metabolic processes and pathways than SEnGMMA (Appendix B, figure 96). A similar pattern was seen with molecular function in addition to a higher percentage of proteins involved in porin and channel activity in the GMMAs, particularly in the SEnGMMA (Appendix B, figure 97). The most apparent difference of all three GO classifications is seen in cellular components with a higher percentage of proteins across the cytoplasm, intracellular and cytosol in bacteria in contrast to the cell periphery and membrane components in the GMMAs (Appendix B, figure 98). See Appendix B for a complete list of GO terms and KEGG Pathways for the bacteria and GMMAs.

## 5.3.2 Identification and Analysis of 118 shared proteins across GMMA and bacterial-origin strains

### Identification of the 118 Shared Proteins

Four approaches were undertaken to identify shared proteins across GMMAs and equivalent bacterial strains. A comparison was made between the most abundant replicate per sample (249 shared proteins); proteins shared by all replicates (44 shared

proteins); proteins shared by all replicates (excluding STm Bacteria replicate 1) [118 shared proteins, figure 59] and proteins present at least once across replicates (excluding STm Bacteria replicate 1) [300 shared proteins].

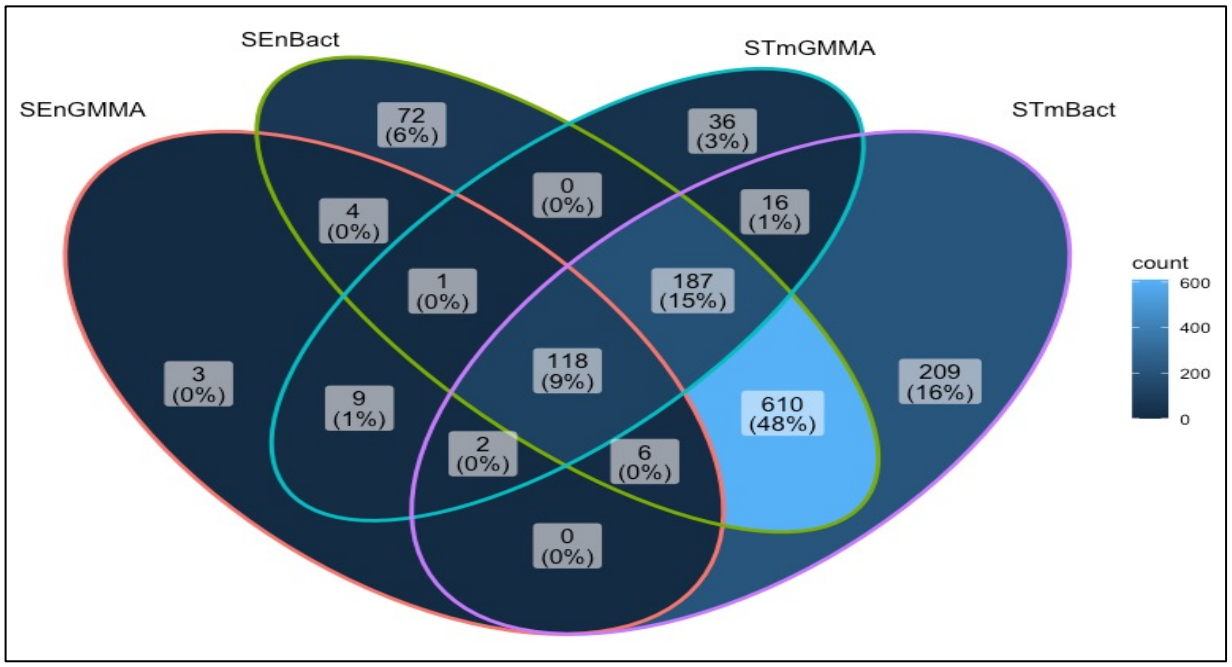



*Figure 59 Venn diagram showing the shared distribution of unique proteins across all four sample types by most abundant replicate (excluding STmBact replicate 1)*

For the identification of immunogenic proteins that could be used as antigens, the most conservative approach for ongoing analysis was chosen: proteins shared by all replicates (excluding STm Bacteria replicate 1) [figure 59]. This balanced a reduction of possible protein antigens in favour of a likelihood of protein antigens present across all active GMMA components at a significant abundance to stimulate an immune response. For a list of all 118 shared proteins, please see Appendix C.

# Differential Abundance Analysis of the 118 Shared Proteins

A differential abundance analysis of the initial 118 proteins using proDA[245] and Limma[246] packages in r[149]/Rstudio[150] was conducted to identify statistically significant differences in protein abundance between GMMA and bacteria sample types. Statistical analysis using empirical Bayes statistics in limma[246] revealed no statistical difference in abundance between bacteria. However, there were significant differences in abundance between GMMAs (figure 60).

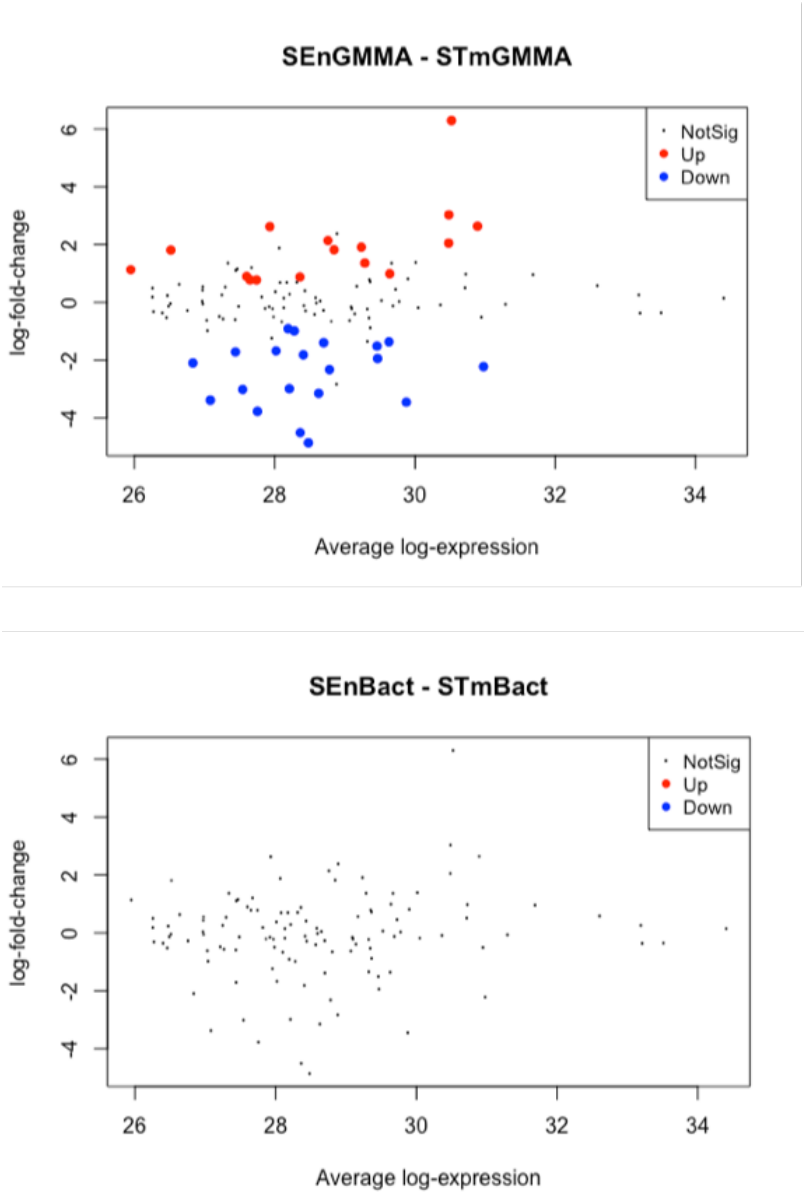



*Figure 60 Differential abundance analysis between SEnGMMA and STmGMMA (Top) and SEn and STm bacteria (Bottom); Statistical analysis using empirical Bayes statistics in limma[246]*

## Higher Order Function of the 118 Shared Proteins

To further explore the 118 shared proteins, an enrichment analysis using STRING[241] to identify protein interactions and gene ontology (GO) terms was implemented to extract a rationale for proteins shared between sample types.

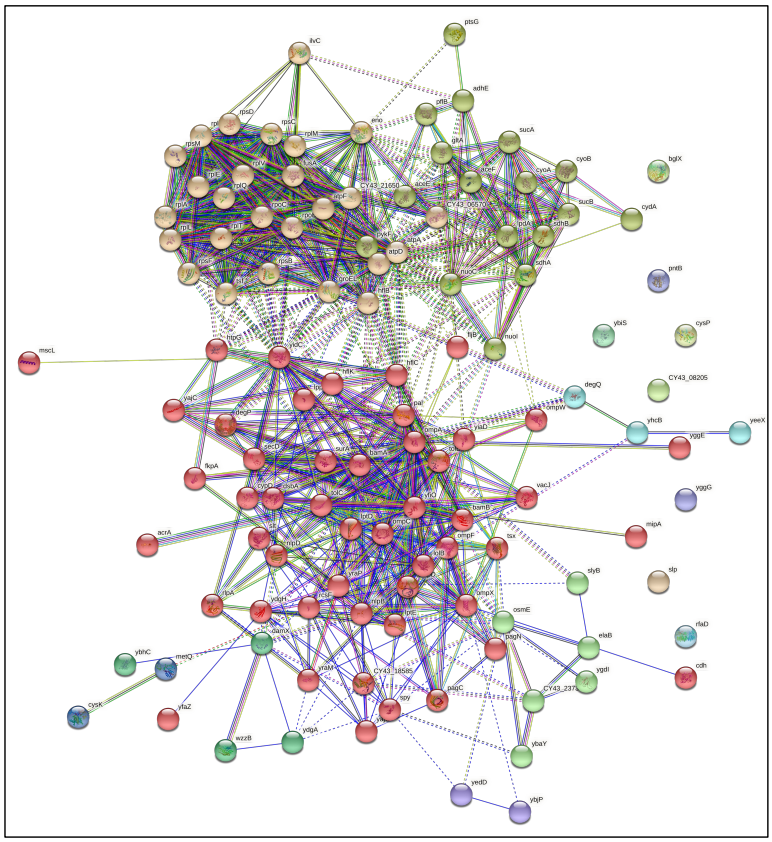


*Figure 61 STRING[241] network analysis of 118 shared protein connections, p-value <1x10-16; Markov Cluster Algorithm (MCL) analysis, with an inflation parameter set at 2, identifying 8 clusters.*

Significant (STRING p value $<1x10^{-16}$) interactions existed between the shared 118 proteins by physical and functional phenotype. Furthermore, clustering analysis by Markov Cluster Algorithm (MCL)[249] with the inflation parameter set at two revealed proteins clustered into 8 groups. The largest three with over ten proteins (figure 61 and 62) were: Cluster 1 (Red Nodes), Cluster 2 (Light Yellow Nodes) and Cluster 3 (Light Green Nodes). Cluster 1 (red) consisted of 50 proteins associated with the membrane, porin and periplasmic proteins involved in the membrane's structure, transport, and localisation. Cluster 2 (light yellow) comprised 20 proteins associated with metabolic functions and protein translation. Cluster 3 (light green) consisted of 17 proteins related to the metabolic, enzymatic, and respiration-based activity.

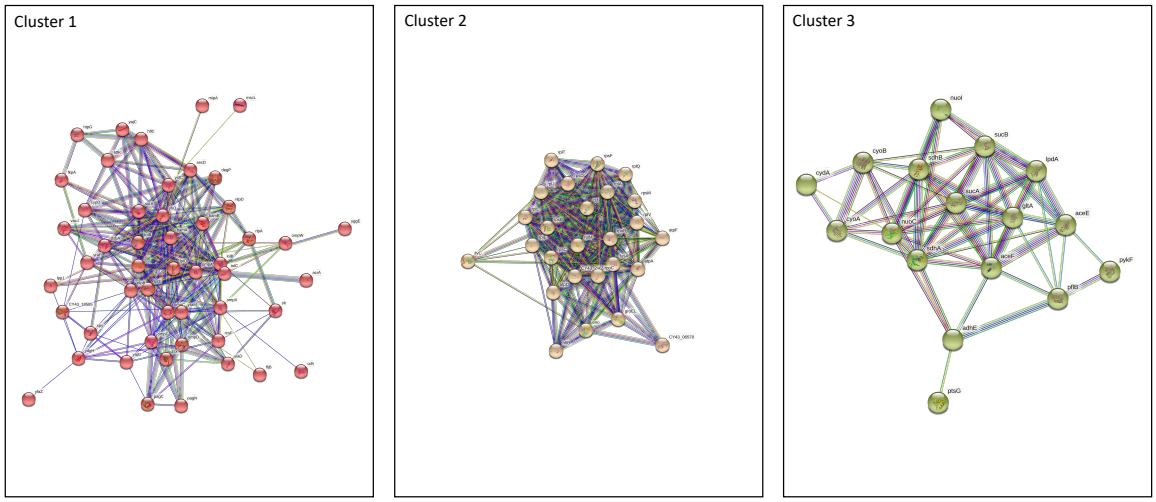



*Figure 62 Clustering Analysis of STRING network of 118 shared proteins, identifying the three most significant clusters (>10 proteins)*

## Subcellular Location of the 118 shared proteins

The subcellular location of the shared 118 proteins was identified using PSortb[226] (See section 5.2.5). Most unique proteins were from the cytoplasm (33%), followed by the outer membrane (22%) [figure 63]. A similar pattern emerged once protein abundance was factored in from the individual sample types (figure 64). However, once the entire outer membrane including the outer membrane, inner/cytoplasmic membrane and periplasm were summated, this accounted for the greatest number of unique and abundant proteins in the shared 118 (figure 63 and 64).

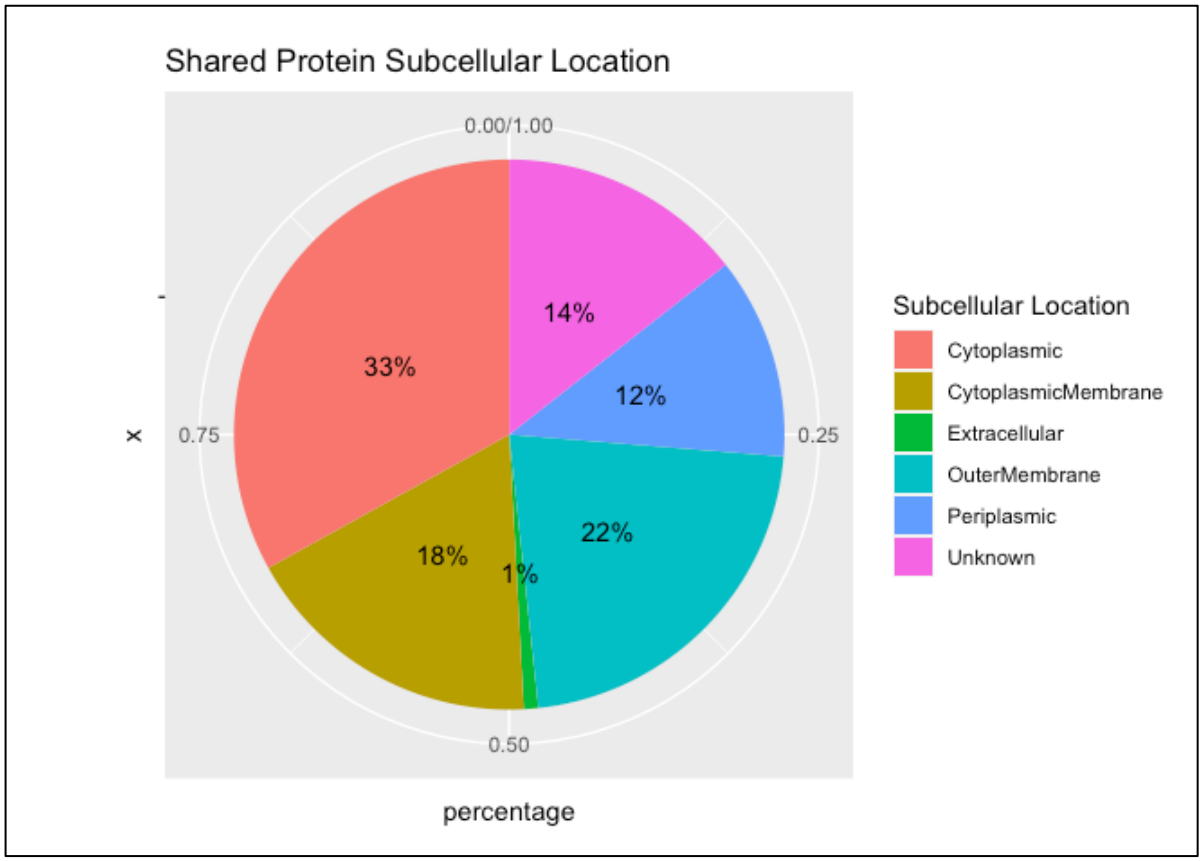



*Figure 63 Distribution of 118 shared proteins by subcellular location*

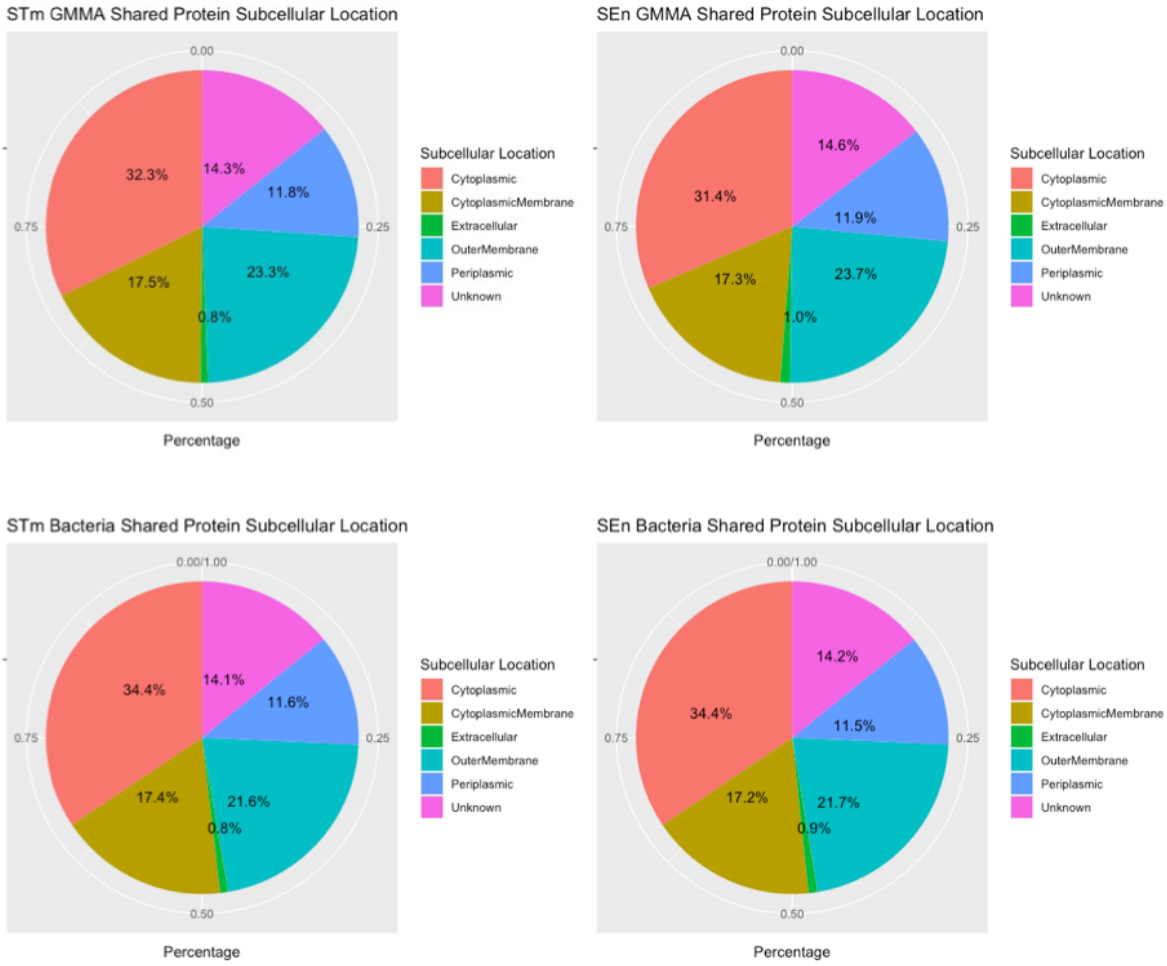



*Figure 64 Distribution of 118 shared proteins by subcellular location and abundance per sample type*

## 5.3.3 From 118 Shared Protein to Identification of Shared Immunogenic Proteins within GMMA Particles and Bacteria

Selection criteria were established to filter protein selection from the shared 118 proteins to potential candidates for in-house production and immunoassay testing (See Section 5.1.4). The schemata for filtering 118 shared proteins to the 8 proteins selected for production is outlined in figure 65. These initial criteria included protein abundance, immunogenicity, and practicalities of production. Finally, to investigate proteins that may stimulate a cross-reactive immunogenic response across *Salmonella* species beyond

*Salmonella* Typhimurium and *Salmonella* Enteritidis homologous proteins to *Salmonella* Typhi were identified. In addition, proteins with responses to *S*.Typhi convalescent sera from protein microarray experiments[239 240] were identified.

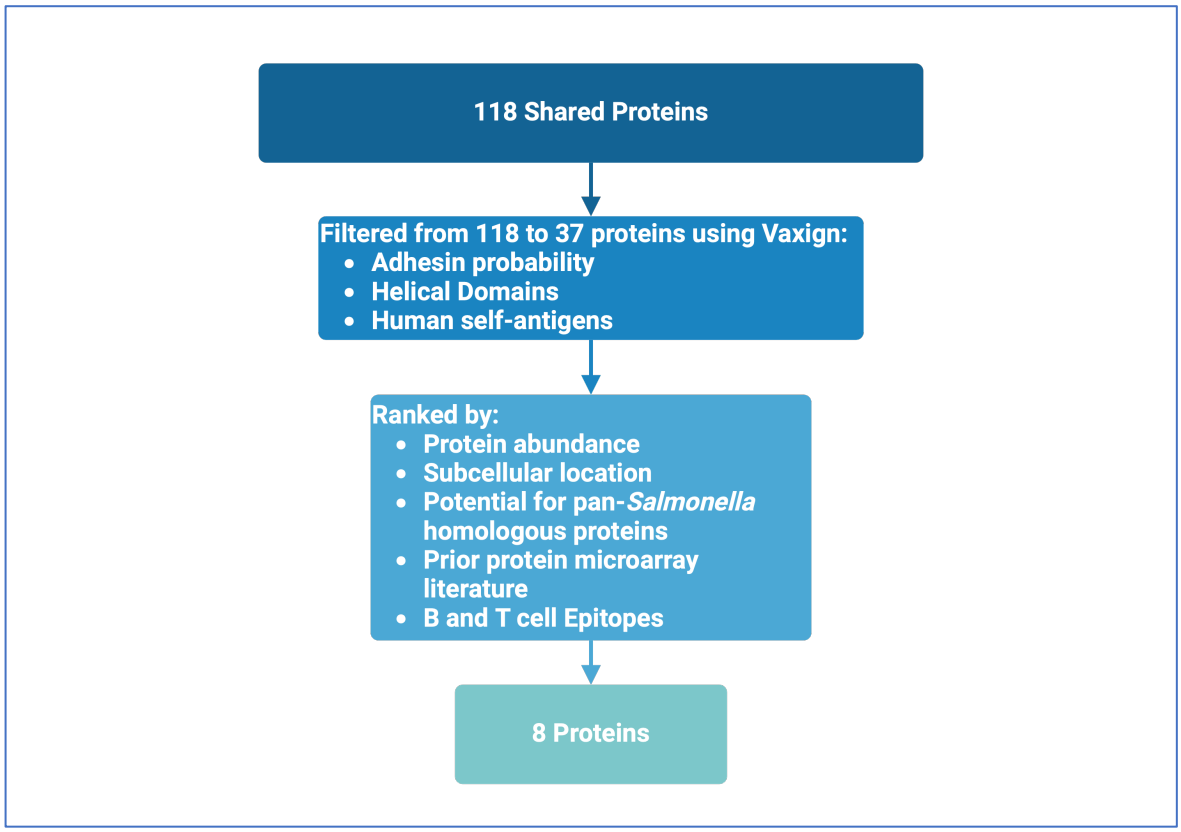



*Figure 65 Schemata for the filtering of the 118 shared proteins between SEn and STm GMMA and their origin bacteria, to 8 proteins selected for production and immunoassay testing*

## Ranking by Helical Domain and Adhesin Probability

Using Vaxign[227], a web-based application that assesses adhesin probability, helical domains, and human self-antigens, filtered the 118 shared proteins to 37 proteins which were further characterised (figure 66).

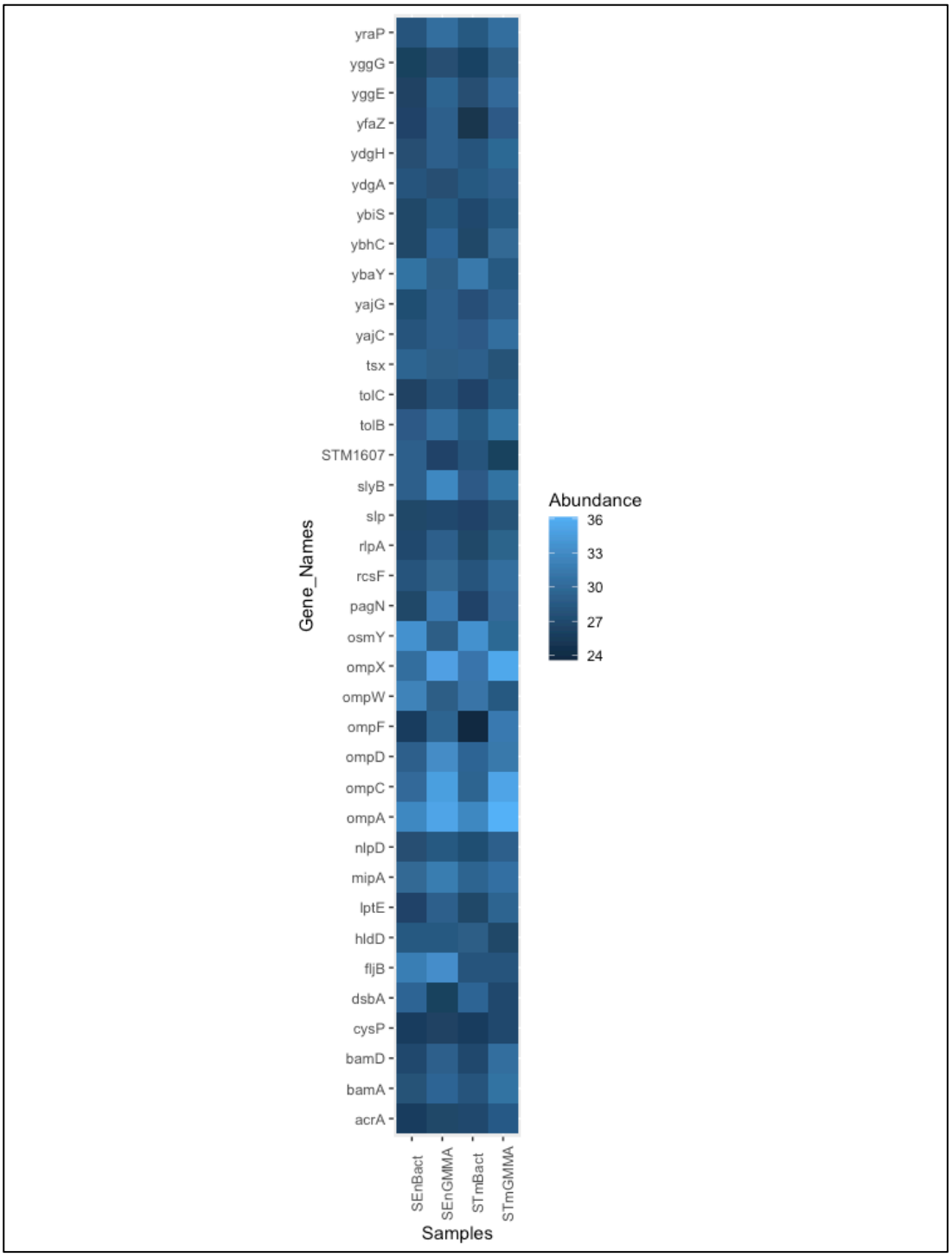



*Figure 66 Heatmap of the abundance of all selected 37 proteins by log10 Label-Free Quantification (LFQ)*

## Ranking by Protein Abundance

Once the 20 most abundant proteins were ranked (table 31) by the abundance of the GMMA and bacteria, ompA ranked highest for the GMMAs and fourth for the bacteria. Multiple outer membrane and extracellular proteins were higher up the index in GMMAs than bacteria, with some proteins in the GMMAs, including outer membrane proteins C and D, not included in the most abundant 20 seen in bacteria. The highest-ranked proteins for the bacteria were tuf, osmY and cypD. Elongation Factor Tu (tuf) is a cytoplasmic protein involved in ribosomal protein translation[237]. The protein osmY is a periplasmic protein of undefined function[237]. Peptidyl-prolyl cis-trans isomerase D (cypD) is both an enzyme and a membrane component[237]. However, all 37 proteins remained abundant within all four sample types as measured by LFQ (figure 66).

| | SEnGMMA | STmGMMA | SEn Bacteria | SEn Bacteria |
|---|---|---|---|---|
| 1 | ompA | ompA | tuf | tuf |
| 2 | ompX | lpp | osmY | osmY |
| 3 | ompC | ompX | cypD | cypD |
| 4 | lpp | ompC | ompA | ompA |
| 5 | fljB | cypD | rplL | rplL |
| 6 | cypD | groEL | ompW | fusA |
| 7 | ompD | pal | eno | eno |
| 8 | slyB | pagC | fljB | rpsD |
| 9 | osmE | osmE | fusA | gapA |
| 10 | mipA | ompF | gapA | osmE |
| 11 | pal | degP | tsf | tsf |
| 12 | pagN | ompD | rpsD | rplJ |
| 13 | ygdI | bamC | elaB | rplE |
| 14 | sucB | aceF | rplJ | rpsM |
| 15 | degP | yiaD | gltA | rpsF |
| 16 | tolB | bamB | rplE | rplA |
| 17 | yiaD | sucB | rpsF | ybaY |
| 18 | yraP | tolB | rpsM | rpsB |
| 19 | bamC | slyB | rplA | ygdI |
| 20 | rcsF | aceE | osmE | rplM |

*Table 31 20 most abundant proteins of 118 shared proteins across all sample types*

## Identification by Subcellular Location

The subcellular location of each protein from the shared 37 proteins was identified, revealing 46% Outer Membrane, 16% Periplasmic, 8% Cytoplasmic Membrane, 3% Cytoplasm, 3% Extracellular and 24% Unknown (table 32).

| Proteins | Subcellular Location | PSortB Score |
|---|---|---|
| ompA | OuterMembrane | 10 |
| ompX | OuterMembrane | 10 |
| ompC | OuterMembrane | 10 |
| fljB | Extracellular | 9.96 |
| ompD | OuterMembrane | 10 |
| slyB | OuterMembrane | 9.92 |
| mipA | OuterMembrane | 10 |
| pagN | Unknown | 2.5 |
| tolB | Periplasmic | 10 |
| yraP | Periplasmic | 9.76 |
| rcsF | OuterMembrane | 9.92 |
| bamA | OuterMembrane | 10 |
| ybhC | OuterMembrane | 9.93 |
| yggE | Periplasmic | 9.76 |
| ompF | OuterMembrane | 10 |
| yajC | CytoplasmicMembrane | 10 |
| yajG | Unknown | 2 |
| bamD | OuterMembrane | 9.92 |
| rlpA | Unknown | 2.5 |
| yfaZ | Unknown | 2.5 |
| ydgH | Unknown | 2.5 |
| lptE | OuterMembrane | 9.92 |
| tsx | OuterMembrane | 10 |
| ybaY | Unknown | 2.5 |
| ompW | OuterMembrane | 10 |
| osmY | Periplasmic | 10 |
| nlpD | OuterMembrane | 8.86 |
| ybiS | Unknown | 2.5 |
| hldD | Cytoplasmic | 9.26 |
| tolC | OuterMembrane | 10 |
| yggG | Unknown | 2 |
| ydgA | CytoplasmicMembrane | 9.82 |
| slp | OuterMembrane | 9.92 |
| acrA | CytoplasmicMembrane | 9.97 |
| STM1607 | Unknown | 2 |
| cysP | Periplasmic | 10 |
| dsbA | Periplasmic | 10 |

*Table 32 Subcellular Location of the 37 filtered proteins*

## Ranking by Homologous *Salmonella* Typhi Proteins

To identify potential cross-reactive antigens, shared proteins were investigated for >95% sequence homology to *Salmonella* Typhi and Paratyphi using BLASTp tool within the

UniProt database[237]. Of the 37 selected proteins, ompA, ompC, ompF, IptE, and slyB, in particular, were identified with homologous sequences in *S*.Typhi. There were fewer proteins with homologous sequences in *S*.Paratyphi, but IptE and fliC were identified. As per the literature ompD was not identified in *S*.Typhi but was present in *S*.Paratyphi[91].

| BLAST S.Typhi | | BLAST S.Paratyphi | |
|---|---|---|---|
| ompA | Q8Z7S0 | x | x |
| ompW | Q8Z7E2 | x | x |
| ompS2 (64.5%) | Q56111 | ompD | Q5PHY0 |
| dsbA | Q9X6N0 | x | x |
| OmpC | P0A264 | ompD (62.5%) | Q5PHY0 |
| tsx | P0A262 | x | x |
| slyB | P0A1X1 | x | x |
| hldD | P67913 | hldD | B5BHZ3 |
| tolB | Q8Z8C0 | tolB | B5BC62 |
| yraP | Q7CPQ6 | x | x |
| x | x | flic | P06178 |
| bamA | Q8Z9A3 | bamA | B5BAN4 |
| nlpD | Q56131 | x | x |
| rlpA | Q8Z8I0 | x | x |
| lptE | B5BCE7 | lptE | Q8Z8H6 |
| ompF | Q56113 | ompD (55.6%) | Q5PHY0 |

*Table 33 S.Typhi and S.Paratyphi homologous proteins identified by the BLAST database from the 37 shared proteins*

## Ranking by *Salmonella* Typhi Protein Microarray Data

Two studies (Darton et al., 2017[240]; Liang et al, 2013[239]) investigated *Salmonella* proteins as antigens across a microarray using human convalescent sera following *Salmonella* Typhi, Paratyphi and non-typhoid *Salmonella* infection. Antigens from these studies were grouped in order of serum *Salmonella* cross-reactivity (tables 34 and 35). Due to the extensive range of antigens tested by Liang, this was the primary antigen source used to cross-reference with the shared proteins from the GMMA and bacteria proteomic data set. Antigens present in Darton et al. (2017) were cross-referenced and marked as 'Y' in

Table 35. OmpA and flagellin were the only two proteins present across both data sets. However, similar proteins to the NTS proteins, nlpD (nlpC, t1224), tolB and tolC from the tol-pal system (ybgF, t2126) were identified. FkpA was identified in group 1 and the 118 shared proteins but not in the 37 filtered due to similarity with human proteins.

| Groups | Rationale |
|---|---|
| 1 | Proteins cross-reactive between convalescent NTS and TS serum - Equivalent Antibody Response |
| 2 | Proteins cross-reactive between convalescent NTS and TS serum - Antibody Response NTS > TS |
| 3 | Proteins cross-reactive between convalescent NTS and TS serum - Antibody Response TS > NTS |
| 4 | Proteins cross-reactive across:<br>SE Asia convalescent NTS **AND** negative control;<br>African convalescent NTS **BUT NOT** negative control |
| 5 | Additional proteins stimulating antibody response from Typhoid challenge convalescent serum* |

*Table 34 Grouping strategy of protein antigens from Liang et al. (2013). *Additional proteins from Darton et al. (2017)*

| Group | ID | Symbol | Product Description | Cross-Reference |
|---|---|---|---|---|
| 1 | t4052 | fkpA | FKBP-type peptidyl-prolyl cis- trans isomerase | N |
| 1 | t3710 | hslS | heat shock chaperone IbpB | N |
| 1 | t1224 | nlpC | putative lipoprotein | N |
| 1 | t3065 | hypA | hydrogenase nickel incorporation protein HybF | N |
| 1 | t0536 | nuoA | NADH dehydrogenase subunit A | N |
| 1 | t4322 | | terminase subunit | N |
| 1 | t4253 | pilK | hypothetical protein t4253 | N |
| 1 | t3904 | yhjJ | putative zinc-protease precursor | N |
| 2 | t1459 | | putative secreted hydrolase | N |
| 3 | t1477 | hlyE | hemolysin E | Y |
| 3 | t1111 | cdtB | putative toxin-like protein | N |
| 3 | t1128 | | putative heat shock protein | N |
| 3 | t3515 | | hypothetical protein t3515 | N |
| 3 | t1285 | ssaP | putative type III secretion protein | N |
| 3 | t2970 | | hypothetical protein t2970 | N |
| 3 | t1814 | hpcG | 2-oxo-hepta-3-ene-1,7-dioic Y acid hydratase | N |
| 4 | t4225 | phoN | Non-specific acid phosphotase precursor | N |
| 4 | t2941 | | hypothetical protein t2941 | N |
| 4 | t2126 | ybgF | tol-pal system YbgF | N |
| 4 | t3119 | | hypothetical protein t3119 | N |
| 4 | t1449 | | hypothetical protein t1449 | N |
| 4 | t1850 | OmpA | outer membrane protein A | Y |
| 5 | t0918 | | flagellin | N/A |
| 5 | t2919 | | 2-acylglycerophosphoethanolamine acyl transferase (aas) | N/A |
| 5 | t1300 | | hypothetical protein t1300 | N/A |
| 5 | t0101 | | IS element transposase | N/A |
| 5 | t1485 | | methyl viologen resistance protein SmvA | N/A |
| 5 | t0596 | | phosphotransfer intermediate protein in two-component regulatory system with RcsBC | N/A |

*Table 35 Protein antigens from Liang et al. (2013) and Darton et al. (2017) grouped by order of preference*

## T Cell Epitope Analysis

CD4 linear epitope analysis (*IEDB, USA*)[229] revealed all 37 proteins contained predicted T cell epitopes with tolB followed by cysD, pagN and bamA containing the highest number with a range of 1-13 epitopes across all 37 proteins (figure 67).

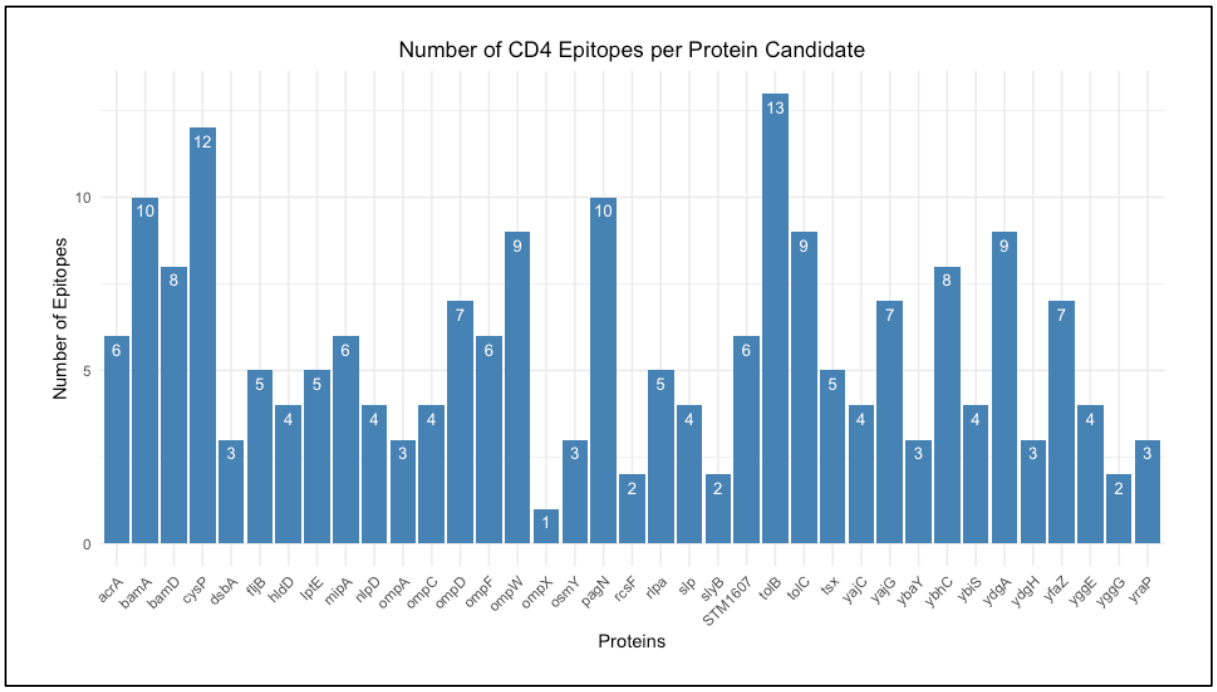



*Figure 67 Distribution of CD4 T Cell epitopes across the selected 37 proteins*

## Linear B Cell Epitope Analysis

Linear B Cell epitope analysis using Bepipred Linear Epitope Prediction 2.0 (*IEDB, USA*)[228] revealed all 37 proteins contained predicted B Cell Epitopes. Overall, there were a greater number of B cell predicted epitopes across the proteins than predicted T Cell epitopes. BamA (28), ydgA and tolC (18) contained the greatest number of epitopes. The next highest were the outer membrane proteins OmpA (13), OmpC (14), OmpD (14) and OmpF(14) [figure 68].

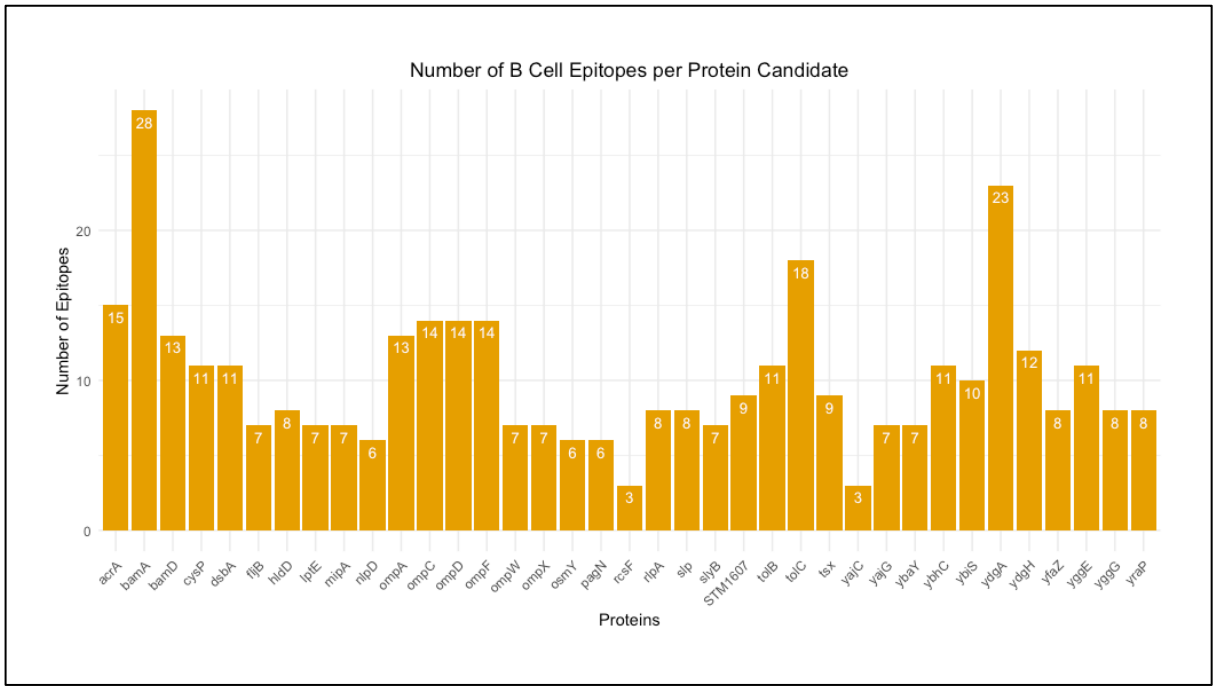



*Figure 68 Distribution of B Cell epitopes across the selected 37 protein*

# Identification of 8 Selected Proteins for Production

| Protein Name | <2 Trans-membrane Helices (Vaxign) | Adhesin Probability (>0.5) (Vaxign) | Non-Human Proteins (Vaxign) | Ranked 118 Proteins by Abundance in SEnGMMA (mean log10 LFQ) | Subcellular Location (PSortb) | Homo-logous S.Typhi and / or S.Paratyphi Proteins (BLAST /UniProt) | Microarray-identified Antigens (Liang et al, 2013; Darton et al, 2017) | B Cell Epitopes | T Cell Epitopes |
|---|---|---|---|---|---|---|---|---|---|
| **OmpA** | Yes | Yes | Yes | 1 | Outer Membrane | S.Typhi | Yes | 13 | 3 |
| **OmpC** | Yes | Yes | Yes | 3 | Outer Membrane | S.Typhi | No | 14 | 4 |
| **OmpF** | Yes | Yes | Yes | > 20 | Outer Membrane | S.Typhi | No | 14 | 6 |
| **SlyB** | Yes | Yes | Yes | 8 | Outer Membrane | S.Typhi | Yes | 7 | 2 |
| **Flagellin** | Yes | Yes | Yes | 5 | Extracellular | S.Paratyphi | Yes | 7 | 5 |
| **FkpA** | Yes | Yes | No | > 20 | Periplasmic | S.Typhi S.Paratyphi | Yes | - | - |
| **IptA/E/D** | Yes | Yes | Yes | > 20 | Outer Membrane | S.Typhi | No | 7 | 5 |
| **OmpD** | Yes | Yes | Yes | 7 | Outer Membrane | S.Typhi | No | 14 | 7 |

*Table 36 Selected Proteins for Production*

## 5.4 Discussion

### 5.4.1 Summary

1. Evaluation of the proteomic components of the iNTS-GMMA vaccine.

Most proteins identified in the SEn and STm GMMA were identified in their origin bacteria. The GMMAs were enriched for membrane proteins, as the single largest group of proteins. Whilst cytoplasmic proteins were the single largest group in whole bacteria, they remained a significant group in the SEn and STm GMMA which was not seen in the *Shigella* GMMA proteomic analysis[122]. Principle component analysis revealed that the proteomic composition across SEn And STm bacteria were highly similar, with the SEn and STm GMMAs more dissimilar from each other in protein composition.

2. Identification of immunogenic homologous *Salmonella* proteins conserved across *Salmonella* serovars.

A stepwise approach was undertaken to identify conserved proteins across *Salmonella* serovars. To begin with a conservative approach to identify proteins conserved across SEn and STm GMMAs and their origin bacteria revealed 118 shared proteins. It was decided that the most efficient immunogenic proteins useful within the iNTS-GMMA and a future iNTS vaccine should be present across both GMMA and their origin bacteria. The initial 118 proteins were then filtered to 37 proteins based on likely immunogenicity (adhesion

probability, helical domains and self-antigens). The proteins were then sorted based on abundance, sub-cellular location, T cell and B cell epitopes and homologous proteins within *S.* Typhi and *S.* Paratyphi. Eight proteins were selected on the basis of these factors and produced. The immunogenicity of these proteins were then tested to determine antibody and T cell reactivity using a series of protein ELISAs (Chapter 6) and T cell proliferation assays (Chapter 7).

### 5.4.2 Differences in the GMMA and Bacteria Sample Types

#### The Protein Composition is Similar between SEn and STm Bacterial Sample Types

The principal component analysis revealed all bacterial replicates (excluding STm bacterial replicate 1) clustered together, suggesting a similar composition in both unique proteins and abundance of each protein. This was confirmed by the identification of 81% of unique proteins seen across both SEn and STm bacterial samples (figure 57), similar most abundant proteins (table 31), and the demonstration of no significant difference in abundance in the 118 shared proteins between these bacteria (figure 60). Whilst 1097 shared proteins were identified between SEn and STm here, an analysis of the core proteomes from multiple sequence types of both SEn and STm across sub-Saharan Africa and Southeast Asia revealed only 465 shared proteins[250]. This might be explained by a variation in proteome across multiple sequence types between geographical and intercontinental regions. The extent to which the efficacy of the iNTS-GMMA vaccine might vary between geographical regions is likely highly multifactorial, with possible

factors including the variation in immunogenic proteins and relative contribution of O-antigen and protein to protective immunity between the iNTS-GMMA vaccine and NTS serovars and sequence types.

## Protein Composition is Dissimilar between GMMAs and Corresponding Bacterial Sample Types

The primary principal component in the PCA identified a variance of over 60% between the GMMAs and corresponding bacteria, which likely reflects the reduction of unique proteins in GMMAs. Shared proteins between GMMA and bacteria were 23% and 36% of total proteins in SEn and STm, respectively. This was underlined by the differences in specific proteins, as seen with the variation in subcellular location (figure 58) and most abundant proteins (table 32). As expected, the GMMAs were enriched for membrane proteins in comparison to cytoplasmic proteins seen in bacteria (figure 58). However, whilst membrane proteins were the single largest group within the SEnGMMA, cytoplasmic proteins remained the largest group in STmGMMA (although reduced compared with STm bacteria). This distribution was not seen in the *Shigella* GMMA proteome with both *Shigella flexneri* and *Shigella sonnei* GMMAs containing predominantly outer membrane and periplasmic proteins with significantly lower cytoplasmic protein content[122].

Few proteins identified in the GMMAs were not seen in the origin bacteria. These proteins might be explained by enrichment during the blebbing process from the bacterial

outer membrane, due the culture conditions used to enhance GMMA production, or possibly due the genetic modification of the origin bacteria resulting in a modification of GMMA protein expression.

## Protein Composition is Dissimilar between SEn and STm GMMAs

Bacterial protein composition appears similar according to the PCA, but principal component two identified variance between GMMAs, suggesting each serovar produces GMMAs with different protein content. This was confirmed following differential abundance analysis of the 118 shared unique proteins, with significant differences seen in protein abundance between SEn and STm GMMAs but not SEn and STm bacteria (figure 60). Plausible explanations include the differences in protein composition: by unique proteins exclusive to each GMMA (256 proteins) [figure 56]; or shared proteins enriched differently in each GMMA, especially if location (outer membrane) and involvement in GMMA production are associated with these proteins; or differences in production of GMMAs by each SEn and STm bacteria under the same conditions, noting differences in protein number, genomic and phenotypic differences between bacteria[25].

Following increasing evidence of *Salmonella* Typhimurium ST313 phenotypic convergence towards *Salmonella* Typhi and *Salmonella* Paratyphi[25], it is plausible that a higher cytoplasmic component in STmGMMAs may be consistent with factors resulting from this convergence, including virulence factors. This is also consistent with further evidence of

OMV function in the transportation of virulence factors, quorum sensing proteins and pathogenic machinery[216]

### 5.4.3 The 118 Shared Proteins

#### Conservative Approach to Identification of the 118 Shared Proteins

118 unique proteins were identified as shared between all replicates (except STm Bacteria 1) and sample types (SEnGMMA, STmGMMA, SEn and STm bacteria). As the most conservative approach, it is possible that potential immunogenic proteins might have been excluded, with shared proteins across at least one replicate of each sample type totalling 300. This approach was chosen due to the certainty of protein presence within the GMMAs and bacteria. Further work may include a further investigation of these 182 remaining proteins with a degree of overlap between sample types.

#### Identification of possible *Salmonella* Cross Reactive Proteins

Proteins that elicit cross-reactive responses and hence potentially offer a degree of cross-serovar protective response may be of benefit to any polyvalent *Salmonella* vaccine. Therefore, any proteins which have the potential to stimulate this response to non-typhoid *Salmonella*, *S*.Typhi and *S*.Paratyphi were prioritised. From the filtered 37 proteins (filtered by adhesin probability, helical domains, and human self-antigens from shared 118), the number of *S*.Typhi homologous proteins outnumbered that of *S*.Paratyphi, however, it is unclear whether the difference is due to a greater number of shared

proteins in *Salmonella* Typhi or the completeness of the S.Paratyphi database. OmpA and FkpA were the only proteins identified from the microarray studies[239] [240] present in the 37/118 filtered proteins.

### 5.4.4 Identification of Selected GMMA Proteins for Production

#### Identification of Type 3 Secretion System Proteins

YggG is the only effector protein from the type 3 secretion system in the filtered 37 proteins. This protein is important in stimulating apoptosis in the *Salmonella*-infected host cell. In mice infected with the yggG knockout *Salmonella* strain, the liver and spleen showed a reduction in *Salmonella* burden compared with mice infected with a wild type strain[251]. The type 3 secretion system is the principal virulence mechanism for *Salmonella* invasion into host cells and evasion of the host immune responses[252]. A vaccine which has the potential to target these effector proteins [within the type 3 secretion system (T3SS)] have been shown in mice models to be effective in stimulating antibody and CD4 responses resulting in survivability following *Salmonella* challenge for the T3SS protein SseI[253] and SseB-Flagellin conjugate[93]. Improvement of the GMMA vaccine components might not only be to increase GMMA production and control of OAg content but also to increase the quantity of immunogenic proteins such as T3SS effector proteins, as has been demonstrated to be achievable by modification of culture conditions[125].

## Outer Membrane Proteins

Outer membrane proteins A (OmpA), C (OmpC), F (OmpF), and D (OmpD) are all porins which have been investigated as possible vaccine components as a result of their ability to stimulate host immunogenicity. OmpA has been identified in many Gram-negative organisms as one of the most abundant proteins in both the bacteria and OMVs[122], confirming the findings presented in this chapter. As a result, it is being investigated as a vaccine antigen[254 255] with an antibody response in mice demonstrated from *E. coli*[256] to *Acinetobacter*[257], with a reduction in mice bacterial burden post-*E. coli* challenge[256]. In addition, it has been shown to increase the virulence of Gram-negative bacteria by its involvement in host cell invasion[255] and survival of bacteria within macrophages[258]; and has been a target of monoclonal antibodies and vaccines across multiple Gram-negative organisms[257 259]. It was the second most abundant protein seen in the *Shigella* GMMAs Sf2a and Ss[122], raising the prospect of cross-reactivity beyond *Salmonella* species, and its homolog has been proposed as a potential vaccine candidate for *Shigella*[260]. OmpC and F are present across multiple *Salmonella* serovars[261] and have been investigated as a possible *Salmonella* vaccine component in pre-clinical testing with evidence of T cell immune responses in mice[262]. Outer Membrane Protein D (OmpD) is another porin protein that is currently in pre-clinical vaccine testing[91]. One study in HIV seropositive individuals found that high antibody titres to OAg were inhibitory to antibody-mediated bacterial killing, but it was antibodies to the OmpD that were bactericidal[64].

### Extracellular Proteins

Flagellin is the principal component of the bacterial flagellum whose function principally includes bacterial motility as well as transmission of bacterial effector proteins[263]. Flagellin was abundant in both SEnGMMAs and bacteria but not the equivalent STmGMMA. However, it was seen in the most abundant 20 of the filtered 37 proteins in all sample types except STmGMMA. Flagellin elicited antibodies in the Typhoid convalescent samples, although analysis using BLAST of the NTS flagellin failed to identify *S.*Typhi flagellin to over 95% sequence homology. The presence of 5 T cell epitopes and 7 B cell epitopes reflects the predicted immunogenicity of Flagellin as seen in multiple *in vitro* and mice studies[93] and for this reason used as the protein component in the NTS bivalent COPS:FliC vaccine candidate[116 264].

### Periplasmic Proteins

FkpA is peptidyl-prolyl isomerase involved in the structural manipulation of envelope proteins[265 266] and is the only periplasmic protein selected. As a protein that both captured antibodies from *Salmonella* Typhi and non-typhoid *Salmonella* convalescent sera in Liang et al., 2013[239], as well as being present in the shared 118 proteins, it was selected for further testing.

## 5.4.5 Limitations

The laboratory culture conditions used for growing the origin bacterial strains and production of GMMAs were not identical and might have affected the type and number of

proteins present in each type of sample[250]. In addition, the behaviour of bacteria, including proteins expressed, whilst cultured *in vitro* might not be the same as *in vivo*[250]. As there were no complete reference proteomes for the SEn and STm GMMA origin strains, closely related strains were chosen, which might have affected protein identification. Finally, whilst the process and tools used to identify potential immunogenic proteins might give an indication of immunogenicity, further testing is required for confirmation, which might include *in vitro* immunoassay testing.

## 5.5 Conclusion

Given the dual burden of iNTS and *Salmonella* Typhi in sub-Saharan Africa, a polyvalent vaccine covering multiple *Salmonella* species would be ideal. The establishment of cross-reactive responses to NTS proteins within GMMAs would be a welcome addition to any future polyvalent vaccine involving OMVs. Further work in this thesis includes the establishment of the immunological response to these proteins using convalescent human samples (See *Chapters 6.3.7* and *Chapter 7.3.3*).

# Non-Typhoid *Salmonella* GMMAs Demonstrate Functional Cross-Reactivity with Antibodies Generated Across other *Salmonella* Serovars

## 6.1 Introduction

### 6.1.1 Cross-Reactive Antibodies

Antibodies are cross-reactive when they react or bind to more than one antigen. These antigens can be from the same organism, different serotypes or entirely different species, which can ultimately be harmful or beneficial to the host. Harmful cross-reactive antibodies include those involved in autoimmunity or molecular mimicry, such as Group A Streptococcus and Rheumatic fever[267]. Hypothetical harmful immunological responses include antibody-dependent enhancement (increase in disease following subsequent infection)[268] or original antigenic sin when an initial antigen primes the immune system such that a subsequent boost by a partially similar but more effective antigen has reduced efficacy for the current variant as the initial antigen immune response is preferentially boosted[269].

However, there are numerous beneficial responses, particularly in vaccinology, where cross-reactivity is the basis of many vaccines, including the first vaccine administered by Edward Jenner, in which the Vaccinia virus was inoculated for protection against disease

caused by the Variola virus in humans[270]. More recent examples of beneficial cross-reactive antibodies include the bivalent HPV vaccine affording some protection against wider HPV serotypes[270 271]. If these cross-reactive antibodies provide a clinical benefit, i.e. ameliorate or prevent disease, they could be defined as cross-protective[270]. The meningococcal B vaccine is the only licensed vaccine based on outer membrane vesicles and was found unexpectedly to reduce the incidence of gonococcal infections in a cohort of individuals who received the vaccine suggesting cross-reactivity can lead to significant cross-protection across two similar bacterial species[272]

### 6.1.2 Current Evidence for *Salmonella* Immunodominant and Cross-Reactive Antibodies

*Salmonella* spp. share similar structural, metabolic and functional components. Changes in the genome in response to internal and external pressures has increased the diversity of these components. Lipopolysaccharide (LPS) is present in the outer membrane of all Enterobacteriaceae as an essential structural component and virulence factor, providing a barrier to hydrophobic antimicrobials[95]. LPS is a potent stimulator of the innate immune system and is recognised as a pathogen-associated molecular pattern (PAMP) via toll-like receptor 4 (TLR4)[95 273]. Whilst the lipid A component of LPS is a particularly strong determinant of the innate septic response seen in Gram-negative bloodstream infections[274], the O-antigen component is also immunogenic across *Salmonella* spp.[275]. However, functional assays using an O-antigen component demonstrate bactericidal antibodies that attenuate rather than protect in *S.* Typhi controlled human infection models[276].

The O-antigen structure is highly variable in length, consisting of chains of monosaccharides with additional modifications, including acetylation and methylation[277]. This high variability caused by the evolutionary response to host immune activation has led to significant differences in antibody binding as demonstrated by the Widal test[278] and forms the basis of the Kauffman White Classification[279] of *Salmonella*. There are 46 serogroups in *Salmonella*[274], with some shared O-antigen serogroups across *Salmonella* serovars. Pakkanen et al. (2019)[280] showed that antibodies and plasmablasts induced by the Ty21a live attenuated typhoid vaccine were cross-reactive to *S.* Enteritidis and *S.* Paratyphi B. The serogroups O:9 and O:12 are conserved across *S.* Enteritidis, *S.* Paratyphi and *S*. Typhi, but the functional activity of OAg-specific antibodies induced by these serovars has been limited to a non-significant elevation in opsonophagocytic activity[281].

Whilst O-antigen may be an important vaccine target[275 282], resistance to targeted antibodies is already emerging. Mutations in the *Salmonella* Typhimurium *wzz* genes increase O-antigen chain length, which has been shown *in vitro* to confer resistance to antibody-dependent complement killing[96]. Such chain-length hypotheses have been linked to the success of *Salmonella* Paratyphi to both evade the immune system and consequently cause enteric fever[283].

The development of serum resistance to O-antigen may make vaccines like the iNTS-GMMA increasingly important by stimulating a polyclonal antibody response and adding breadth to the vaccine-induced immune response. Multiple *Salmonella* components have been investigated as vaccine antigens, including Vi polysaccharide, O-antigen and flagella

for *S*.Typhi[239], O-antigen[92] for *S*. Paratyphi, and O-antigen[275], flagellin[92], SSeB[94] for non-typhoid *Salmonella*. Whilst there is evidence for T cell cross-reactivity between *Salmonella* proteins, particularly involving intracellular proteins[106], evidence for antibody cross-reactivity is limited to protein microarray experiments[239], and very few studies, have investigated vaccine-stimulated serum cross-reactivity to *Salmonella* sp.[280]. In addition to testing GMMA-induced antibodies to O-antigen, selected proteins identified in Chapter 5 (Proteomic Analysis of the GMMAs) have been tested to determine the contribution to the primary or cross-reactive immune response.

### 6.1.3 Aims and Objectives

This chapter aims to identify whether typhoidal *Salmonella* sp. generate antibodies which show functional cross-reactivity with the SEn and STm GMMAs and could, therefore, contribute to or interfere with a non-typhoid *Salmonella* (NTS) immune response to GMMAs as a surrogate of whole bacteria or as a constituent of a multivalent vaccine. Secondly, this chapter aims to measure the potential for GMMAs to stimulate pan-*Salmonella* sp. responses and finally, to identify specific antigens contributing to a direct or cross-reactive antibody response to the GMMAs. Evidence from non-typhoid *Salmonella*, *S*. Typhi and *S*. Paratyphi exposed convalescent and iNTS-GMMA vaccine sera tested across GMMA-based ELISAs and functional antibody assays including antibody-dependent monocyte phagocytosis (ADMP) and antibody-dependent NK cell activation (ADNKA) are presented. In addition, these sera were tested on ELISAs using potential immunogenic proteins originally identified in *Chapter 5*.

Sera obtained following NTS, *S.* Typhi and *S.* Paratyphi infection and iNTS-GMMA vaccination were used to address the following objectives:

1. Evaluation of serum IgG and IgA antibody response to SEnGMMA and STmGMMA, generated following infection with non-typhoid *Salmonella* using a validated SEnGMMA and STmGMMA IgG and IgA ELISA.
2. Evaluation of serum IgG and IgA cross-reactive antibodies to SEnGMMA and STmGMMA, generated following infection with *S.*Typhi and *S.* Paratyphi using a validated SEnGMMA and STmGMMA IgG and IgA ELISA.
3. Evaluation of serum IgG and IgA functional primary and cross-reactive antibody response to SEnGMMA and STmGMMA, generated following infection with *S.*Typhi, *S.* Paratyphi or non-typhoid *Salmonella* using antibody dependant monocyte phagocytosis (ADMP) and NK activation (ADNKA) assays.
4. Evaluation of the functional cross-reactivity of iNTS-GMMA generated antibodies beyond non-typhoid *Salmonella.*
5. Identification of primary immunodominant IgG and IgA SEnGMMA and STmGMMA specific antigen following iNTS-GMMA vaccination or non-typhoid *Salmonella* infection.
6. Identification of the cross-reactive IgG and IgA SEnGMMA and STmGMMA specific antigen following *S.* Typhi and *S.* Paratyphi infection.
7. Identify protein antigens in the GMMA which may contribute to the direct and cross-reactive antibody response.

## 6.2 Methods

See *Chapter 2 Material and Methods* for further details on the GMMA-based ELISAs (*Chapter 2.6*), ADMP (*Chapter 2.8*), and ADNKA (*Chapter 2.9*) assays.

### 6.2.1 *Salmonella* Challenge Studies (VAST, PATCH, P1)

Serum samples from *S*.Typhi (n = 24 ) [VAST[83]] and *S*.Paratyphi (n = 24) [PATCH[155], P1[154]] controlled human infection studies were tested across ELISAs and functional antibody assays; including at D0 (day of the challenge) and D28 following microbiological diagnosis of *Salmonella* infection.

### 6.2.2 Severe Typhoid Fever Surveillance in Africa (SETA) Study

A subset of samples (n=20) from the SETA[151] study collected as a sub-study to investigate the seroepidemiology of invasive non-typhoid *Salmonella* were tested across ELISAs and functional antibody assays. Samples were collected across multiple time points following initial diagnostic bacteraemia, then at D14, 1, 3 and 6 months.

### 6.2.3 Protein Enzyme-Linked Immunosorbent Assay

For further details on selected proteins and production, see *Chapter 5.3.9* and *Chapter 2.4*. The protein ELISAs were performed as per the GMMA-based ELISAs, except the Nunc Maxisorp™ 96-well flat-bottom plates (*Thermofisher Scientific, Massachusetts, USA;* 243656*)* were coated overnight with 50µL per well of 2µg/mL protein (OmpA, OmpC, OmpD, OmpF, SlyB, FkpA, Flag, LptS, YnCE, HlyE) in carbonate-bicarbonate buffer at pH 9.6 *(Sigma-Aldrich, Dorset, UK;* C3041*)*.

Pooled *Salmonella* sp. convalescent sera (used as a serum standard across all assays in this thesis) demonstrated reactivity with all tested proteins coated at 2µg/mL (figure 69). *Salmonella* sera demonstrated lower OD450 values for all single proteins compared with SEn and STm GMMA responses. Antibodies binding to HlyE were the highest of the single proteins. The remaining protein antibody levels failed to reach the coated proteins' saturation point, confirming fewer antibodies in sera than for HlyE and GMMA.

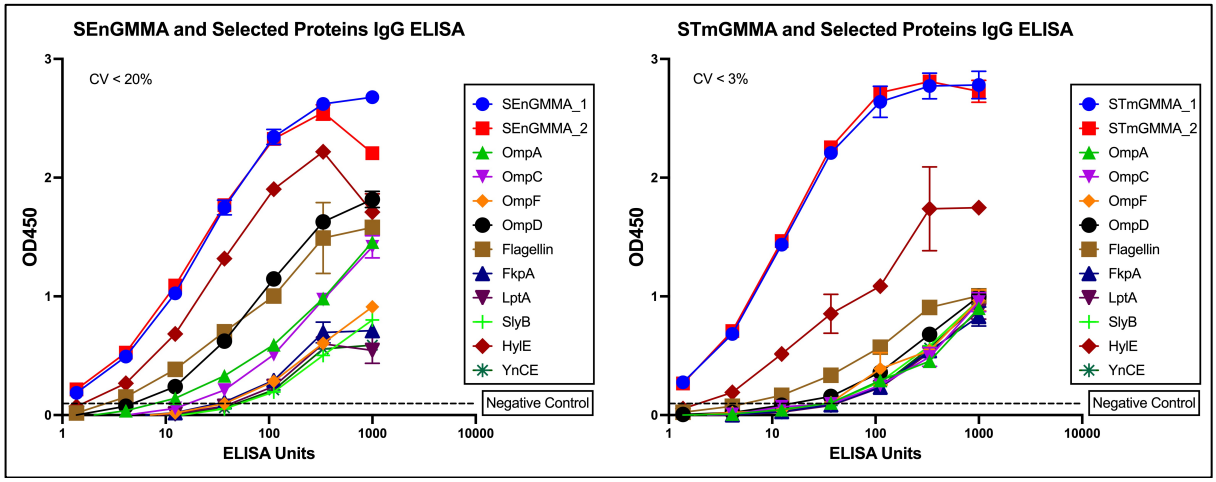



*Figure 69 Salmonella sp. pooled convalescent sera tested in serial dilutions across SEnGMMA and the ten selected protein ELISAs (L) and STm GMMA and the ten selected protein ELISAs (R)*

## 6.2.4 O-Ag Pre-adsorption Assay

Serum samples were diluted in 1% BSA (*Sigma-Aldrich, Dorset, UK;* A7030) in PBS (*Sigma-Aldrich, Dorset, UK;* P2272) at 1:500 and incubated for 1 hour at 25°C with serial dilutions of either SEn O:4, 5 antigens or STm O:9 antigen between 100µg/mL and 0.1 µg/mL similarly to Mohamed et al(2020)[284]. The assay was performed as per the SALVO GMMA ELISA except for the addition of Serum: O-antigen sample at the serum sample addition step.

### 6.2.5 Statistical Analyses

Paired T-test, Spearman Correlation, and Linear Regression, including visualisation, were performed using Prism (*Version 10, Graphpad™ Software, Boston, USA*) and R *(R Core Team, Vienna, Austria)*[149] / R Studio *(Version 2023.03.0+386, RStudio Team, Boston, USA)*[150].

Statistical analysis for the ELISA and ADMP was performed using a t-test. For the GMMA-specific ADMP, this was a sample mean comparison of median fluorescent intensity across paired D0 and D28 sera. For the O-antigen-bead ADMP, this was a sample mean comparison of phagocytic score across paired D0 and D28 sera. Statistical analysis for the ADNKA was performed using a Wilcoxon signed rank test, comparing D0 and D28 (following infection) paired samples.

## 6.3 Results

### 6.3.1 Characterisation of the *Salmonella* sp. stimulated IgG Antibody Response to GMMA-Specific Antigens

#### Natural infection by *Salmonella* spp. elicits antibodies that bind to SEn and STm GMMA *in vitro*

SEn / STmGMMA-specific IgG ELISAs were performed on convalescent sera from children presenting to medical units in sub-Saharan Africa at day 14 – 6 months following *Salmonella* bacteraemia [Burkina Faso and Ghana; Appendix D, *Demographics of SETA*[151] *study samples* with laboratory-confirmed *Salmonella* spp. bacteraemia (iNTS)]. This demonstrated that antibodies that bind to GMMAs are generated following an iNTS and *S*.Typhi infection. The highest responses were detected at D14 post-laboratory-confirmed bacteraemia and subsequently waned over six months (figures 70 and 71). In addition, antibody responses against SEn / STmGMMAs were seen after infection with each of *S*. Typhi, *S*. Typhimurium and *S*. Enteritidis. Of note, convalescent *S.* Typhi sera had the highest response to both GMMAs. Finally, the GMMA-specific antibody response positively correlated with the respective O antigen-specific IgG (SEn, O:9; STm O:4, 5) – O-Antigen ELISA performed by collaborator Sean Elias (both unpublished and published data[98]) [figure 72].

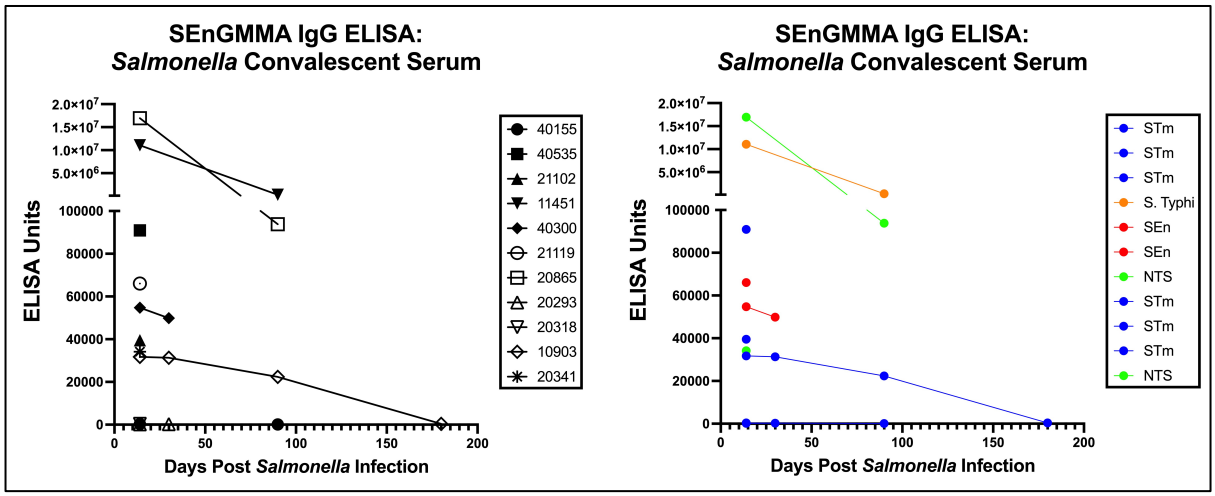



*Figure 70 iNTS convalescent sera (SETA Study) tested in the SEnGMMA ELISA: by laboratory ID [demographic characteristics of human participants in Appendix D] (L); by specified infecting Salmonella serovar (R)*

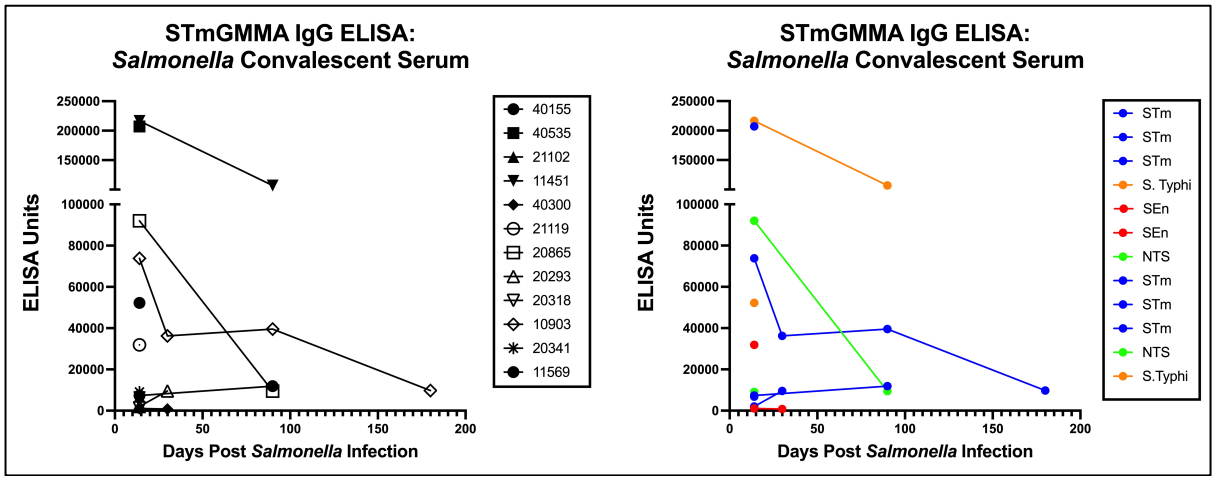



*Figure 71 iNTS convalescent sera (SETA Study) tested in the STmGMMA ELISA: by laboratory ID [demographic characteristics of human participants in Appendix D] (L); by specified Salmonella sera (R)*

## IgG Antibody Responses to SEnGMMA and STmGMMA Correlate with SEn (O:9) and STm (O:4, 5) (ELISA)

Both SEn GMMA(r = 0.810, p=<0.001) and STm GMMA (r = 0.8361, p=<0.001) positively correlate with OAg IgG ELISA (Spearman correlation analysis), suggesting that O-antigen

may be a significant component of the GMMA binding antibodies generated following natural infection by *Salmonella* spp (figure 72).

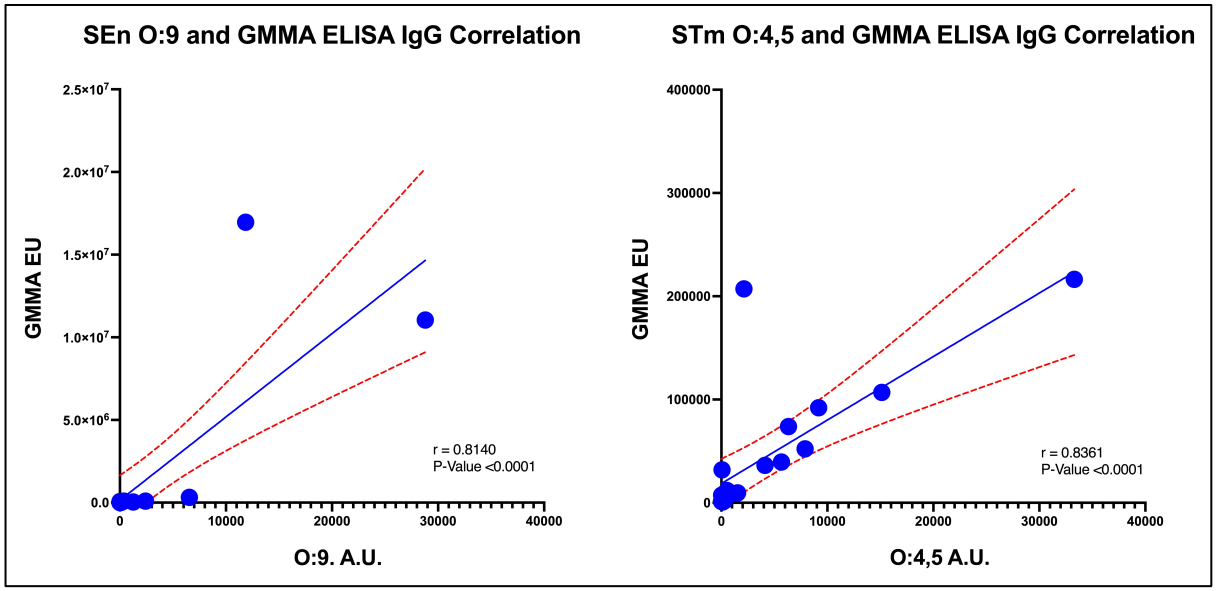



*Figure 72 Correlation of SEn O:9 Antigen and SEn GMMA IgG ELISA (L) and STm (0:4,5) Antigen and STm GMMA IgG ELISA (R); Spearman correlation analysis between corresponding SEn (0:9), STm (O:4, 5) Arbitrary Units (A.U.) and GMMA ELISA Units (EU).*

## Infection by *S.* Typhi elicits antibodies that bind to SEn / STm GMMA *in vitro*

To investigate cross-reactivity to the GMMAs, sera collected following infection by *S*. Typhi (VAST, controlled human infection study) was tested in the IgG GMMA-based ELISAs. A significant increase in SEnGMMA-specific (D0: GM 565.4 [138.8-2303]; D28: 7789 [3927-15451]; p=0.010) and STmGMMA-specific (D0: 1092 [607.7-1961]; D28: 5759 [2137-15523]; p=0.01) binding antibodies was seen at day 28 (post microbiological enteric fever diagnosis) compared with baseline (day 0, pre-*S.* Typhi challenge) in the paired samples tested (t-test, comparison of sample means; SEnGMMA, n=12; STmGMMA, n=8) [figure 73].

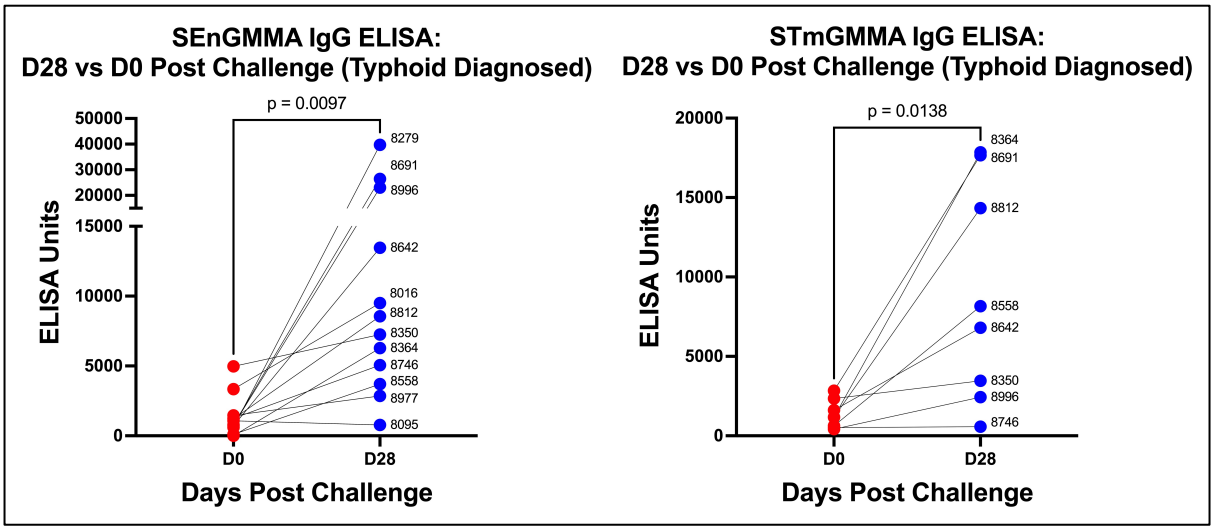



*Figure 73 IgG GMMA cross-reactive antibodies at baseline (pre-challenge, D0) and following S.Typhi infection (D28) in human adult participants, SEnGMMA (L) and STmGMMA (R); Statistical test = Paired t-test of D0 (pre-challenge) versus D28 (post S.Typhi infection)*

## Infection by *S.* Paratyphi A elicits antibodies that bind to STm / SEn GMMA *in vitro*

Following the demonstration of generated cross-reactive antibodies to SEn / STm GMMA following *S.*Typhi infection, sera obtained following *S.* Paratyphi A infection (PATCH / P1 controlled human infection studies) were tested in the GMMA-specific IgG ELISAs.

A significant increase in SEnGMMA-specific (D0: GM 1672 [1019-2745]; D28: 4959 [2396-10263]; p=0.004) and STmGMMA-specific (D0: 2962 [1924-4561]; D28: 11302 [4736-26969]; p=0.006) binding antibodies was seen at day 28 (post microbiological enteric fever diagnosis) compared with baseline (day 0, pre-*S.* Paratyphi A challenge) in the paired samples tested (t-test, comparison of sample means; SEnGMMA, n=12; STmGMMA, n=12) [figure 74].

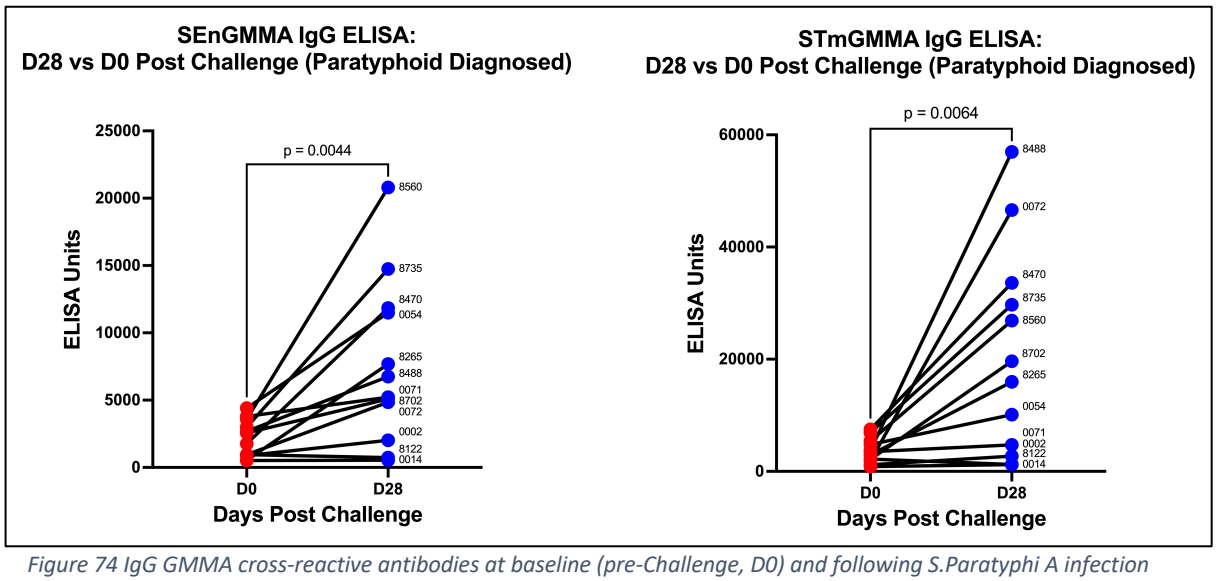



*Figure 74 IgG GMMA cross-reactive antibodies at baseline (pre-Challenge, D0) and following S.Paratyphi A infection (D28) in human adult participants, SEnGMMA (L) and STmGMMA (R); Statistical Test = Paired t-test of D0 (pre-challenge) versus D28 (post S .Paratyphi A infection)*

### 6.3.2 Functional activity of the *Salmonella* sp. stimulated Antibody Response GMMA and O-Antigen

SEn/ STm GMMA binding IgG levels increase following iNTS, *S*. Typhi and *S*. Paratyphi A infection and after vaccination with the iNTS-GMMA vaccine (See *Chapter 4 Section 3.1*). To identify whether these cross-reactive antibodies are functional and could contribute to protection across *Salmonella* spp., the sample sets were tested using antibody-dependent monocyte phagocytosis (ADMP) and antibody-dependent NK activation (ADNKA) assays.

### 6.3.3 GMMA-based and OAg Bead-based Antibody-Dependent Monocyte Phagocytosis (ADMP) Assays

#### *Salmonella* spp. Generate Functional Antibody to GMMA Following Human Infection

Sera obtained from children following natural *Salmonella* infection demonstrated functional antibodies as measured using a GMMA-based ADMP assay (figures 75 and 76). Antibody-dependent phagocytosis of the GMMA was highest at D14 post bacteraemia and waned after that for the individuals with sera collected at later time points. All serovars [SEn, STm, *S.*Typhi, NTS (unknown)] were seen to generate NTS GMMA-specific and cross-functional antibodies following infection.

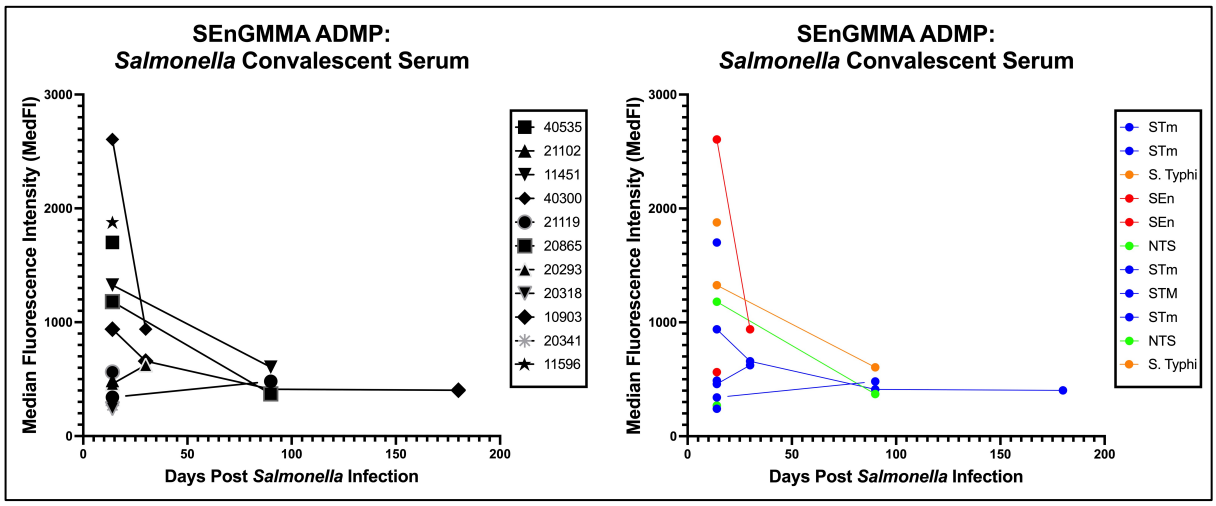



*Figure 75 Antibodies from iNTS convalescent sera (SETA Study) against the SEnGMMA ADMP: by laboratory ID [demographic characteristics of human participants in Appendix D] (L); by specified Salmonella sera (R)*

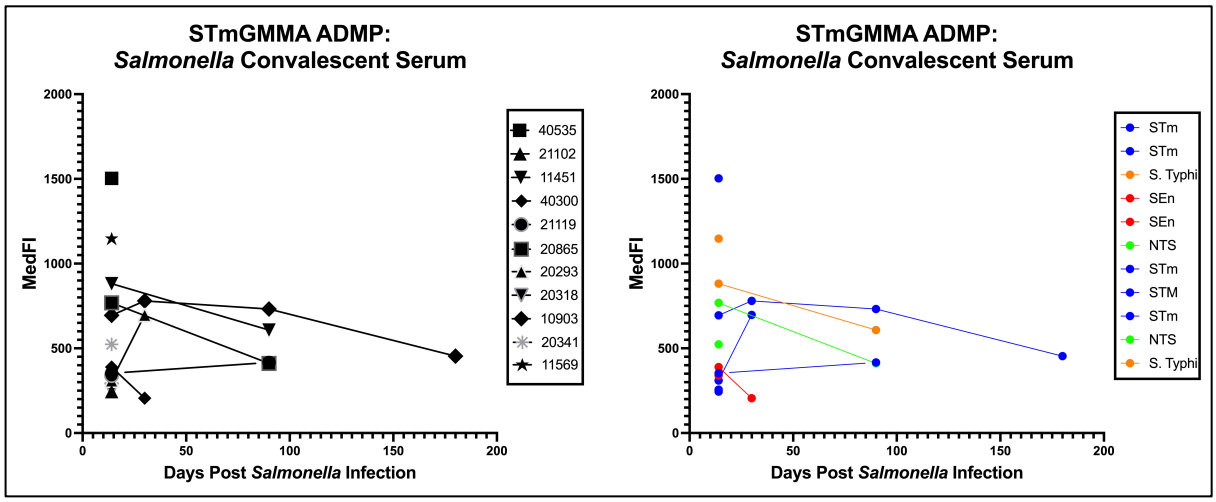



*Figure 76 Antibodies from iNTS convalescent sera (SETA Study) against the STmGMMA ADMP: by laboratory ID [demographic characteristics of human participants in Appendix D] (L); by specified Salmonella sera (R)*

# *Salmonella* Typhi Generates Functional Antibody to GMMA-Specific Antigen and O-specific Antigen Following Human Infection

A significant increase in antibody-dependent phagocytosis of the SEnGMMAs was seen *in vitro* at D28 (figure 77) after a confirmed *S.*Typhi infection, compared with D0, pre-*S.*Typhi challenge)[D0: 414.4 [245.9-698.4]; D28: 640.6 [548.1-748.6]; p=0.048]. In addition, a non-significant increase in phagocytic activity of sera at D28 post-infection (D0: 566 [417.7-767.1]; D28: 1026 [712.0-1480]; p=0.06) as measured using SEn O-Antigen conjugated beads was observed, compared with baseline (figure 77).

In comparison, a non-significant increase in antibody-dependent phagocytosis of the STmGMMAs was seen *in vitro* at D28 (figure 77) after a confirmed *S.*Typhi infection, compared with baseline (D0, pre-*S.*Typhi challenge)[D0: 724.6 [690.6-760.5]; D28: 810.5 [729.5-900.4]; p=0.10]. However, a significant increase in phagocytic activity of sera at D28 post-infection (D0: 549.7 [405.5-745.2]; D28: 775.6 [426.3-1411]; p=0.03) as measured using STm O-Antigen conjugated beads was observed, compared with baseline.

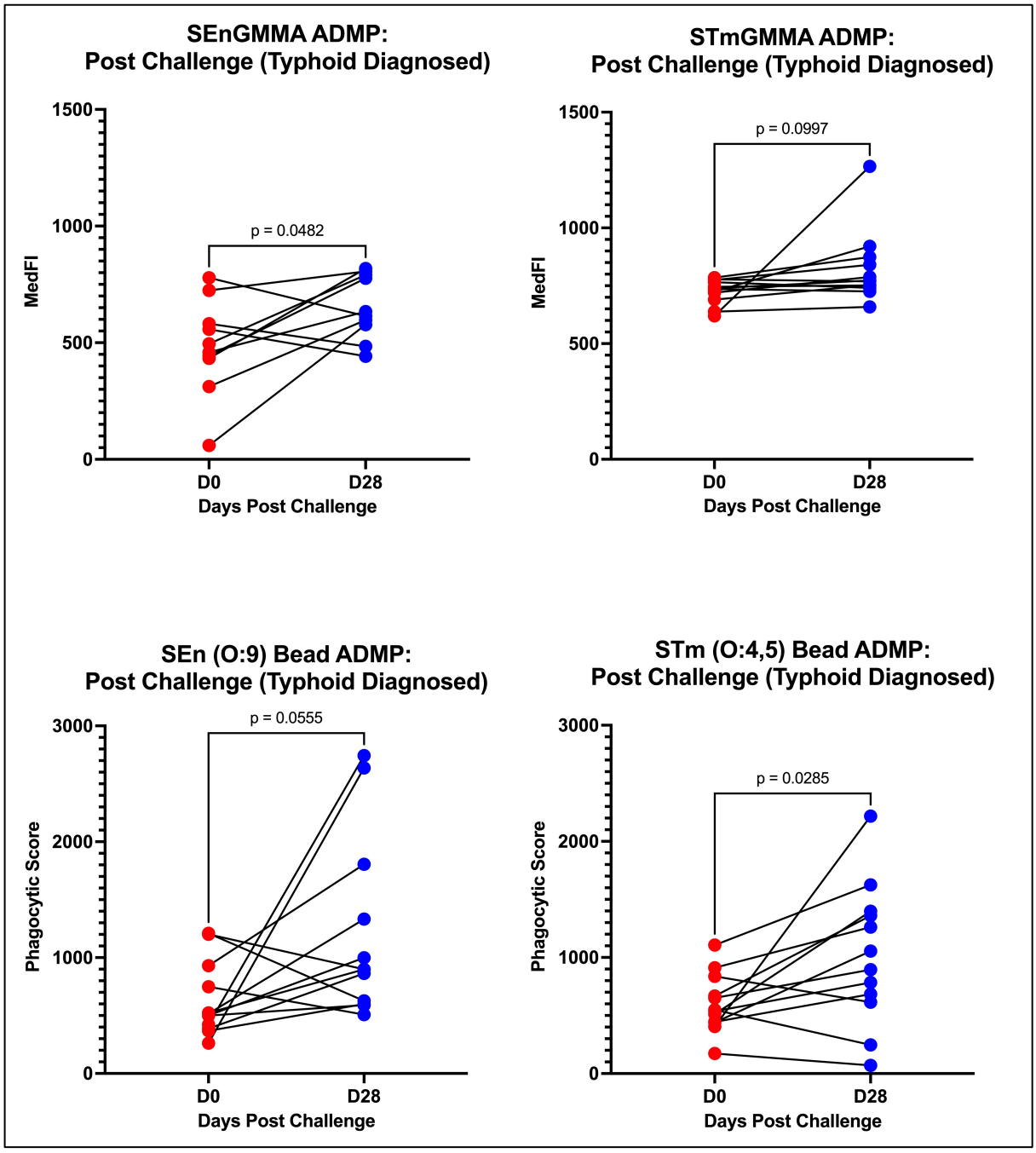



*Figure 77 Demonstration of functional antibodies generated 28 days following S.Typhi infection in human participants compared with baseline (pre-challenge, D0) to SEnGMMA, STmGMMA, SEn (O:9) and STm (O:4 5) measured using GMMA and bead-based ADMP assays. Statistical test = Paired t-test comparing sera at D0 (pre-challenge) and D28 (following S.Typhi infection). NB: GMMA y-axis MedFI (Median Fluorescent Intensity) and Bead y-axis phagocytic score (this difference is based on assay parameters as discussed in Chapter 2.8)*

# *Salmonella* Paratyphi A Generates Functional Antibody to GMMA-Specific Antigen O-specific Antigen Following Human Infection

A significant increase in antibody-dependent phagocytosis of SEnGMMA (D0: 499.8 [444.0-562.7]; D28: 692.9 [583.9-822.3]; p=0.02), SEn OAg (D0: 633.7 [504.1-796.7]; D28: 1060 [683.4-1645]; p=0.04) and STm OAg (D0: 859.3 [762.7-968.2]; D28: 1273 [875.1-1851]; p=0.03) was seen in sera obtained from D28 after a confirmed *S.*Paratyphi A infection, compared with baseline (D0). However, a non-significant increase in phagocytosis of the STmGMMA from the same sera 28 days following a *S.*Paratyphi A infection was seen, compared with baseline (D0: 738.0 [712.3-764.6]; D28: 813.9 [736.9-898.9]; p =0.07) [figure 78]. Statistical analysis was performed as in section 6.2.5.

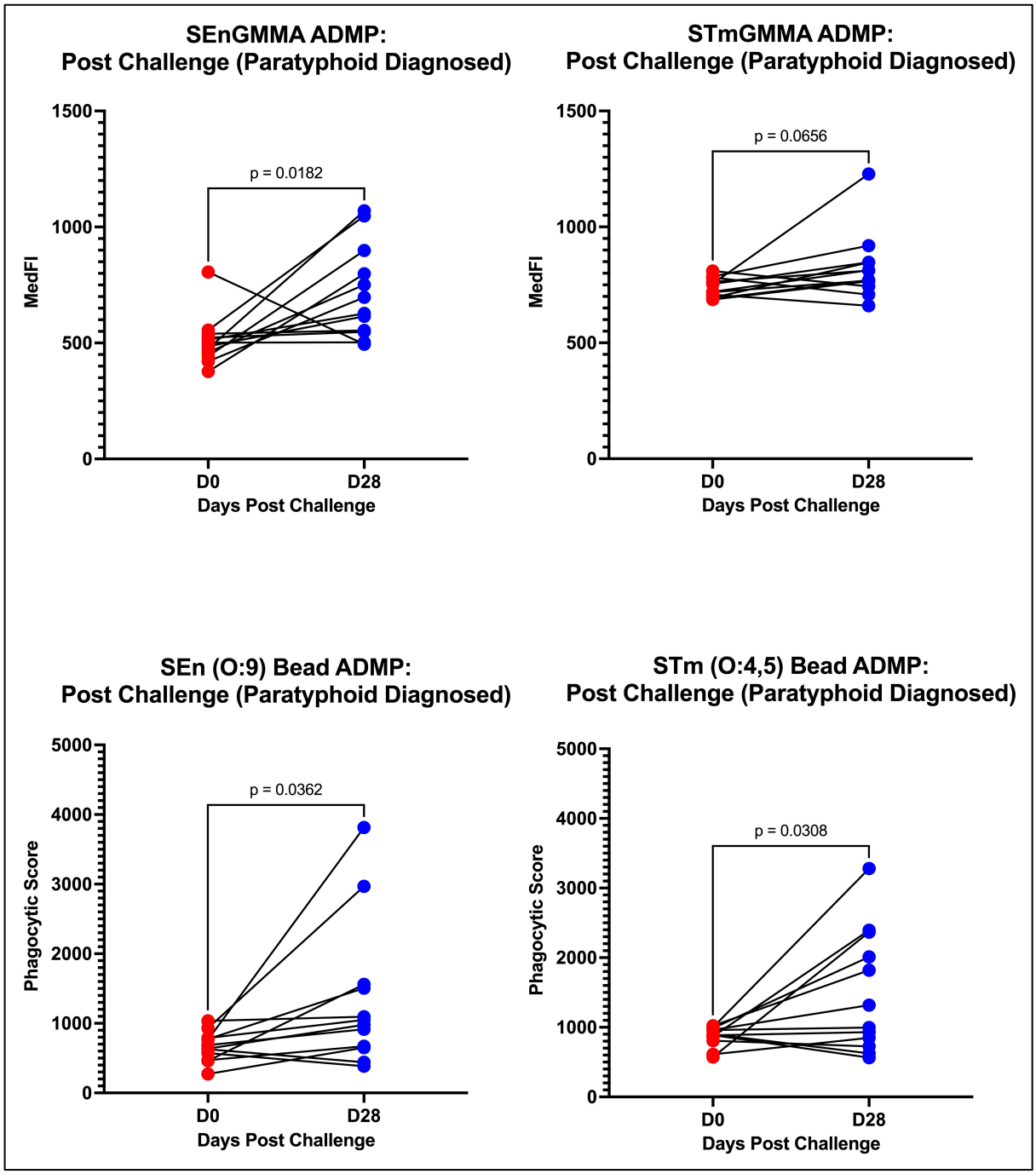



*Figure 78 Demonstration of functional antibodies generated 28 days following S.Paratyphi A infection in human participants compared with baseline (pre-challenge, D0) to SEnGMMA, STmGMMA, SEn (O:9) and STm (O:4 5) measured using GMMA and bead-based ADMP assays. Statistical test = Paired t-test, comparing sera at D0 (pre-challenge) and D28 (following S.Paratyphi A infection). NB: GMMA y-axis MedFI (Median Fluorescent Intensity) and Bead y-axis phagocytic score (this difference is based on assay parameters as discussed in Chapter 2.8)*

## 6.3.4 iNTS-GMMA Vaccine Stimulates Functional Cross-Reactive Antibody to *Salmonella* Paratyphi A

*Chapter 4.3.2* demonstrates that the iNTS-GMMA vaccine induces functional antibodies to the SEn / STm GMMA. *Chapter 6.3.3 and 6.3.3* establish that antibodies generated following infection with *S.*Typhi and *S.*Paratyphi A infection are functionally active against the GMMAs *in vitro*. The next step was establishing whether the iNTS-GMMA vaccine might stimulate a functional antibody response to other serovars beyond SEn and STm. To investigate this, a SALVO sample subset (full dose, n=12; lower dose, n=3; and placebo, n=2) consisting of paired baseline and D28 following vaccination or placebo was tested on an optimised *S.* Paratyphi A bacterial-based ADMP assay. A significant increase in phagocytosis of *S.* Paratyphi bacteria was observed at D28 from sera following full dose (D0: 3114 [2077-4668]; D28: 8627 [5673-13118]; p=0.02) and lower dose (D0: 2680 [ 1214-5915]; D28: 13559 [5775-31836]; p=0.0497) vaccination but not placebo (D0: 3190 [0.2300-44241630]; D28: 2613 [0.0009438-7234089757]; p=0.75). Statistical analysis was performed using a t-test, comparing means of D0 and D28 paired samples for full dose, lower dose vaccination and placebo (figure 79).

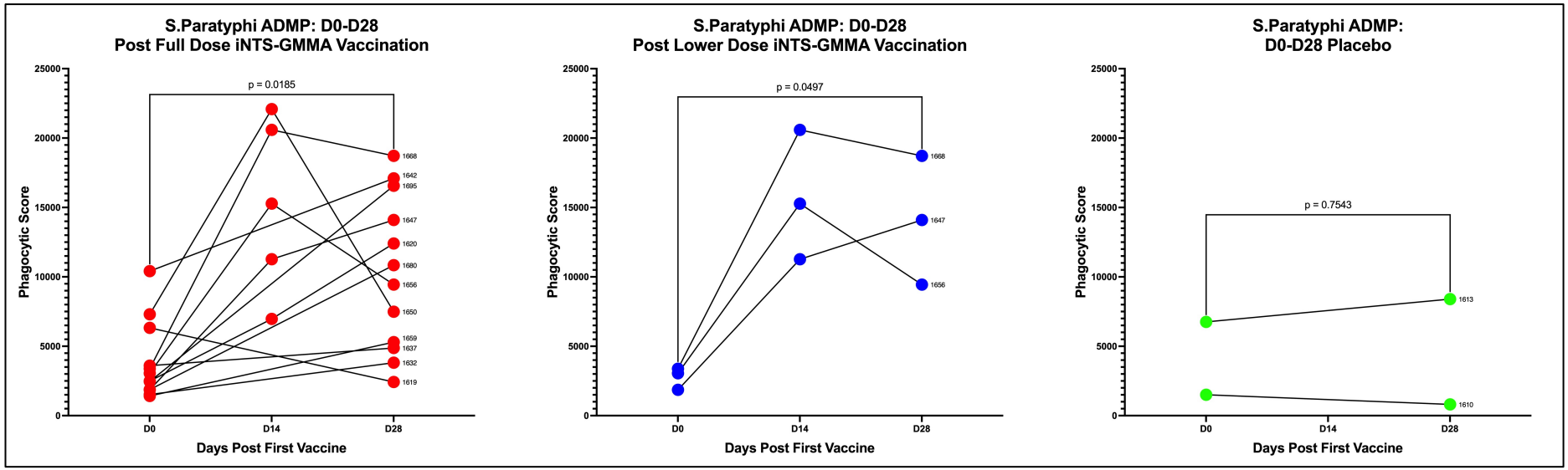



*Figure 79 iNTS-GMMA vaccine generates functional antibody against S.Paratyphi A in human adult participants from Day 0 (baseline) to D28 (following active vaccines or placebo) measured using a whole bacteria ADMP. (Statistical pest = Paired t-test, comparing sera at D0 (pre-vaccination) and D28 (following vaccination) [Conception, sample selection and preparation including dilution, data analysis performed by B. Hanumunthadu; S.Paratyphi ADMP developed and performed by E. Jones]*

### 6.3.5 Antibody-Dependent NK Activation Assay (ADNKA)

#### Non-Typhoid *Salmonella* and S. Paratyphi A Generates Functional Antibody-Dependent NK Activity to SEn / STm GMMA Following Human Infection

Elevated antibody-dependent NK activity above the negative control, as measured by upregulation of CD107a, in response to the SEn / STmGMMAs, was seen with sera tested from 14 days following initial *Salmonella* sp. bacteraemia (figure 80 a, b, e and f).

Sera 28 days following confirmed *S.* Paratyphi A infection significantly increased antibody-dependent NK activity compared with baseline in response to SEnGMMA (D0: GM 5.617 [3.255-9.692]; D28: GM 12.13 [8.082-18.20]; p=0.04) and STmGMMA (D0: GM 3.113 [2.650-3.658]; D28: GM 6.717 [3.926-11.49]; p=0.002) [figure 80].

#### No detectable Functional Antibody-Dependent NK Activity to SEn / STm GMMA Following Human *S.* Typhi Infection

Sera from 28 days following confirmed *S.* Typhi infection showed no significant antibody-dependent NK activity compared with baseline in response to SEnGMMA (D0: GM 1.404

[0.1475-13.36]; D28: GM 2.094 [0.3804-11.52]; p=0.84) and STmGMMA (D0: 3.739 [2.311-6.049]; D28: GM 5.442 [2.795-10.59]; p=0.56) [figure 80c and g].

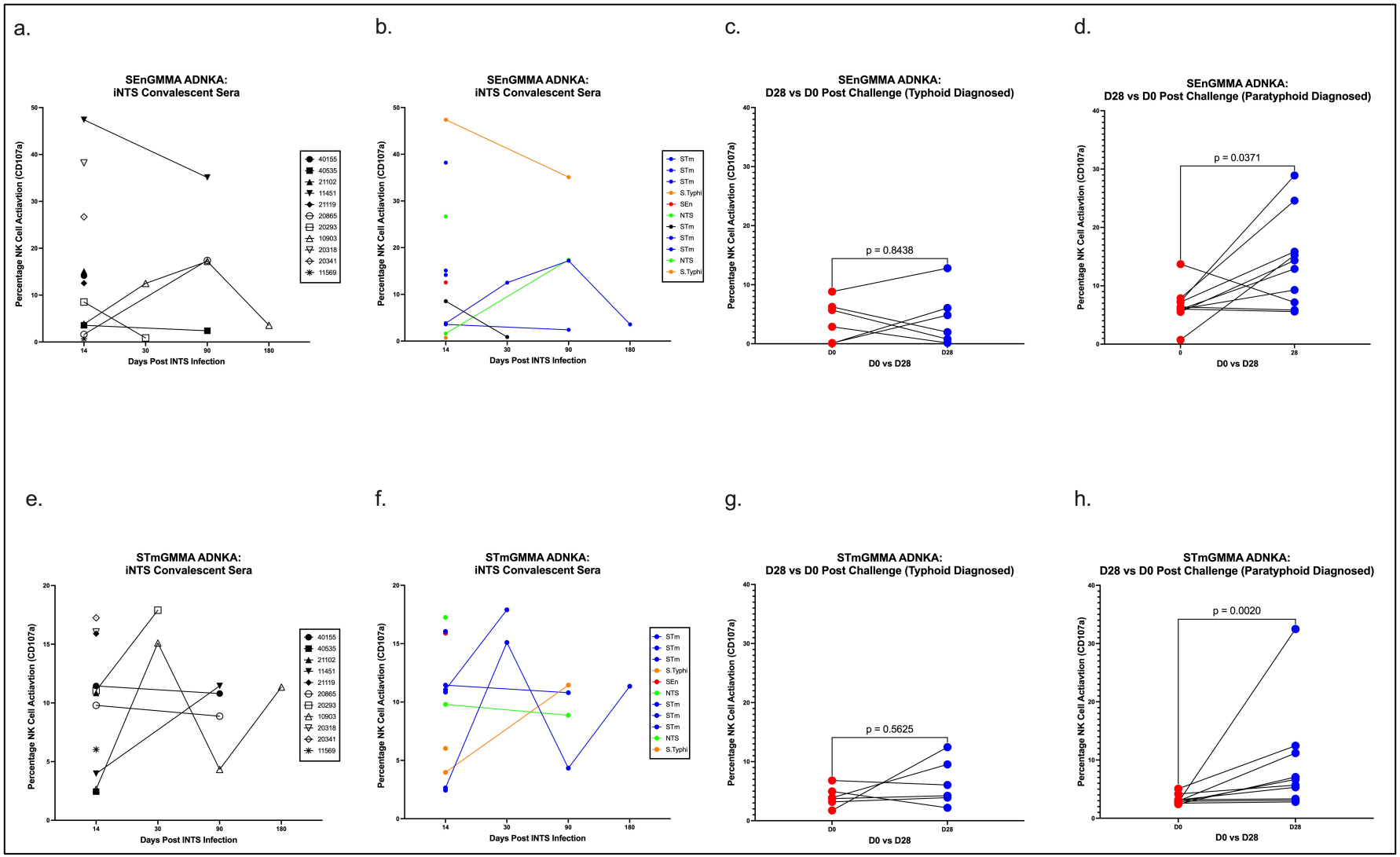



*Figure 80 SEnGMMA and STmGMMA functional antibodies identified in human iNTS convalescent sera [Lab ID (a, e), specified Salmonella sera (b, f)], following S.Typhi infection (c, g) and S.Paratyphi A infection (d, h) as measured by a SEn/STm-GMMA specific ADNKA assays. Wilcoxon Test was used to compare the significance in NK Cell CD107a Percentage from Day 0 (pre-challenge) to Day 28 (post S. Typhi or S.Paratyphi A challenge). Statistical tests were not performed for the iNTS convalescent sera due to unavailability of baseline sera as a result of the original study design*

### 6.3.6 O Antigen is the immunodominant and primary cross-reactive antibody target in both SEnGMMA and STmGMMA

#### OAg Pre-adsorption Assay

A series of pre-adsorption assays were established to determine whether O-antigen generated, or alternative binding antibodies are either immunodominant or the primary cross-reactive antibody. Convalescent NTS (day 14-90 post-infection), post-challenge *S.* Typhi (day 28 post-infection), *S.* Paratyphi A (day 28 post-infection) and full dose iNTS-GMMA vaccine sera (day 28 post-vaccination) were pre-adsorbed with serial dilutions from 0.1 to 100µg/mL of either SEn O-Antigen (O:9) or STm O-Antigen (O:4, 5) before testing in the SEnGMMA and STmGMMA IgG and IgA ELISAs.

#### Comparison of response at D28 post-*Salmonella* spp infection and iNTS-GMMA vaccination)

Anti-SEnGMMA IgG levels were similar for both *S.*Typhi and *S.* Paratyphi A post-infection sera to the NTS convalescent samples (IgG: *S.*Typhi, p>0.99; *S.* Paratyphi A, p=0.82) and samples tested from iNTS-GMMA vaccination (SEnGMMA: *S.*Typhi, p=0.22; *S.* Paratyphi A, p=0.58). However, the anti-STmGMMA IgG levels for both *S.*Typhi and *S.* Paratyphi post-infection sera were significantly lower than convalescent NTS sera (*S.*Typhi, p=0.004; *S.* Paratyphi A, p=0.008) and sera from iNTS-GMMA vaccine recipients (SEnGMMA: *S.*Typhi,

p=0.22; *S.* Paratyphi A, p=0.58; STmGMMA: *S.*Typhi, p=0.004; *S.* Paratyphi A, p=0.007) [figure 81].

## OAg is the immunodominant antigen following iNTS-GMMA Vaccination

To investigate the relative contribution of OAg to the immune response, antibody responses were measured in sera following OAg pre-adsorption. When sera from day 28 post-full dose iNTS-GMMA vaccination were pre-adsorbed with SEn OAg (O:9) at the lowest OAg concentration an almost complete blocking of SEnGMMA IgG and IgA binding was observed (IgG: 0.1 µg/mL, p=0.0708, IgA: 0.1 µg/mL, p<0.001). Complete or near complete blocking was observed for all OAg pre-adsorption concentrations tested. In contrast, pre-adsorbed sera exhibited incomplete blocking of STmGMMA IgG and IgA at the lowest STm OAg (O:4,5) concentration tested (IgG: 0.1 µg/mL, p=0.33, IgA: 0.1 µg/mL, p=0.71) and significant partial blocking only at the highest concentration tested (IgG: 0.1 µg/mL, p=0.006, IgA: 0.1 µg/mL, p<0.001) compared with no OAg pre-adsorption [figure 81 d, e, I and j]. Antibodies to SEn / STmGMMA plateaued at 1-10 µg/mL, above the negative control, with no further pre-adsorption of antibodies despite increase pre-adsorption antigen, suggesting other antigen-specific antibodies contribute to a polyclonal response, which is more evident for STmGMMA antibodies (IgG SEnGMMA vs STmGMMA fold change difference 0 to 100µg/mL: -0.5651, p=0.16; IgA SEnGMMA vs STmGMMA fold change difference 0 to 100µg/mL: -0.7133, p=0.02).

## OAg is the immunodominant antigen following iNTS infection

Similarly, SEnGMMA IgG responses in convalescent NTS sera were significantly blocked by pre-adsorption with 0.1 µg/mL to 10 µg/mL SEn OAg (p=0.001). STmGMMA IgG within convalescent NTS sera was significantly blocked from 0.1 µg/mL to 10 µg/mL STm OAg (p=0.02). As for the full dose iNTS-GMMA vaccine, OAg only exhibited partial blocking of STmGMMA IgG (figure 81f, i, and j). O-antigen is the major antibody target following NTS natural infection that is present in the SEnGMMA and STmGMMA and is the primary induced antibody target by the iNTS-GMMA vaccine.

## OAg is a major cross-reactive antigen following *S.* Typhi and *S.* Paratyphi A infection

Sera from 28 days following *S.* Paratyphi A infection exhibited a significant decrease in SEnGMMA and STmGMMA IgG with corresponding SEn and STm OAg pre-adsorption concentration increase from 0.1 (SEnGMMA, p=0.36; STmGMMA, p=>0.99) to 10 µg/mL (SEnGMMA, p<0.001; STmGMMA, p<0.001) [figure 81c and h] compared with no OAg pre-adsorption. However, this reduction of GMMA-specific IgG remained above the negative control for the final OAg concentration (10µg/mL), suggesting other less prominent antigens may contribute as antibody targets. Sera from 28 days following S.Typhi infection exhibited a significant decrease in SEn / STmGMMA IgG with corresponding SEn and STm OAg pre-adsorption concentration increase from 0.1 (SEnGMMA, p=0.10; STmGMMA, p=0.74) to 10 µg/mL (SEnGMMA, p=0.02; STmGMMA, p=<0.001)[figure 81b and g] compared with no OAg pre-adsorption. Overall, OAg is the major S*almonella* sp. cross-

reactive antigen in both GMMAs. However, there may be other significant antigens, particularly within SEnGMMA and to *S.* Typhi convalescent sera.

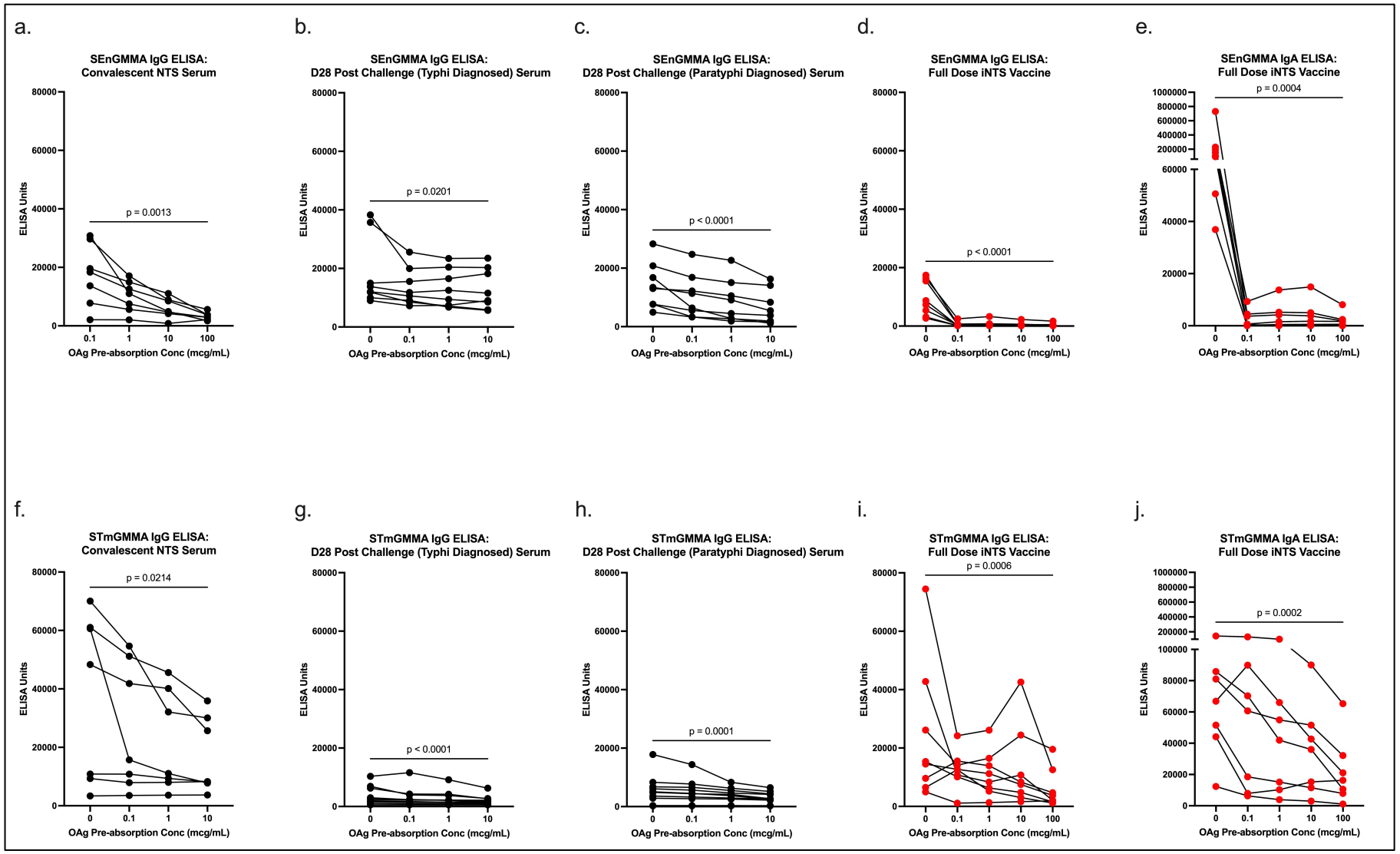



*Figure 81 GMMA-based O-Antigen pre-adsorption assay. Serial dilutions of SEn and STm O-antigen (0.1-100µg/mL) IgG pre-adsorbed with convalescent iNTS Sera (a, f); S.Typhi infection sera (b, g); S.Paratyphi A infection sera (c, h); full dose iNTS-GMMA Vaccine IgG (d, i) and IgA (e,j) tested for IgG and IgA GMMA antibodies. Statistical test: Friedman Test with Dunn Multiple Comparisons Test from 0-0.1 µg/mL to 10-100 µg/mL OAg*

### 6.3.7 GMMA Protein Antigens Contribute to the Primary and Cross-reactive Antibodies

To test the immunogenicity of the 8 proteins selected following proteomic analysis of the GMMAs (Table 38, *Chapter 5*) and determine whether these could contribute to the primary or cross-reactive polyclonal antibody response to the GMMAs and *Salmonella* sp., the protein antigens were tested in a series of protein ELISAs with sera following NTS, *S.*Typhi, *S.*Paratyphi infection and iNTS-GMMA vaccination.

All proteins exhibited binding of antibodies above the negative control from sera obtained following infection with NTS (D14)*, S.* Typhi (D28)*,* and *S.* Paratyphi (D28). No significant difference was seen between sera post-infection from NTS, *S.* Typhi and *S.* Paratyphi (OmpA p=0.79; OmpC, p=0.86; OmpD, p=0.74; OmpF, p=0.76; SlyB, p=0.79; FkpA, p=0.79; Flag, p=0.93; LptA, p=0.71; YnCE, p=0.55; HlyE, p=0.76). However, baseline serum was not tested, and evidence of pre-existing antibodies could not be established (figure 82).

All proteins exhibited binding of antibodies at baseline (above negative control) prior to administration of vaccine or placebo. No significant difference in baseline antibody was seen across all proteins (p=0.40) and between full dose, lower dose vaccines or placebo across individual proteins (OmpA p=0.60; OmpC, p=0.59; OmpD, p=0.18; OmpF, p=0.21;

SlyB, p=0.52; FkpA, p=0.54; Flag, p=0.42; LptA, p=0.48; YnCE, p=0.45; HlyE, p=0.17)[figure 83].

No significance difference between D28 post full dose iNTS-GMMA administration compared with baseline, (OmpA, GM OD450 0.6928 [0.3820-1.256], p=0.08; OmpC, GM 0.6940 [0.3998-1.205], p=0.04; OmpD, GM 0.8525 [0.6200-1.172], p=0.34; OmpF, GM 0.4149 [0.1996-0.8627], p=0.16; SlyB, GM 0.4205 [0.1843-0.9591], p=0.10; FkpA, GM 0.7107 [0.3696-1.367], p=0.13; Flag, GM 0.5025 [0.2528-0.9986], p=0.56; LptA, GM 0.5669 [0.2719-1.182], p=0.07; YnCE, GM 0.6536 [0.3095-1.380], p=0.07; HlyE, GM 0.8381 [0.5832-1.205], p=0.12) except for OmpC. Only OmpC demonstrated a significant increase in antibody level at D28 compared with baseline following full dose iNTS-GMMA vaccination. However, although not significant, most proteins exhibited a rise in antibody levels in 3-4 participant samples, with a large variation within the full dose-iNTS vaccination sample sets [figures 83 and 84].

There was no significant difference in fold change from day 0 to day 28 between proteins (full dose iNTS-GMMA, p=0.52, ANOVA), demonstrating minimal variation between proteins, with individual samples having similar antibody levels across proteins. If an individual sample exhibited a high response to one protein antigen, the remaining proteins did also exhibit high antibody responses.




*Figure 82 Heatmap showing protein IgG levels across the selected ten proteins following iNTS-GMMA vaccination (full dose: 1696, 1636; lower dose: 1668, 1656, 1647; placebo: 1678), and iNTS, S.Typhi, and S.Paratyphi Infection as measured by Log EU at D0 (pre- vaccination) and D28 (following infection or vaccination); except for iNTS convalescent sera including D14 and D180 following bacteraemia only*




*Figure 83 Heatmap showing protein IgG levels across the selected ten proteins following lower dose (n=3), full dose (n=10) iNTS-GMMA vaccine and placebo (n=4) as measured by OD450 at D0 (pre- vaccination) and D28 (following vaccination)*

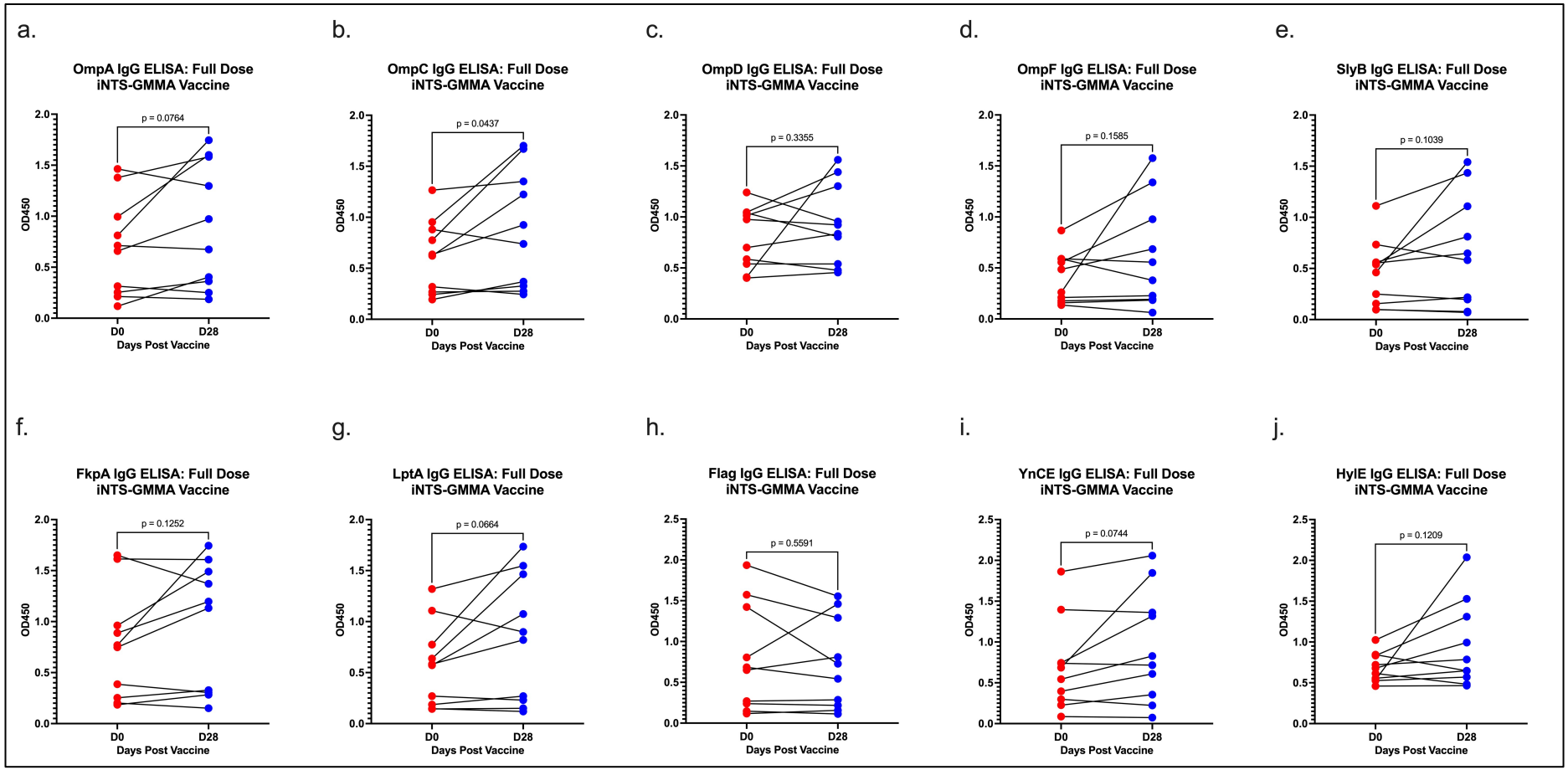



*Figure 84 Protein IgG antibody response at baseline (pre-vaccination) and at 28 days following Full Dose iNTS-GMMA vaccination (D28); Of the ten selected proteins, only OmpC shows a significant increase in antibody levels following vaccination. Statistical test = Paired t-test, comparing sera at D0 (pre-vaccination) and D28 (following full dose iNTS-GMMA vaccination).*

## 6.4 Discussion

### 6.4.1 Antibodies with functional activity toward SEn and STm GMMA are generated following NTS natural infection in children

Natural SEn and STm infection in children from sub-Saharan Africa elicited both binding and functional antibody responses by GMMA IgG ELISA and ADMP. Whilst it has been demonstrated that an increased serum bactericidal activity following *Salmonella* Typhimurium infection coincides with a reduction in cases of invasive non-typhoid *Salmonella*[46], investigation of broader functional responses is yet to be undertaken. The opsonophagocytic response to the GMMAs (as a surrogate of *Salmonella* infection) suggests a combined functional response (including serum bactericidal activity) may contribute to the immune response following natural infection in the under-five age group. Data from a greater number of samples, including longitudinal samples are required to assess the extent of the wider functional response and its duration. Field or challenge studies are needed to identify correlates of protection, including whether these functional responses contribute to protection against *Salmonella* infection.

Variation in ages in the under-fives infected with NTS may have contributed to variation in responses detected as the immune system matures[192]. In addition, the difference in age between the NTS infection group (under 5 years) and iNTS-GMMA vaccinated, and *S.*Typhi

and *S.*Paratyphi A infected groups (adults) may have confounded comparisons. Finally, not all convalescent sera were identified to the specific infecting serovar, and some sera were later re-categorised following whole genome sequencing analysis, in particular with one isolate re-categorised as *S.* Typhi.

However, despite these limitations, the initial response to *Salmonella* infection was determined and the presence of functional antibodies to the GMMAs following natural infection was demonstrated. The identification of these antibodies suggests the presence of antigens within the GMMAs that may generate an immune response similar to the antibody response following natural infection. Determination of the O-Antigen as immunodominant following natural infection and iNTS-GMMA vaccination provides further confirmation of similar responses. Further testing of convalescent sera from adults and children is required to determine, although unlikely, if the immunodominant antigen changes as the immune system matures. Conversely, testing of the iNTS-GMMA vaccine in children is required to determine the immunodominant antigen in this age group.

### 6.4.2 Infection with *Salmonella* Typhi and Paratyphi A generate functional cross-reactive antibodies to SEn and STm GMMA

Antibodies generated at D28 following *S*. Typhi and *S*. Paratyphi infection display functional cross-reactivity with both SEn and STm GMMA measured using ADMP. Confirmation of a functional response suggests that infection with either *Salmonella* sp. could contribute to some degree of attenuation or protection to infection with non-typhoid *Salmonella*. NTS infection in sub-Saharan Africa peaks in the under-fives and

declines thereafter[285] suggesting that protective responses develop in childhood or that exposure patterns change as children get older. The extent to which cross-reactive antibodies from *S.* Typhi or *S.* Paratyphi A infection may contribute to this protection is unclear. Given the high incidence of *S*. Typhi and *S.* Paratyphi A peaking globally in the 5-9 age group[286], this response may be significant and warrants further investigation. To test this hypothesis further cross-reactive sero-epidemiology and observational data could be gathered from large scale field studies or challenge models to determine either the protective effect of NTS or S.Typhi / S.Paratyphi infection on subsequent heterologous serovar re-exposure. Whilst the oral Ty21a vaccine has demonstrated cross-reactive antibodies and a non-significant increase in opsonophagocytic activity to SEn and STm bacteria[280], significant functional cross-reactive antibody responses have been seen here. Further evidence in the literature of this humoral functional cross-reactive response is limited except for CD4 (as a surrogate of T cell-dependent B cell response) cross-reactive responses following Ty21a vaccination[287] and pan-*Salmonella* sp. clonal T Cell responses following *Salmonella* Typhi controlled human infection studies[106].

It is possible this observed functional cross-reactive response due to prior exposure to *S.* Typhi or *S.* Paratyphi A may lead to a subsequent blunted response[269] to NTS following iNTS-GMMA vaccination. Antigenic sin[269], where a reduced antibody response to a second antigen occurs following prior exposure to similar antigens, could lead to hyporesponsiveness to the vaccine in an area of high *S.* Typhi and *S.* Paratyphi A incidence. The planned second phase I study in adults in an area of NTS and S.Typhi high endemicity should provide a clearer picture of whether either a cross-reactive boosted response or interference will occur. However, the likelihood of *Salmonella* prior exposure[3]

in children aged under two who are the intended recipients of a potential iNTS vaccine could make this concern of lesser importance.

### 6.4.3 Functional Antibodies to *S*. Paratyphi A bacteria are generated following iNTS-GMMA vaccination

Whilst prior discussion has involved antibody generated from *S.* Typhi, *S.* Paratyphi and NTS infection, the next section describes the functional antibodies to *S.* Paratyphi A generated in human participants following iNTS-GMMA vaccination.

Antibodies elicited following iNTS-GMMA vaccination exhibited functional cross-reactivity with live *S* Paratyphi A using an ADMP assay *in vitro*. Whilst the degree of protection or attenuation of *S.* Paratyphi A infection is unclear, further investigation in planned iNTS-GMMA vaccine trials may be warranted to examine if the level of functional cross-reactivity is boosted in a highly endemic population. To determine whether the level of functional cross-reactivity of the iNTS-GMMA provides protection against *S.* Paratyphi A infection, either large field efficacy trials or S.Paratyphi A controlled human infection studies would be required. Alternatively, the immune response following iNTS-GMMA vaccination could be examined against a correlate of protection for *S.*Paratyphi vaccines when this is determined.

Whilst the iNTS-GMMA vaccine sera were not tested on a functional *S.*Typhi ADMP, the protection that an iNTS-GMMA vaccine could offer may be of greater value for this

*Salmonella* serovar considering the epidemiology of both infections often overlap in sub-Saharan Africa[2 3 137]. However, *S.* Typhi occurs well into adulthood in Africa [286] suggesting that antibodies generated during childhood to NTS either wane or do not protect against *S.* Typhi either entirely or in the longer term.

Should a benefit be observed, the GMMA could be an essential additive component in any multivalent pan-*Salmonella* sp. vaccine with iNTS and *S.* Paratyphi vaccine of more benefit in SE Asia and an iNTS and *S.* Typhi vaccine of greater benefit in sub-Saharan Africa[2 3].

### 6.4.4 O-Antigen is the immunodominant antigen following natural infection and iNTS-GMMA Vaccination

The GMMA particles used in the SALVO vaccine clinical trial have been quantified based on the O-antigen content as this is the presumed immunodominant antigen based on antibody responses to the O-antigen tested across multiple studies[98 282 288].

The results presented in this chapter demonstrate a significant reduction in antibody binding to SEn / STm GMMA once the O-antigen-specific antibody was pre-adsorbed. However, whilst the antibody binding response to SEnGMMA was almost wholly blocked following pre-adsorption with O-antigen, the antibody response to STm GMMA, although significantly reduced, was not completely blocked. This suggests O-antigen is the primary immunodominant antigen in GMMAs and, by extension, the *Salmonella* bacterial membrane although other antigens may play a role in GMMA immunogenicity,

particularly in the STmGMMA. This difference in STm versus SEn could be explained by a greater number and abundance of unique proteins in the STmGMMA identified in the proteomic analysis in *Chapter 5.3.1* which may contribute to the humoral response to STmGMMA. Whilst cross-reactivity to the GMMA between non-typhoid *Salmonella* sp. and Typhoid *Salmonella* sp. convalescent sera and GMMA-generated binding antibodies to pan-*Salmonella* sp. has been established, the cross-reactivity between SEn and STm in humans has not yet been established. Should there be a dominance of one GMMA serovar in the immune response to vaccination, optimisation of the GMMA ratios could be adjusted to optimise immunogenicity.

In summary, SEn O-antigen is the primary target of IgG and IgA antibodies to the SEnGMMA, stimulated by the iNTS-GMMA vaccine. In comparison, STm O-antigen is a major but non-exclusive target of IgG and IgA antibodies to the STmGMMA, stimulated by the iNTS-GMMA vaccine.

### 6.4.5 The role of O-Antigen-specific antibodies as the primary cross-reactive antibody across *Salmonella* sp.

The antibody response to GMMA following *S.* Typhi and *S.* Paratyphi A challenge was lower than for natural infection or following iNTS-GMMA vaccination. However, a significant reduction in GMMA binding antibody was identified following pre-adsorption with O-antigen for both SEn and STm GMMA, suggesting significant O-antigen involvement in the cross-reactive response. Residual GMMA antibody binding at the highest OAg concentration together with the absence of a plateau in IgG antibodies at

increasing OAg pre-adsorption concentrations (particularly in the SEnGMMA IgG ELISA) indicates the potential for other antigen-specific antibody involvement. Given the significant responses to O-antigen demonstrated in this chapter, the O:12 antigen shared between STm and *S.* Paratyphi A[280] should be the first antigen to investigate further. In addition, *Salmonella* sp. shares multiple protein homologs, as described in *Chapter 5.3*, which should also be investigated.

### 6.4.6 Antibodies are generated to protein antigens following natural NTS infection and iNTS-GMMA vaccination

8 GMMA proteins identified in Chapter 5 predicted to stimulate human immune responses were tested with a subset of NTS, *S.*Typhi, *S.* Paratyphi A convalescent, and iNTS-GMMA vaccine-induced sera. D14 convalescent NTS sera and D28 sera following *S.*Typhi and *S.*Paratyphi exhibited antibodies to all proteins tested. However, baseline responses prior to NTS, *S.*Typhi and *S.* Paratyphi A infection were not available for testing, and therefore, an increase in cross-reactive antibodies in response to these *Salmonella* sp. could not be established due to an inability to determine the presence of pre-existing antibodies at baseline.

There was no significant difference in antibody level across the full dose iNTS-GMMA vaccine baseline (D0) sera tested between proteins. However, there were differences between participant samples. Where high baseline responses were observed this applied to all 8 proteins tested suggesting that pre-existing antibodies were polyclonal across

multiple proteins and dependent on the participant exposure. There may be several reasons this could have varied between participants. Prior exposure to *Salmonella* sp. or cross-reactive Enterobacteriaceae antibodies may have been present. Many of the proteins tested exist in many Enterobacteriaceae as homologs[257 260], and their existence in gut microbiomes may have stimulated pre-existing antibodies. Further work on the gut microbiome and mucosal responses in SALVO participants is planned to delineate this.

Sera 28 days following full and lower dose iNTS vaccination were compared with baseline, revealing at least three antibody response scenarios. The first was a low baseline and D28 antibody response, suggesting minimal prior cross-reactive antigen exposure and protein elicited antibody response following the iNTS-GMMA vaccine. These coincided with the lowest baseline GMMA IgG antibody level in the full dose vaccination group but all produced significant increases in GMMA IgG at D28. The second was moderate-high baseline and D28 response suggesting more significant prior *Salmonella* sp. or cross-reactive Enterobacteriaceae antigen exposure, which could account for the high baseline GMMA responses seen in the IgG and IgA ELISAs in *Chapter 4* as well as the residual STm GMMA antibodies present following O-Antigen pre-absorption across iNTS-GMMA vaccine and iNTS convalescent sera. The final scenario demonstrated an increase in antibody levels above baseline following the first vaccination, which was significant only for the OmpC proteins but approached significance for some of the remaining proteins, including OmpD and OmpF.

YnCE (Accession Number: NHK45680.1) and HlyE (Accession Number: AAO69115), a marker of carrier status and acute Typhoid infection[35], respectively, demonstrated a non-

significant increase in antibody reactivity in 3 of 10 participants who received the full dose iNTS-GMMA vaccine. Whilst both proteins are expressed in STm, neither was identified in the GMMA proteomic analysis. These may have been present in the GMMA at low levels or have been bound by antibodies cross-reactive to a structurally similar antigen.

Finally, further testing of sera at later time points might be beneficial to determine if affinity maturation reduces possible cross-reactive responses to the ten proteins under investigation.

### 6.4.7 Comparison of SEn and STm GMMA

SEnGMMA bound more cross-reactive IgG antibodies from sera following *S.*Typhi and *S.* Paratyphi infection than STmGMMA. A clear pattern of greater binding to SEnGMMA was not seen with the NTS convalescent sera, nor were sera observed to show greater binding to GMMA from the serovar corresponding to the infection; some SEn sera exhibited greater antibody binding to STmGMMA and some STm sera displayed greater binding responses to SEnGMMA. While greater sample numbers are required to confirm this finding, initial data suggest cross-reactivity amongst SEn and STm antibodies to STm and SEn GMMA antigens may occur. Furthermore, the most apparent difference between SEn and STm GMMAs in the responses toward them was the almost complete eradication of the SEnGMMA IgG and IgA with SEn OAg (O:9) pre-adsorption of sera but only a partial loss of STmGMMA antibody binding following pre-adsorption with STm OAg (O:4,5) suggesting other antibodies to the STmGMMA may contribute to the antibody response following NTS infection and full dose iNTS-GMMA vaccination. Alternative antigens which may stimulate antibodies to STmGMMA include the outer membrane proteins. However,

since in the experiments described, sera were pre-adsorbed with OAg matching the serovar of the GMMA in the ELISA, differing degrees of cross-reactivity between OAgs or GMMAs may also provide an explanation. It is possible that antibodies to SEn (O:9) cross-react or bind to STmGMMA to an extent not seen to SEnGMMA. The lower effect of STm OAg pre-adsorption seen in STmGMMA may reflect a degree of interference by these SEn OAg antibodies. To test this further, SEn OAg pre-adsorbed sera could be tested on the STmGMMA to confirm cross-reactivity. The next step might be to alter the SEnGMMA: STmGMMA component ratio to determine differential immune response. However, ultimately the iNTS-GMMA vaccine stimulated a binding IgG and functional response to both SEn and STm GMMA as shown in chapter . The degree to which cross-reactivity plays a role in this joint response remains to be determined.

Finally, a clear difference between SEnGMMA and STmGMMA in phagocytosis or NK activation was not discernible in either ADMP or ADNKA tested with *S.* Typhi or *S.* Paratyphi A sera, although the medium fluorescent intensity was higher for SEnGMMA ADMP following NTS infection.

### 6.4.8 Future Considerations

Identifying a conserved cross-protective antigen is the goal for any pan-*Salmonella* vaccine, but it may be the case that a multivalent vaccine could provide equal or better protection whilst minimising impact on the infant vaccination schedules. These results suggest that GMMAs might be used as an immunogenic component which may provide some non-serovar specific protection in a multivalent *Salmonella* sp. vaccine similar to the

cross-reactive responses seen in *Neisseria* outer membrane vesicles[128]. The GMMAs stimulate cross-reactive functional responses; the second phase I trial will help to confirm that these responses are boosted rather than causing interference[35] and whether these results are reproduced in an endemic population who may have pre-existing immunity to *Salmonella*. In addition, any multivalent vaccine would require the measurement of the individual vaccine components to confirm response, determine the presence of interference and adjust the component ratio accordingly. Ultimately responses would be determined by efficacy against specific *Salmonella* serovar infections, which may lead to correlates of protection such as serum bactericidal assays or opsonophagocytic assays used in the determination of individual serovar response.

## 6.5 Conclusion

Both SEn and STm GMMA bind antibodies generated from sera following *S.*Typhi and *S.*Paratyphi infection, suggesting the possibility of attenuation of NTS disease following prior exposure to these serovars. The iNTS-GMMA vaccine stimulates a significant functional cross-reactive antibody response following the first vaccination and may offer additional benefits as a component in a multivalent pan-*Salmonella* sp. vaccine. Further testing is required to confirm a beneficial role for these cross-reactive NTS responses, and, conversely determining that this response does not interfere with the iNTS-GMMA vaccine response. In addition, this work suggests O-antigen is the primary immunodominant antigen in GMMAs with potential for cross-serovar responses. However, other protein antigens may also contribute to cross-serovar responses, particularly those elicited by STmGMMA .

# Non-Typhoid Salmonella GMMAs Stimulate T Cells Generated Across *Salmonella* Serovars

## 7.1 Introduction

### 7.1.1 The Role of T Cells in the *Salmonella* Immune Response

T cells provide regulatory feedback and direct effector immune function[204]. Varied and functionally distinct, these cells can be classified by effector function and cytokine production. Co-stimulatory molecules are required for T cell receptor and downstream activation, including the CD3 complex, CD4 and CD8, and are also used for identification[289].

$CD3^+CD4^-CD8^-$ consists of the rarer T cells with a role in the innate and adaptive response, including MAIT and gamma delta T cells[204 289]. $CD3^+CD4^+$ cells define T helper cells whose primary role is to co-stimulate B cells to promote antibody class switching and affinity maturation[204 289 290]. $CD3^+CD8^+$ provide direct cytotoxic effector function by identifying infected host cells and stimulating apoptosis[204 289].

$CD3^+CD4^+$ and $CD3^+CD8^+$ T cells cannot directly bind to antigens. Instead, they require the processing of peptide epitopes following phagocytosis and antigen-presenting within the major histocompatibility complex (MHC I and II)[57]. MHC molecules form a complex with

the peptide epitope and present bound peptide to the corresponding $CD3^+CD4^+$ and $CD3^+CD8^+$ cells[57].

*Salmonella* is an intracellular pathogen which is typically phagocytosed by dendritic and monocyte cells and survives intracellularly in *Salmonella*-containing vacuoles (SCV)[291]. Consequently, T cell immunity, specifically CD3+CD8+ cells, was presumed to be the primary immune component in *Salmonella* infection[67]. However, recent evidence suggests antibodies play a crucial role in clearance, particularly during the extracellular phase of *Salmonella* pathogenesis[46 67]. Therefore, the ability of any iNTS vaccine to stimulate $CD3^+CD4^+$ cells is essential. The SEn and STm GMMA contain multiple proteins, which may act as carriers for surface O polysaccharides or stimulate $CD3^+CD4^+$ cells. Identifying proteins in the GMMAs that may stimulate these cells allows the prediction of significant and prolonged antibody immunity, in addition to identifying possible conjugating proteins and antigens for iNTS vaccine development. Once $CD3^+$ cells are activated, memory subsets are established, which rapidly respond to a secondary infection[204]. This chapter investigates evidence for these memory CD3+ subsets in the GMMA-stimulated T-cell proliferation assays.

## 7.1.2 Current Evidence of *Salmonella* T Cell Cross-Reactivity

Much of the recent data surrounding T Cell cross-reactivity to different *Salmonella* serovars have been established through *Salmonella* challenge and vaccine studies[287 292]. Participants who received the attenuated oral vaccine Ty21a[292] were shown to develop CD8 T memory cells that recognise cells infected with either *S.* Paratyphi A, B or *S.*Typhimurium, suggesting this vaccine produced a cross-reactive T cell memory

response. Participants challenged with *S.*Typhi were found to have $CD4^+$ and $CD8^+$ cells that recognised peptides presented by immortalised lymphoblastoid cell lines infected with *S.*Paratyphi or *S.*Typhimurium ST313[287]. Peptide-specific T cell effector clones identified following *Salmonella* challenge studies were tested against *S.* Paratyphi A, STm and SEn infected cells *in vitro*, identifying intracellular peptides (e.g., PhoN) as the most common antigen to stimulate $CD3^+CD4^+$ [106]. However, a mouse model of *S.* Typhi infection testing both intracellular and surface antigens identified surface antigens such as *IroN* and *CirA* as essential antigens in prolonging mice survival times following *Salmonella* challenge[105].

### 7.1.3 T Cells and Outer Membrane Vesicles

The T-cell response to bacterial outer membrane vesicles (OMV) has been well-established in animal models. Mice studies have demonstrated that outer membrane vesicle antigens from another facultative intracellular bacterium *Burkholderia* outer membrane vesicles are presented by dendritic cells followed by T-cell responses in CD4 and CD8 subsets[293]. However, the evidence of the T cell response to OMVs in humans is limited, with most of the data generated from pre-clinical testing using proliferation assays and meningococcal serogroup B OMV vaccines administered to human participants confirming T cell responses previously seen in pre-clinical testing[184 294].

### 7.1.4 Aims

This chapter aims to determine whether SEn and STm GMMAs are recognised by and stimulate the innate response via monocytes and, therefore, present GMMA peptides to $CD3^+$ T Cells. Secondly, to determine whether the GMMAs stimulate an adaptive memory

$CD3^+$ T cell cross-reactive response to *S.* Typhi and *S.* Paratyphi. Finally, to determine whether the individual proteins identified from the GMMA proteomic analysis (*Chapter 5*) stimulate the $CD3^+$ T cell response following iNTS-GMMA vaccination.

Frozen peripheral blood mononuclear cells (PBMCs) obtained following *S.* Typhi and *S.* Paratyphi infection and iNTS-GMMA vaccination were used to address the following objectives:

1. Evaluate the GMMA-specific monocyte activation by the expression of IL-1 and IL-6.
2. Evaluate the cross-reactive responses of *S.* Typhi and *S.* Paratyphi post-infection PBMCs using SEn / STm GMMA-specific T cell proliferation assays.
3. Identify proteins in the GMMAs which may contribute to the direct $CD3^+CD4^+$ and $CD3^+CD8^+$ response following iNTS-GMMA vaccination.

## 7.2 Methods

### 7.2.1 Sample Collection

PBMCs were collected at D0 and D28 following *S.* Typhoid and *S.* Paratyphoid A diagnosis as part of the human challenge studies P1[154], PATCH[155] and VAST[83]. PBMCs were collected at baseline (Vaccine 1), D14, D28, D56 (Vaccine 2) and D84 following iNTS-GMMA vaccination as part of the SALVO trial (*Chapter 3*). These PBMCs were frozen in liquid nitrogen and thawed on the day of use.

Fresh whole blood from donors was used on the day of collection for the monocyte activation assays.

The 8 selected proteins identified in *Chapter 5* were tested: OmpA, OmpC, OmpD, OmpF, SlyB, LptA, FkpA and Flagellin; as well as HlyE, CDt and YnCE.

### 7.2.2 Monocyte Activation Assay

The SEn and STm GMMA-based monocyte activation assay (including PBMC isolation, leucocyte isolation, stimulation, fixation, staining, flow cytometric analysis and statistical analysis) was performed as detailed in *Chapter 2.10*.

## 7.2.3 T Cell Proliferation Assay

The GMMA and protein-based T cell proliferation assay (PBMC CFSE staining, stimulation, fixation, surface marker staining, flow cytometric and statistical analysis) was performed as detailed in *Chapter 2.11*.

Day 0 (baseline) and D28 PBMCs collected following *S.* Typhoid and *S.* Paratyphoid A diagnosis as part of the human challenge studies P1[154], PATCH[155] and VAST[83] were used to establish *Salmonella*-exposed T cell cross-reactivity to SEn and STm GMMAs. 500,000 PBMCs per well were used in this experiment consistent with the T cell proliferation assays in *Chapter 4*.

The 8 selected proteins (OmpA, OmpC, OmpD, OmpF, SlyB, LptA, FkpA and Flagellin) and additional proteins (HlyE, CDt and YnCE ) at 2 mcg/mL were tested in the T cell proliferation.

In order to maximise the availability of samples for analysis day 0 and day 14 PBMCs following the first full dose iNTS-vaccination were used. In additions 250,000 PBMCs per well were used instead of 500,000 PBMCs per well to maximise proteins and duplicates tested.

## 7.2.4 Statistical Analysis

Statistical analysis was performed for the T cell proliferation assay as detailed in *Chapter 2.11.5.*

## 7.3 Results

### 7.3.1 GMMAs stimulate Monocyte Activation

SEn / STm GMMA stimulate monocyte activation above the negative control and positive lipopolysaccharide control, identifying GMMAs as potent stimulators of the innate response, which corroborates the non-antibody baseline phagocytosis seen in the antibody dependent monocyte assays (*Chapter 4.3.2*) [figures 85 and 86]. In addition, this confirms that monocyte can be stimulated *in vitro* by the GMMAs and could act as antigen-presenting cells to CD3+ T cells within the sample PBMCs, allowing progression to the T cell proliferation assays. As a result of this analysis 10 and 100 micrograms of GMMA were taken as the stimulatory dose for the T cell proliferation assays.

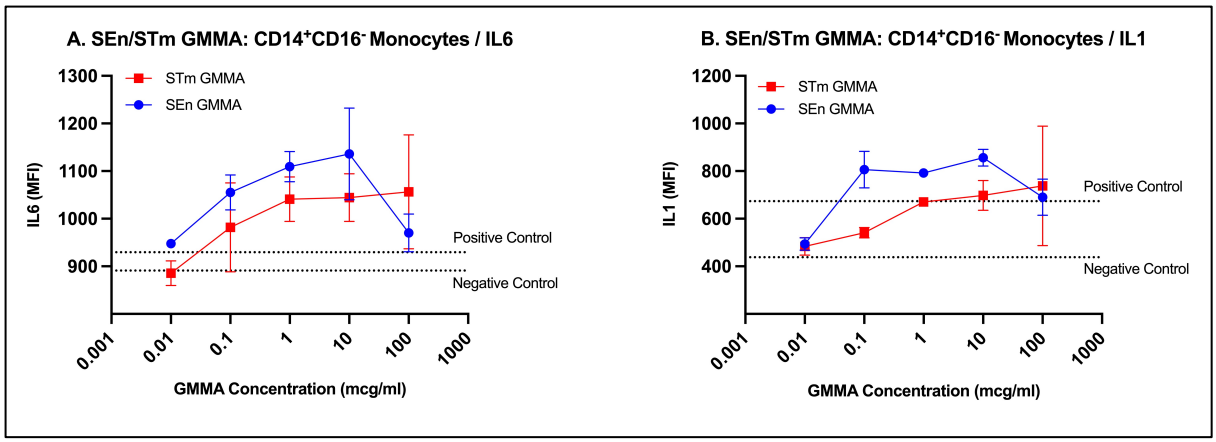



*Figure 85 IL-6 monocyte activation assay (a); IL-1 monocyte activation assay (b) testing increasing concentrations of SEn and STm GMMAs from 0.01 to 100 µg/mL stimulation of fresh donor Salmonella naïve human PBMCs; Mean Fluorescence Intensity (MFI) +/- SD*

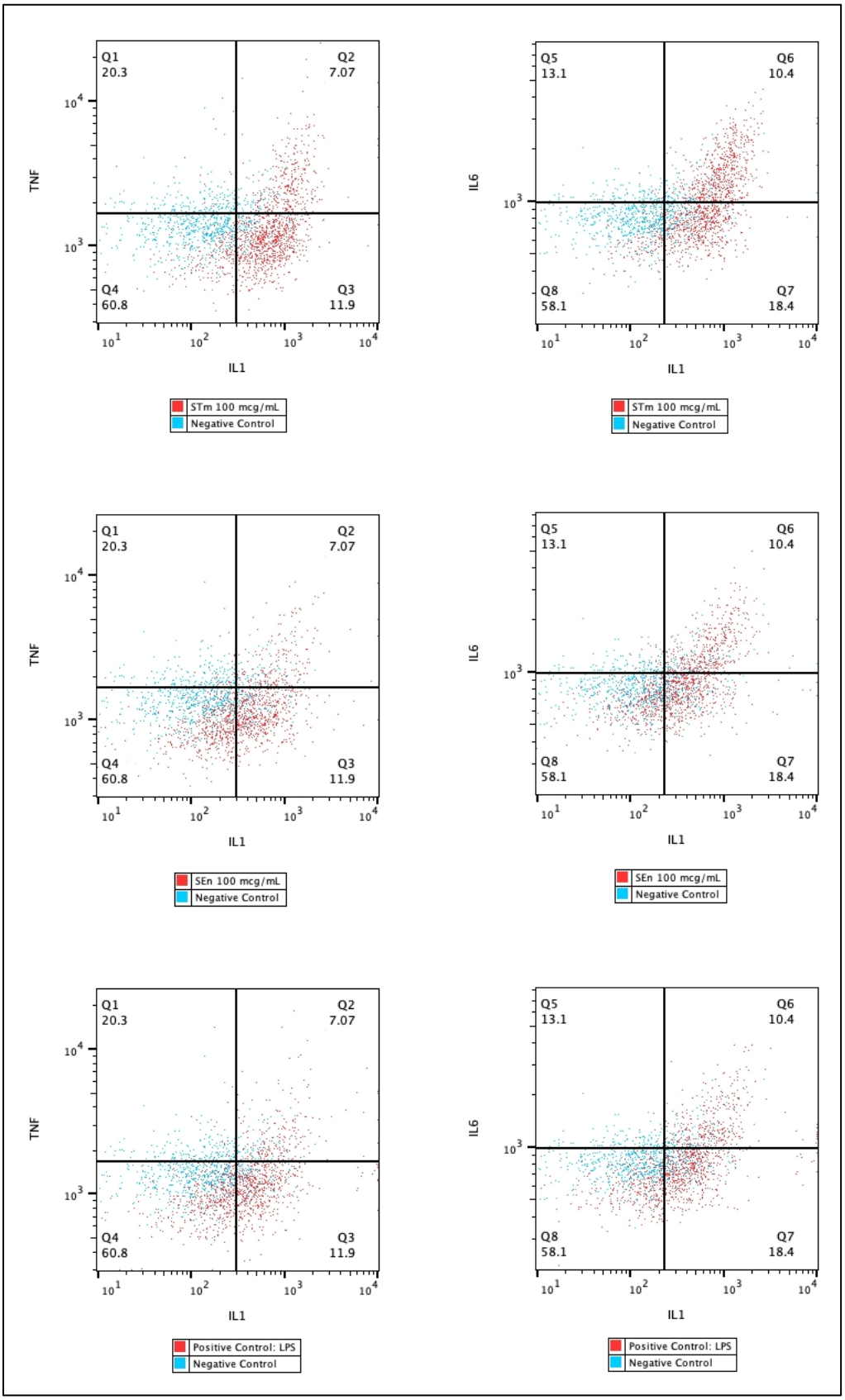



*Figure 86 2D scatterplots showing monocyte activation by release of IL-1, IL-6 and TNF in response to SEn/STm GMMA 100µg/mL: TNF versus IL-1 (L) and IL6 vs IL1 (R); STmGMMA 100 µg/mL vs negative control (Top Row); SEnGMMA 100 µg/mL vs negative control (Middle Row); and positive control (LPS) vs negative control (bottom row)*

### 7.3.2 Characterisation of *Salmonella* Naïve, *Salmonella* Typhi, and *Salmonella* Paratyphi T cell response to GMMAs

#### $CD3^+CD4^+$ Response

$CD3^+CD4^+$ cells from participants before *S.* Typhi and *S.* Paratyphi oral challenge demonstrated no stimulation in response to either SEn or STm GMMA. The only exception was the 100 µg GMMA stimulation in the D0 S.Typhi PBMC sample set.

$CD3^+CD4^+$ cells from participants 28 days post *S.* Paratyphi challenge demonstrated an increase in proliferation following stimulation with GMMA particles (100µg SEnGMMA: mean 3.859 [SD 5.526], p= 0.15; 10µg SEnGMMA: mean 1.173 [SD 0.3241], p=0.10; 100µg STmGMMA: mean 2.568 [SD 4.014], p=0.16; 10µg STmGMMA: mean 0.9967 [SD 1.309], p=0.52) compared with D0 (100µg SEnGMMA: mean 0.6605 [SD 1.012]; 10µg SEnGMMA: mean 0.3309 [SD 0.3241]; 100µg STmGMMA: mean 0.4578 [SD 0.4816]; 10µg STmGMMA: mean 0.7020 [SD 0.7784]), although this increase was not statistically significant [paired t-test] (figure 87).

$CD3^+CD4^+$ cells from participants challenged with *S.* Typhi demonstrated a mixed response at D28 post challenge (100µg SEnGMMA: mean 1.319 [SD 0.7534], p=0.69; 10µg SEnGMMA: mean 0.5266 [SD 0.4853], p=0.78; 100µg STmGMMA: mean 0.7736 [SD 0.5946], p=0.99; 10µg STmGMMA: mean 1.055 [SD 0.7443], p=0.36) compared with D0 (100µg SEnGMMA: mean 1.675 [SD 1.918]; 10µg SEnGMMA: mean 0.4436 [SD 0.4065];

100µg STmGMMA: mean 0.7707 [SD 0.9961]; 10µg STmGMMA: mean 0.5879 [SD 0.6819])

with no statistical significance [paired t-test] (figure 88).

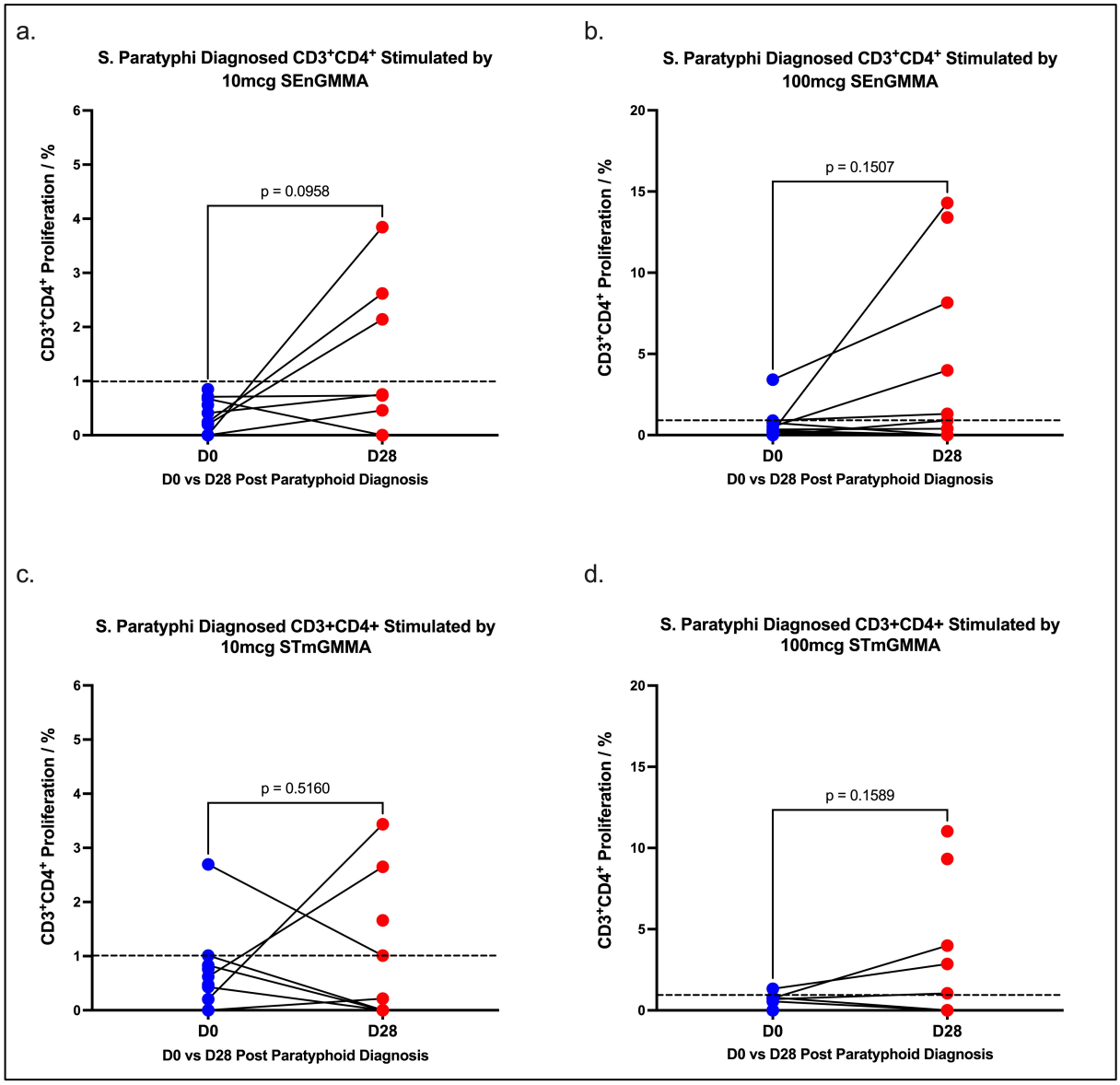



*Figure 87 CD3+CD4+ proliferation in response to stimulation from SEn GMMA (a, b) and STm GMMA (c, d) at 10 and 100µg/mL concentration in samples before (D0) and 28 days (D28) after oral challenge with live S. Paratyphi in healthy adult participants. Statistical test: Paired t-test, comparing sample mean between Day 0 (pre-challenge) and Day 28 (post challenge)*

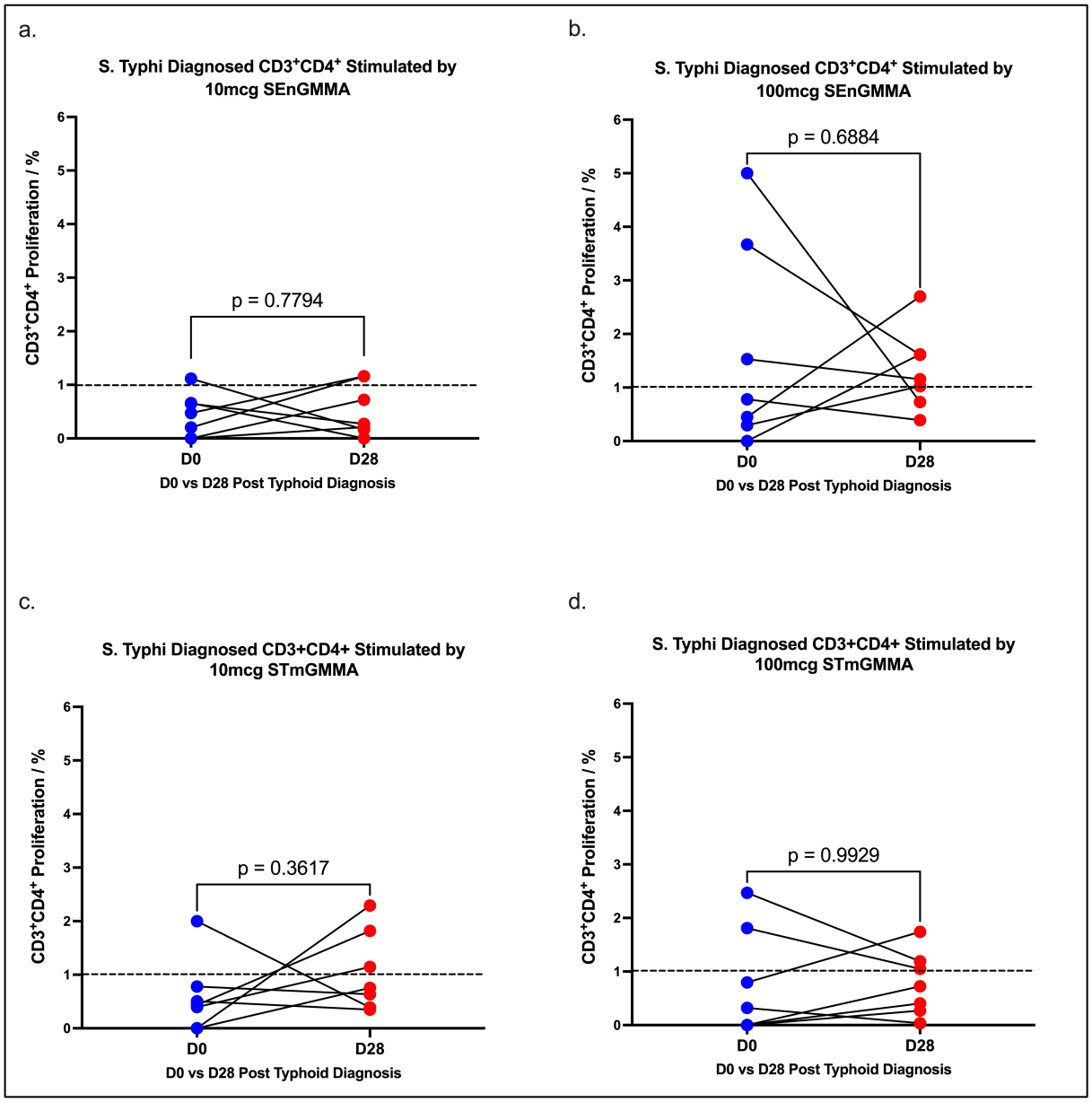



*Figure 88 CD3+CD4+ proliferation in response to stimulation from SEn GMMA (a, b) and STm GMMA (c, d) at 10 and 100µg/mL concentration in samples before (D0) and 28 days (D28) after oral challenge with live S. Typhi in healthy adult participants. Statistical test: Paired t-test, comparing sample mean between Day 0 (pre-Challenge) and Day 28 (post challenge)*

## $CD3^+CD8^+$ Response

$CD3^+CD8^+$ cells from *Salmonella* naïve participants prior to challenge demonstrated detectable proliferation at baseline (D0): *S.* Typhi (100µg SEnGMMA: mean 0.7257 [SD 0.7605]; 10µg SEnGMMA: mean 1.279 [SD 1.305]; 100µg STmGMMA: mean 3.169 [SD 3.343]; 10µg STmGMMA: mean 1.265 [SD 1.842]); and *S.* Paratyphi (100µg SEnGMMA:

mean 0.5950 [SD 0.5785]; 10µg SEnGMMA: mean 1.049 [SD 1.054]; 100µg STmGMMA: mean 0.7944 [SD 0.7922]; 10µg STmGMMA: mean 1.040 [SD 1.025]) [figure 89 and 90].

$CD3^+CD8^+$ cells from participants 28 days post challenge with *S.* Paratyphi demonstrated an increase in proliferation (100µg SEnGMMA: mean 2.531 [SD 2.678], p=0.08; 10µg SEnGMMA: mean 2.656 [SD 2.244], p=0.09; 100µg STmGMMA: mean 3.041 [SD 3.008], p=0.06; 10µg STmGMMA: mean 1.441 [SD 1.827], p=0.65) compared with D0, although this increase was not statistically significant [paired t-test] (figure 89).

$CD3^+CD8^+$ cells from participants challenged with *S.* Typhi demonstrated a mixed response at D28 post challenge (100µg SEnGMMA: mean 1.356 [SD 1.749], p=0.44; 10µg SEnGMMA: mean 0.9600 [SD 0.8783], p=0.54; 100µg STmGMMA: mean 1.588 [SD 2.084], p=0.29; 10µg STmGMMA: mean 1.672 [SD 1.969], p=0.60) compared with D0, with no statistically significant change identified [paired t-test] (figure 90).

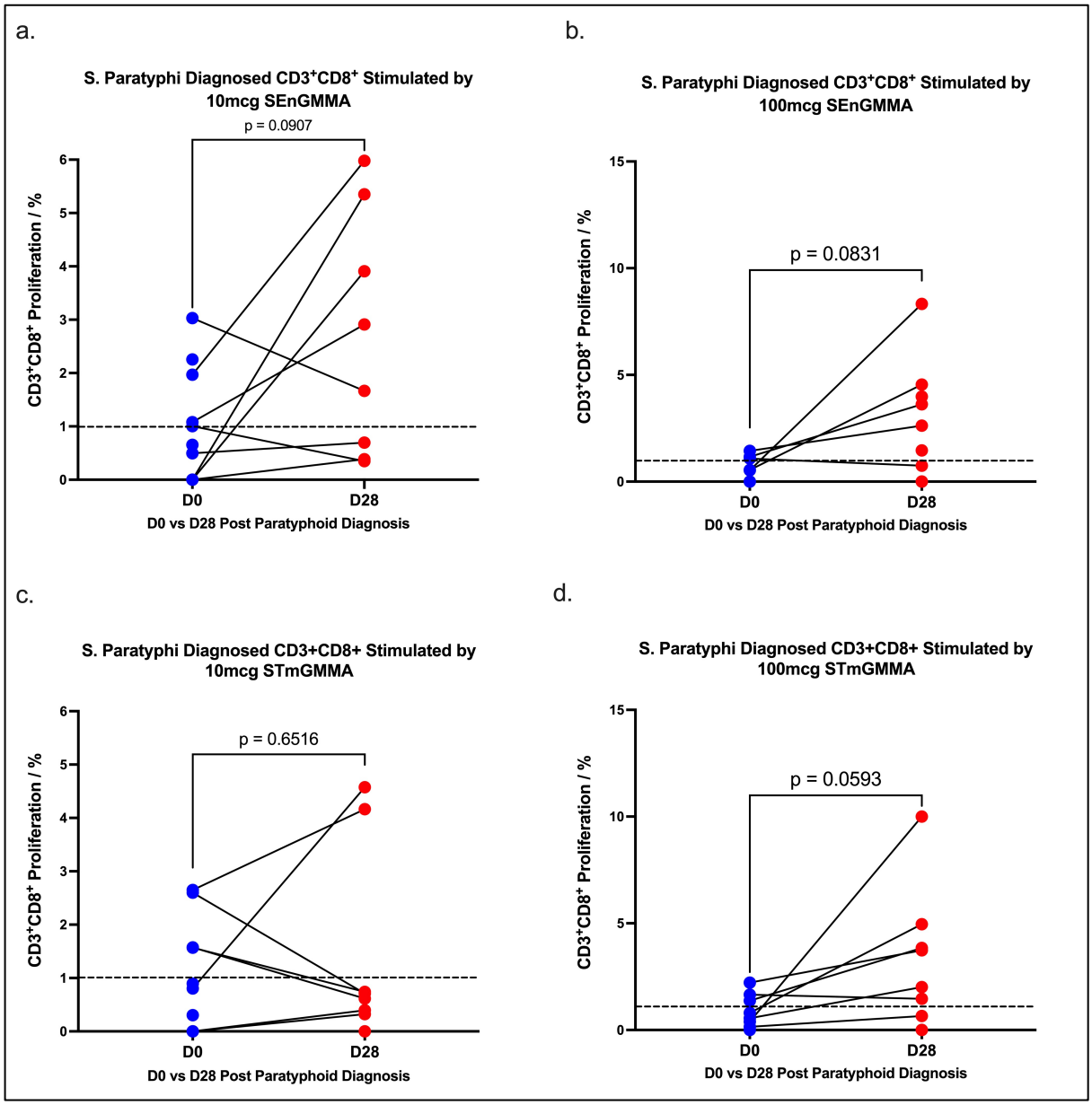



*Figure 89 CD3+CD8+ proliferation in response to stimulation from SEn GMMA (a, b) and STm GMMA (c, d) at 10 and 100µg/mL concentration in samples before (D0) and 28 days (D28) after oral challenge with live S. Paratyphi in healthy adult participants. Statistical Test: Paired t-test, comparing sample mean between Day 0 (pre-challenge) and Day 28 (post challenge)*

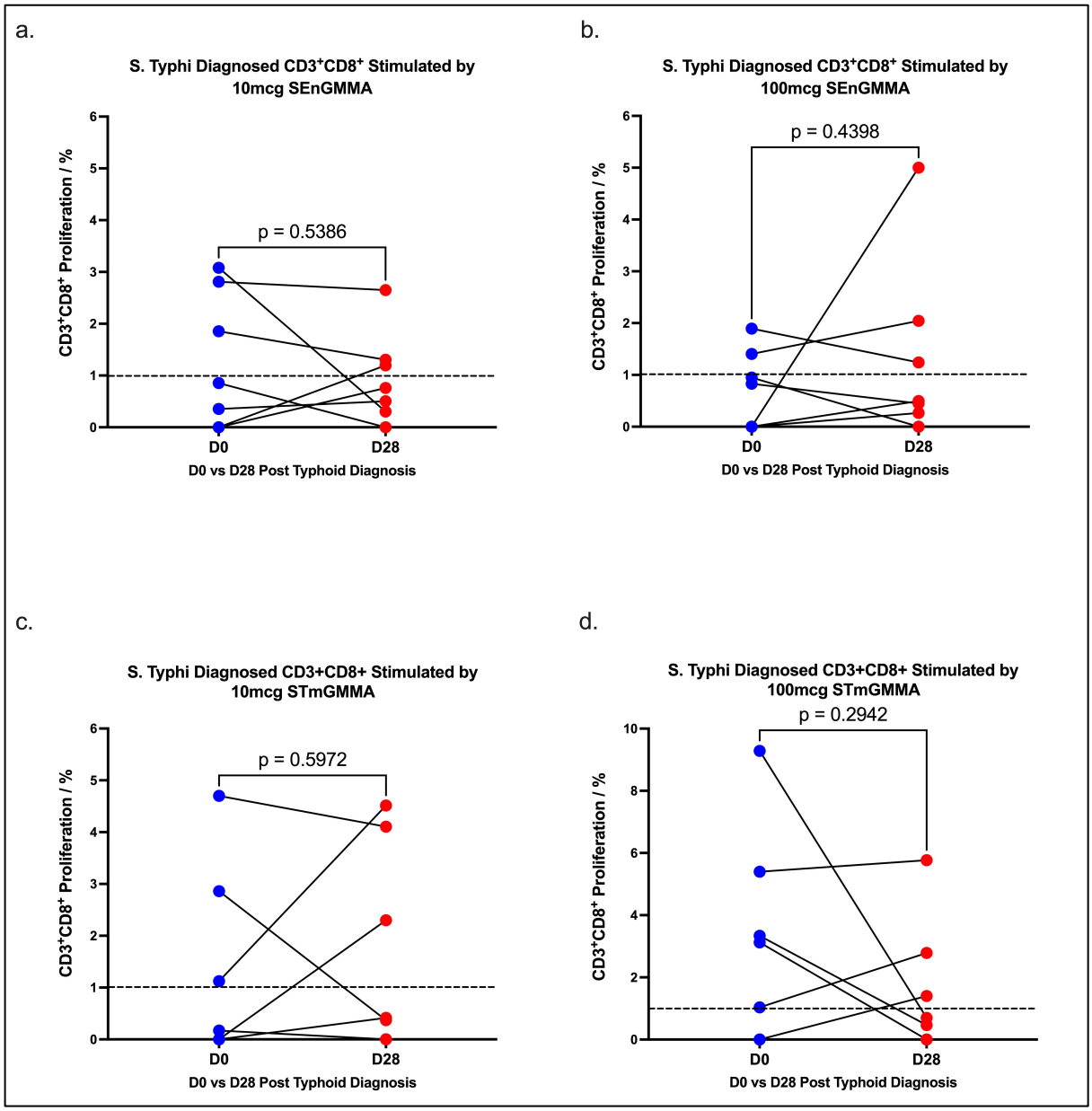



*Figure 90 CD3+CD8+ proliferation in response to stimulation from SEn GMMA (a, b) and STm GMMA (c, d) at 10 and 100µg/mL concentration in samples before (D0) and 28 days (D28) after oral challenge with live S.Typhi in healthy adult participants. Statistical test: Paired t-test, comparing sample mean between Day 0 (pre-challenge) and Day 28 (post challenge)*

### 7.3.3 Characterisation of iNTS-GMMA Vaccinated T cell Response to Selected GMMA Proteins

#### $CD3^+CD4^+$ Response

No GMMA protein specific $CD3^+CD4^+$ cells were detectable at baseline before vaccination (figures 91 and 92).

SEn and STm GMMA stimulated certain D14 full dose iNTS-GMMA vaccine PBMCs, with more CD3+CD4+ proliferation seen with the 100 micrograms/mL than the 10 micrograms/mL GMMA. However, a comparison of means between D0 and D14 samples revealed no significant difference across both GMMAs and concentrations (10µg SEnGMMA, p= 0.15; 100µg SEnGMMA, p=0.91; 10µg STmGMMA, p=0.16; 100µg STmGMMA, p=0.10).

An increase in $CD3^+CD4^+$ proliferation was seen in some D14 full dose iNTS-GMMA vaccine PBMCs following protein-specific stimulation (OmpA, OmpC, OmpD, OmpF, SlyB, LptA, FkpA and Flagellin), except for SlyB, which did not stimulate any D14 samples beyond 1% proliferation. However, a comparison of means between D0 and D14 sample sets for each protein was not significant (OmpA, p=0.19; OmpC, p=0.26; OmpD, p=0.29; OmpF, p=0.2623 SlyB, p=0.15; LptA, p= 0.21; FkpA, p=0.1955; Flag, p=0.28). The participants (1650, 1659) who produced a rise in $CD3^+CD4^+$ were similar across most proteins. OmpD, Flag and LptA stimulated more proliferation in $CD3^+CD4^+$ cells in the greatest number of samples.

The T cell data in this chapter could not be directly compared with the D28 GMMA T cell stimulation analysis in *Chapter 4* due to the disparity in PBMC cell number between the time points: 500,000 cells in the D28 and 250,000 cells in the D14 assay. The lower PBMC count per well was chosen to accommodate all proteins and GMMAs per participant sample.

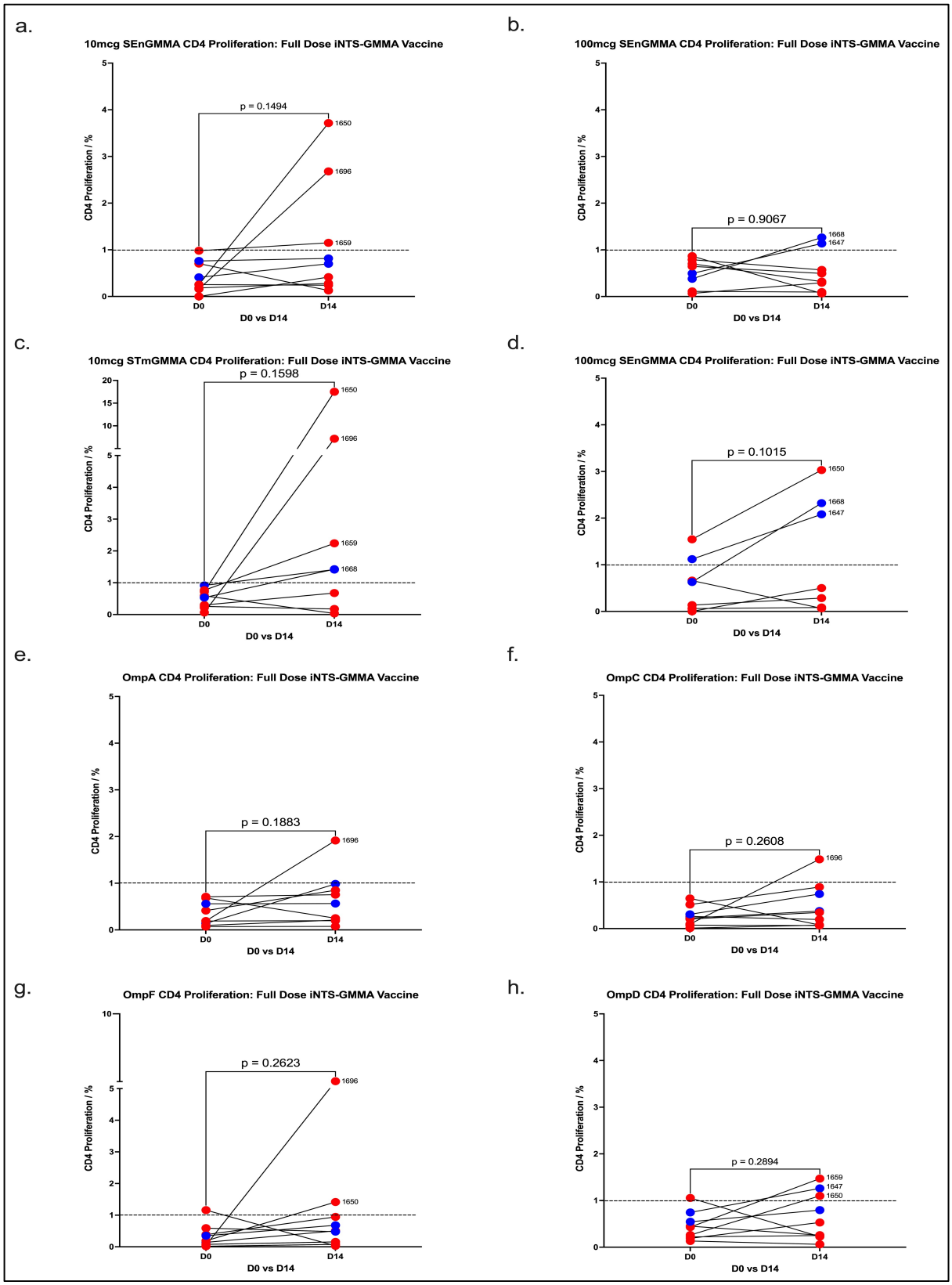



*Figure 91 CD3+CD4+ following iNTS-GMMA vaccine in healthy adult participants stimulated ex-vivo by 10µg/mL SEnGMMA (a), 100µg/mL SEnGMMA (b), 10µg/mL STmGMMA (c), 100µg/mL STmGMMA (d), 2µg/mL OmpA (e), 2µg/mL OmpC (f), 2µg/mL OmpF (g), 2µg/mL OmpD (h); Statistical test: Paired T test comparing sample mean between D0 (pre-iNTS-GMMA vaccine) and D14 (post-iNTS-GMMA vaccine) [full dose, red; lower dose, blue]*

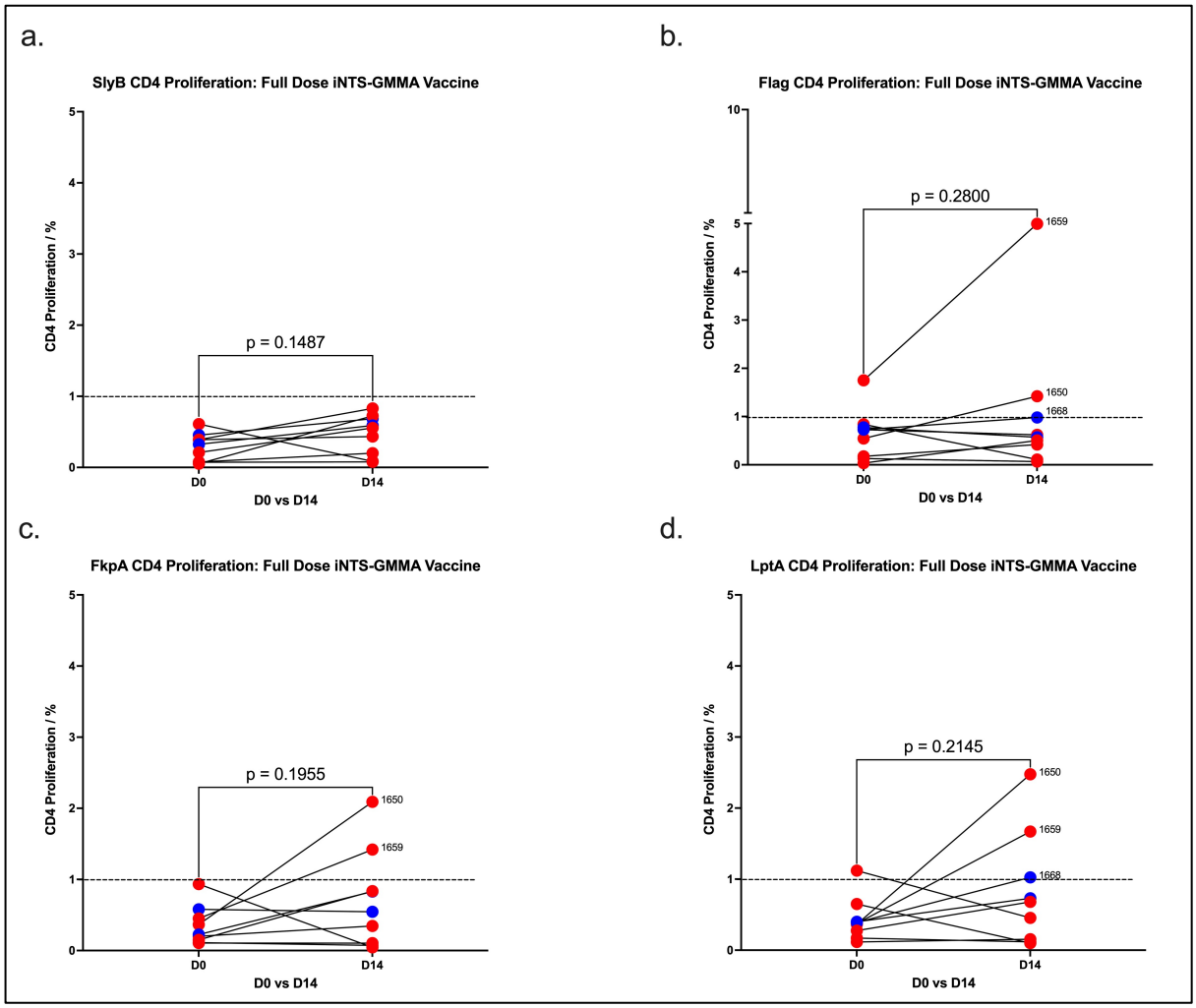



*Figure 92 CD3+CD4+ following iNTS-GMMA vaccine in healthy adult participants stimulated ex-vivo by 2µg/mL SlyB (a), 2µg/mL Flag (b), 2µg/mL FkpA (c) and 2µg/mL LptA (d); Statistical test: Paired T test comparing sample mean between D0 (pre-iNTS-GMMA vaccine) and D14 (post-iNTS-GMMA vaccine)[full dose, red; lower dose, blue]*

## $CD3^{+}CD8^{+}$ Response

The $CD3^{+}CD8^{+}$ response to the proteins was equivocal, with no statistical significance between D0 and D14 in any of the proteins tested (10µg SEnGMMA, p=0.33; 100µg SEnGMMA, p=0.53; 10µg STmGMMA, p=0.39; 100µg STmGMMA, p=0.07; OmpA, p=0.70; OmpC, p=0.59; OmpD, p=0.85; OmpF, p=0.70; SlyB, p=0.27; LptA, p=0.11; FkpA, p=0.36; Flag, p=0.65)[figures 93 and 94].

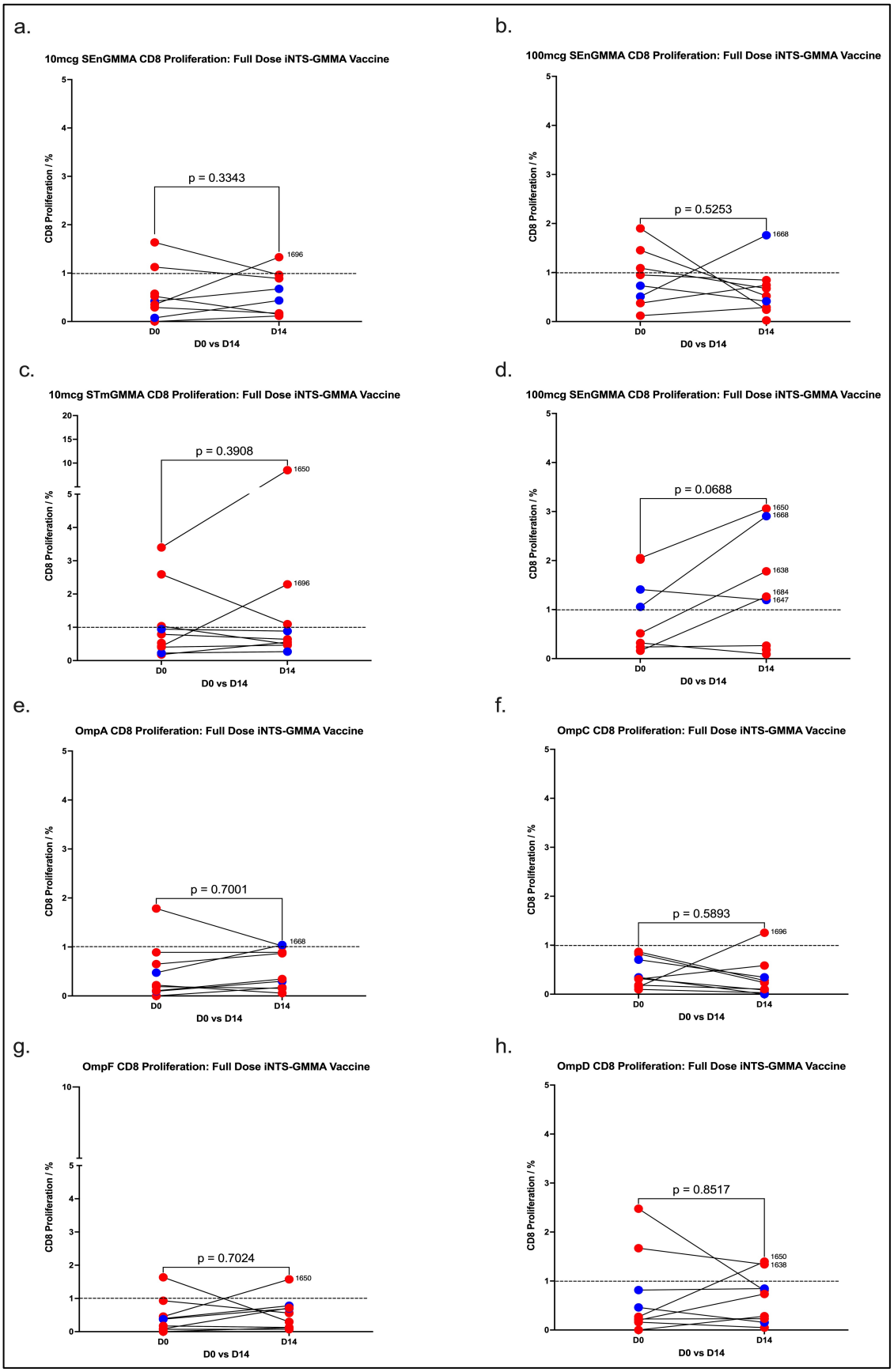



*Figure 93 CD3+CD8+ following iNTS-GMMA vaccine in healthy adult participants stimulated ex-vivo by 10µg/mL SEnGMMA (a), 100µg/mL SEnGMMA (b), 10µg/mL STmGMMA (c), 100µg/mL STmGMMA (d), 2µg/mL OmpA (e), 2µg/mL OmpC (f), 2µg/mL OmpF (g), 2µg/mL OmpD (h); Statistical test: Paired T test comparing sample mean between D0 (pre-iNTS-GMMA vaccine) and D14 (post-iNTS-GMMA vaccine)*

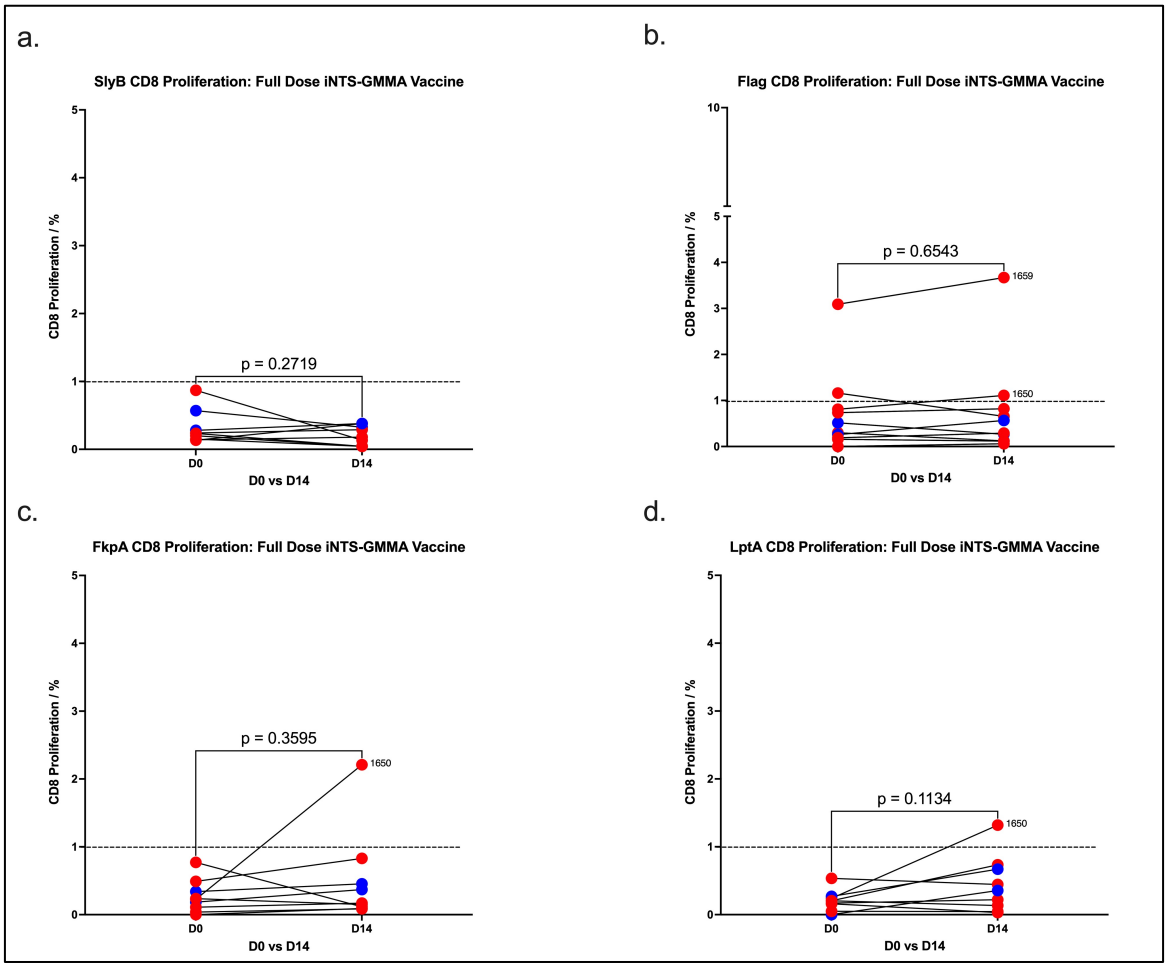



*Figure 94 CD3+CD8+ following iNTS-GMMA vaccine in health adult participants stimulated ex-vivo by 2µg/mL SlyB (a), 2µg/mL Flag (b), 2µg/mL FkpA (c) and 2µg/mL LptA (d); Statistical test: Paired T test comparing sample mean between D0 (pre-iNTS-GMMA vaccine) and D14 (post-iNTS-GMMA vaccine)*

## 7.4 Discussion

### 7.4.1 SEn / STm GMMAs Stimulate Monocyte Activation

Monocytes ($CD14^{+}CD16^{-}$) from healthy donors were activated by both SEn and STm GMMAs alone, confirming the presence of pathogen-associated molecular patterns (PAMPs) within the GMMAs. Whilst lipopolysaccharide is the most likely candidate for stimulating PAMPs, further testing, including the testing of lipoproteins and flagella, would be necessary to confirm the most significant PAMPs present, particularly as directed mutations applied to the origin bacteria have already modified the GMMAs to reduce the reactogenicity of the Lipid A component of LPS[7]. This testing may include a comparative analysis of the individual components of GMMAs most likely to activate monocytes.

IL-1 and IL-6 released by activation of monocytes have a significant role in acute inflammation, innate response and activation of the adaptive response via lymphocytes[295]. The immune system following infection is balanced between inflammation leading to a protective or harmful response. As a candidate vaccine, the iNTS-GMMAs must stimulate immune memory without leading to excessive inflammation and, consequently, reactogenicity or toxicity. Here we identify that GMMAs stimulate the release of pro-inflammatory cytokines *in vitro*. This translated to limited clinical reactogenicity in the clinical safety data, although some participants experienced significant inflammation at the vaccine site (*Chapter 3.3*).

### 7.4.2 SEn and STm GMMAs do not stimulate proliferation in *Salmonella* Naïve $CD3^+CD4^+$ cells

Neither SEn nor STm GMMAs stimulate $CD3^+CD4^+$ proliferation in presumed *Salmonella*-naïve PBMCs, as confirmed in the T cell stimulation experiments at baseline before iNTS-GMMA vaccination (*Chapter 4.3.4*), *S.* Typhi and *S.* Paratyphi challenge. However, $CD3^+CD4^+$ from the *S.* Typhi challenge at 100µg GMMA stimulation and $CD3^+CD8^+$ from the *S.* Typhi and *S.* Paratyphi challenges at both 10 and 100µg GMMA stimulations did show some baseline proliferation in the cells, which may be consistent with the background antibody response to the SEn and STm GMMAs (*Chapter 4.3.2)*, baseline response before *Salmonella* challenge (*Chapter 6.3.2)* and GMMA protein antibody responses seen *(Chapter 6.3.7)*. However, it is likely that low levels of prior *Salmonella* exposure in the UK[98] may explain the overall minimal baseline T cell memory response.

### 7.4.3 *Salmonella* Paratyphi T memory cells trended to proliferate in response to SEn and STm GMMAs

*S.* Paratyphi exposed T cells appeared to proliferate in response to both SEn and STm GMMA. However, this response above baseline was not statistically significant in $CD3^+CD4^+$ and $CD3^+CD8^+$ subsets. The lack of statistical significance could be explained by several factors, including sample number and reduction in PBMC viability due to long-term storage and transportation. Alternatively, this may be explained by the importance of intracellular proteins for T cells and cross-reactive response as described in the whole *Salmonella* specific antigen cross-reactivity analysis in Napolitani et al. (2018)[106]. This

study tested *Salmonella* peptide-specific T cell clones generated following *Salmonella* Typhi challenge studies against EBV immortalised cell lines infected with either *S.* Typhi, *S.* Paratyphi or STm. They found that intracellular proteins such as *PhoN* were amongst the most immunodominant direct and cross-reactive *Salmonella* T cell antigens. Therefore, GMMAs may lack the most important proteins to stimulate a cross-serovar T-cell immune response [*PhoN* not observed in abundance in the GMMAs (see proteomic analysis in Chapter 5.3.3)].

Despite the lack of significant $CD3^+CD4^+$ proliferation across all the D28 samples following *S.* Paratyphi infection compared with baseline (D0), a trend towards higher responses was seen in some instances. *Chapter 5* identifies homologous proteins in the SEn and STm GMMAs and *S*. Paratyphi, which may stimulate a T cell response explaining the proliferation following GMMA stimulation in certain *S.* Paratyphi exposed PBMCs. This $CD3^+CD4^+$ proliferation is in concordance with the *S.* Paratyphi convalescent sera testing for cross-reactivity in the SEn and STm GMMA ELISAs and ADMP (*Chapter 6.3.2 and 6.3.3*). Whether this response could contribute to attenuation or protection in iNTS remains unclear. Given that iNTS-GMMA vaccine-induced antibodies demonstrate functional activity against *S.* Paratyphi A bacteria (*Chapter 6.3.4*), this may suggest a concordant iNTS-GMMA vaccine T cell response against *S.* Paratyphi A, with further testing required for confirmation. This testing might include using iNTS-GMMA vaccine exposed PBMC stimulated by whole *S.* Paratyphi bacteria or specific *S.* Paratyphi proteins in a series of T cell proliferation assays or T cell ELISpots.

### 7.4.4 *Salmonella* Typhi T memory cells are not stimulated to proliferate by SEn and STm GMMAs

Despite *S.* Typhi containing more homologous proteins identified in both SEn and STm GMMA than *S.* Paratyphi, no T cell proliferation was seen in the *S*. Typhi-exposed PBMCs in response to GMMAs. Similar factors may have contributed to this lack of significance, as in *S.* Paratyphi testing.

In addition, the antibody cross-reactivity to SEn and STm GMMA from sera following S. Typhi infection was lower than for *S.* Paratyphi (*Chapter 6.3.2*), which coincides with the lower response seen in *S.* Typhi exposed PBMCs. If this is indeed the case, this would be unfortunate given the higher incidence of *S*. Typhi in sub-Saharan Africa[3] and the potential benefit of a vaccine that generates cross-reactive responses to both NTS and *S.* Typhi.

In the Napolitani study[106], T cells from individuals challenged with *S.* Typhi are activated by antigen-presenting cells infected with *S*. Paratyphi and *S.* Typhimurium ST313[287]. Whole bacteria were tested for T cell cross-reactivity, suggesting that the STmGMMA may not have contained the causative proteins to elicit the T cell responses seen in this previous study. Although GMMA contains some intracellular proteins, these form a relatively small component of total protein content as compared with whole bacteria (*Chapter 5.3.1*). As such, this may suggest that intracellular proteins may be more important than surface proteins for *S.*Typhi cross-reactivity, as seen in Napolitani et al. (2018)[106].

### 7.4.5 Selected Proteins stimulates T cell proliferation in PBMCs following full dose iNTS-GMMA vaccination

The increase in T cell proliferation at D14 in response to both SEn and STm GMMAs was non-significant in contrast to the D28 PBMCs following full dose iNTS-GMMA vaccine. Whether the disparity between time points represents an actual immunological difference or an effect of fewer cells tested at D14 (250,000 cells) is unclear. Given T cell responses typically peak between day 7-14 following vaccination, as seen in the oral *S.* Typhi vaccination (Ty21a)[296], this may suggest that lower cell number may be a major contributory factor. Although it is possible that the T cell response to the proteins may have been higher at D28 post vaccine, only testing of PBMC at this time point will provide confirmation. The GMMAs stimulated a greater T-cell response than the individual proteins themselves. This could be explained by a polyclonal additive immune response to the GMMAs rather than individual GMMA proteins. Alternatively, the contribution of PAMPs within GMMA may contribute to a more efficient recognition and presentation of GMMA proteins than single proteins, resulting in a greater T-cell response. Finally, of the selected proteins tested, the most immunodominant T cell antigen may not have been tested e.g PhoN.

Although not statistically significant, many proteins (ompA, ompC, ompF, ompD, Flag, FkpA, LptA) tested stimulated a trend to increase in $CD3^{+}CD4^{+}$ response in some samples. Prior pre-clinical studies involving many of these proteins have shown promising antibody

and T-cell responses. Mice studies have demonstrated T cell-independent B cell responses to OmpD, OmpC and OmpF[91]. Further studies have shown improved antibody levels dependent on $CD3^+CD4^+$ response to OmpC and OmpF[103]. OmpA has been shown to be a potent pathogen-associated molecular pattern (PAMP) via TLR2[297]. Further testing of pooled peptides of GMMAs and selected proteins may be helpful to confirm the stimulation of $CD3^+CD4^+$ cells in response to both GMMA and proteins.

### 7.4.6 Limitations

Higher sample numbers in the *S.* Typhi and *S.* Paratyphi T cell cross-reactivity experiments might have increased the likelihood of statistical significance being established. PBMCs from more recent studies may have accounted for some of the degradation seen in cell viability and functional activity from long-term storage. Variation may have also been due to the inconsistent number of monocytes within donor PBMCs. Whilst monocytes in the samples may have been sufficient to present and stimulate T cells, pre-digested GMMA protein or GMMA-specific peptide pools might have accelerated antigen presentation and T cell stimulation. Using comparative PBMCs following iNTS infections (if available) might have provided greater insight into the level of cross-reactive response to the GMMAs. In addition, comparison between PBMCs following *S.* Typhi and *S.* Paratyphi may have been affected by the heterogeneity in the D28 time point post infection, given that the time following initial infection to diagnosis may have varied between 1-14 days (P1[154], PATCH[155] and VAST[83]).

## 7.4.7 Implications for future iNTS GMMA Vaccine

*Chapter 4* demonstrates functional antibody responses stimulated by the iNTS-GMMA vaccine in human participants and provides direct and indirect evidence for significant $CD3^{+}CD4^{+}$ T cell memory response to the GMMAs. In this chapter, cross-reactivity was seen in specific individuals exposed to *S.* Paratyphi but not *S.* Typhi; overall, neither was statistically significant. While significance for *S.* Paratyphi might have been met with more sample numbers, this may not be the case for *S.*Typhi. Therefore, any boost by the iNTS-GMMA vaccine to an existing T cell immune response to typhoidal serovars remains unlikely.

Whilst T cell cross-reactivity to boost responses to *S.* Typhi following the iNTS-GMMA vaccine may be desirable in sub-Saharan Africa, cross-reactivity to *S.* Paratyphi remains important, particularly in southeast Asia due to higher incidence seen in the region[3]. Further testing of a T cell response to either *S.* Typhi or *S.* Paratyphi using corresponding antigen stimulation would be required to confirm a boosted T cell response.

*Chapter 4.3.2 and 4.3.4* provides evidence of a functional antibody response and CD3+CD4+ response stimulated by the iNTS-GMMA vaccine. *Chapter 6.3.2* provides evidence for a functional antibody cross-reactive response stimulated by the GMMAs. Should there not be a CD3+CD4+ cross-reactive response stimulated by the GMMAs, the conjugating ability of the proteins within the GMMAs remain present, and additional antigen-specific T-cell response may not be necessary for a cross-protective *Salmonella* vaccine.

## 7.5 Conclusion

The monocyte assay confirmed activation by GMMAs, the antibody-dependant monocyte phagocytosis (ADMP) assay confirmed phagocytosis by THP-1 monocytes in the absence of antibodies (*Chapter 4.3.2*), and the proliferation of $CD3^+$ cells confirmed antigen presentation of peptide sequences.

Whilst $CD3^+CD4^+$ and $CD3^+CD8^+$ cells primed following the full dose iNTS-GMMA vaccines exhibited evidence of T cell memory, there was a non-significant increase in protein-specific T cells in some but not all tested proteins. Several factors may have contributed to non-significance, including the requirement of pathogen-associated molecular patterns within the GMMA, PBMC cell numbers, the cumulative T cell response to many proteins and/or an untested protein being immunodominant.

*S.* Paratyphi but not *S.* Typhi primed $CD3^+$ cells exhibited possible cross-reactivity with both SEn and STm GMMA. However, this was not statistically significant. Ultimately, the T cell cross-reactive *Salmonella* serovar response demonstrated here was not conclusive. Therefore, the STm and SEn GMMAs alone may not be sufficient for a pan-*Salmonella* vaccine and specific *S*. Typhi and *S.* Paratyphi components may need to be incorporated into a potential broadly specific protective vaccine should serovar-specific T cell response be required for protection. This is the current strategy for trivalent and quadrivalent vaccines in development[137].

# Discussion

## 8.1 Introduction

Invasive non-typhoidal *Salmonella* (iNTS) is a neglected infectious disease. Bacterial identification is often delayed due to diagnostic availability and clinical uncertainty. Whilst ameliorating risk factors for the disease might reduce the incidence in a population, eradicating malaria, HIV, and malnutrition is unlikely to be achieved immediately[5]. The emergence of STm ST313 and the convergence to a human-adapted pathogen over the last three decades[19 24 31], together with the rise of antimicrobial resistance[24], make direct action against this pathogen necessary to reduce morbidity and mortality in the present. Whilst there are no licensed vaccines, three iNTS vaccine candidates have recently entered human trials, a live attenuated (*WT05*[114]), a trivalent conjugate (CVD[298]) and an outer membrane vesicle (iNTS-GMMA) vaccine. There has been minimal progression of the oral live attenuated vaccine in the last ten years, and the trivalent conjugate vaccine phase I results are yet to be formally published.

In this thesis, I describe the design and conduct of the third vaccine candidate, the iNTS-GMMA vaccine trial (*Salmonella* Vaccine Study in Oxford [SALVO]). I have presented the safety and immunogenicity data up to day 28 following the second vaccine (primary course) and I investigate four broad themes centred on the investigation and development of the iNTS-GMMA vaccine candidate:

i. The reactogenicity of the vaccine up to 28 days following the primary vaccination course (first and second vaccination).
ii. The immunogenicity of the iNTS-GMMA vaccine following the primary vaccine course, including the development and use of multiple immunoassays to determine the overall immune response.
iii. The investigation of the active GMMA components to identify the immunogenic molecules within the GMMA, including proteomic analysis and immunoassay confirmation.
iv. The determination of cross-reactivity of a broad range of *Salmonella* sp. convalescent sera to and stimulated by the GMMAs.

This chapter summarises the evidence garnered from this thesis for each theme and discusses the implication for the iNTS-GMMA vaccine, other iNTS vaccines and future research.

## 8.2 Summary of findings

### 8.2.1 Low reactogenicity of the iNTS-GMMA Vaccine

The lower (10.6µg) and full (40µg) dose iNTS-GMMA vaccines were investigated during the SALVO trial. Blinded safety data up to 28 days following the second vaccine is presented in this thesis. Only 3-4 participants received the lower dose iNTS-GMMA vaccine, with 15 participants receiving the full dose iNTS-GMMA vaccine. At the time of writing, the study remains participant-observer blind and thus, the safety data presented are blinded to participants who have received placebo vs active vaccine. From the blinded data presented, the vaccine at both lower and full doses appeared provisionally safe and well tolerated within the limited time points assessed (day 0 to 84), and no concerns were identified prohibiting progression to third vaccination and ongoing assessment up to day 350 within the trial, as confirmed by the unblinded Data and Safety Monitoring Committee (DSMC). There was no SAE or SUSARS that were identified. The most common side effect was injection site reactions, such as pain, tenderness, and inflammation, particularly in the full-dose/placebo iNTS-GMMA vaccine groups. Most adverse events, if present, were transient and self-limiting.

The COVID-19 pandemic had multiple effects on the SALVO trial. The study protocol included a COVID-19 pandemic risk assessment, additional study procedures for staff and participant safety and measures to ensure participants could still receive COVID-19 vaccines should they be offered on the national schedule. In addition, the pandemic

delayed the trial due to vaccine manufacturers and logistics. Many participants experienced upper respiratory tract infections, particularly during the winter months, some with a confirmed SARS-CoV-2 infection by antigen testing.

### 8.2.2 Immunogenicity of iNTS-GMMA vaccine

The full dose iNTS-GMMA vaccine stimulates a significant immune response consisting of monocyte activation (*Chapter 7.3.1*), functional antibodies (*Chapter 4.3.2*), $CD3^+CD4^+$ (*Chapter 4.3.4*) and $CD3^+CD8^+$ cells (*Chapter 4.3.4*), comprising multiple immune system components (figure 95). The broad immune response suggests that proteins within the GMMAs stimulate antibodies (*Chapter 6.3.7*), T cells (*Chapter 7.3.3*) and provide natural T cell help to the immunodominant O-antigen (*Chapter 6.3.6*).

Additional work is ongoing using more standard tests of immunogenicity – O antigen ELISA and SBA which will further inform further clinical development of the iNTS-GMMA vaccine.

Whilst the ability of the iNTS-GMMA vaccine to stimulate a human immune response can be assessed *in vitro*, whether the response demonstrated in this thesis shows real-world protection against disease cannot be determined without a protective immunological correlate, a challenge model or field efficacy studies.

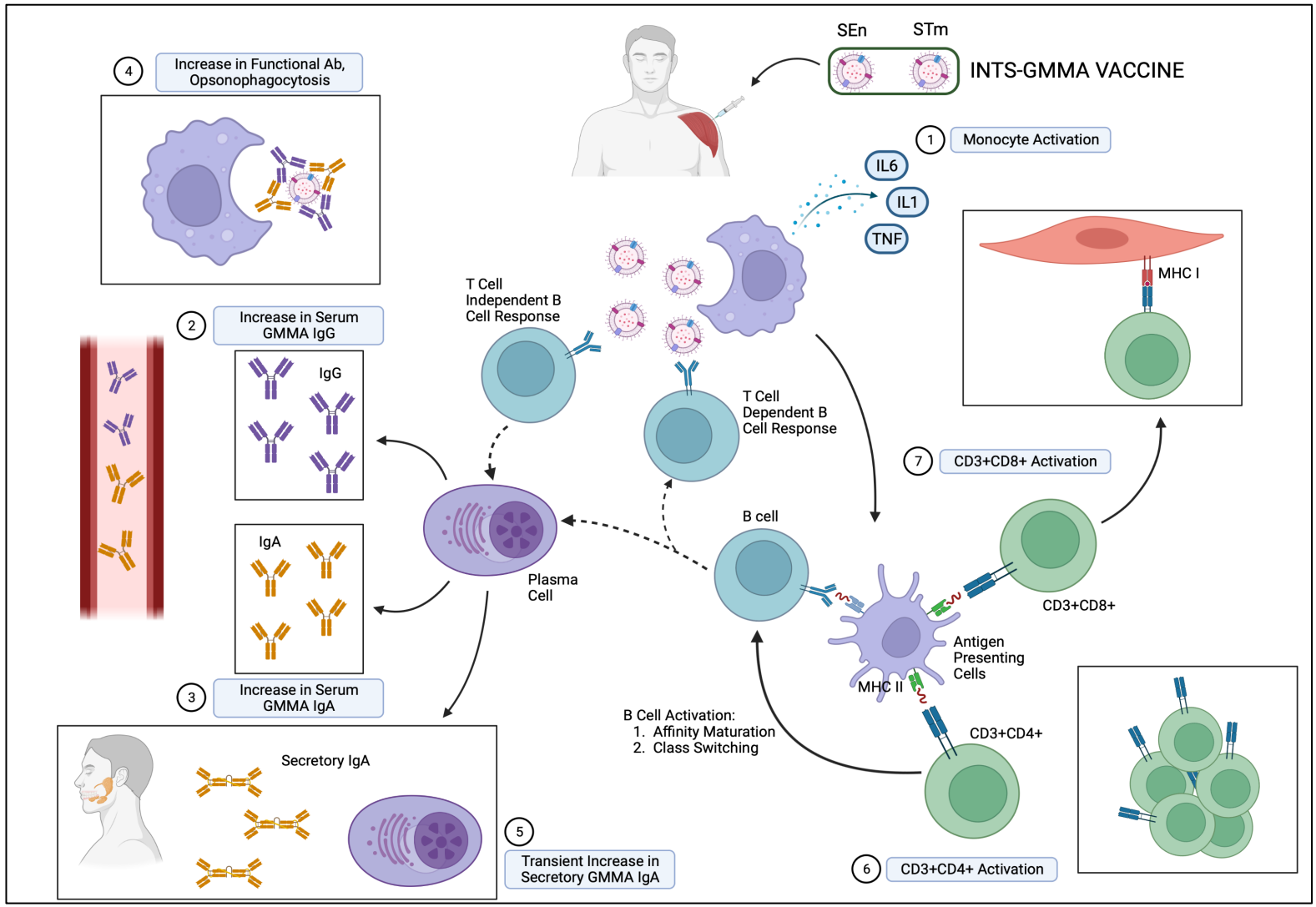



*Figure 95 Summary of Immune Response to the full-dose iNTS-GMMA Vaccine identified in this thesis: Monocyte activation (release of IL-1, IL-6 and TNF) following SEn / STm GMMA stimulation (1); Elevation in serum SEnGMMA- and STmGMMA-specific IgG (2); Elevation in serum SEnGMMA- and STmGMMA-specific IgA (3); Elevation in SEnGMMA, STmGMMA, SEn OAg and STm OAg antibody-dependent opsonophagocytosis (4); Transient elevation in SEnGMMA-specific oral fluid IgA (5); Selection of CD3+CD4+ (6) and CD3+CD8+ (7) Memory Cells. (Created with BioRender.com)*

## 8.2.3 Monocyte activation in response to the SEn / STm GMMA

Activated monocytes release cytokines that recruit immune cells and directly present vaccine antigens to B and T lymphocytes[75]. However, activation can lead to both local and systemic side effects due to elevated cytokine production, which can reduce the tolerability of the vaccine[77]. I have demonstrated that monocytes are activated by SEn / STmGMMA (*Chapter 7.3.1* ) despite the modification of lipid A in the origin bacterial strains to reduce toxicity of the vaccine[7 126]. A reduction in activation in human participants *in vivo* is evident in the limited reactogenicity seen in the safety data after the first and second vaccines compared with published data for other outer membrane vaccines[171]. The extent of this reduction *in vivo* could not be measured unless a 'wild type' OMV and GMMA vaccine were also tested in human participants, but this would not be possible due to concerns over lipid A toxicity[126].

The ability of the GMMA to be phagocytosed by monocytes and be recognised by B and T cells was shown *in vitro* by the elevated antibody-dependant monocyte phagocytosis and $CD3^+CD4^+$ and $CD3^+CD8^+$ proliferation of vaccine-exposed PBMCs in response to the GMMAs.

## 8.2.4 Elevated SEn / STm GMMA IgG and IgA response to the full dose iNTS-GMMA Vaccine from Day 0 to Day 84

The full dose iNTS-GMMA vaccine stimulated a significant rise in serum GMMA-specific IgG and IgA antibody levels within 7-14 days after the first vaccination. Antibodies were

sustained to both SEnGMMA and STmGMMA until 28 days after the second vaccination. The level of antibody cross-reactivity needs further clarification across SEnGMMA and STmGMMA IgG and IgA ELISAs, considering SEn and STm convalescent sera testing suggests substantial cross-reactivity between SEn and STm. IgG and IgA serum antibody levels were tested following the first and second vaccination, demonstrating a rise in antibody levels commensurate with those seen in children under five years 14 days after positive NTS blood cultures. Unexpectedly, serum IgA antibody response was particularly elevated following vaccination which may reflect the response to an enteric pathogen. The literature on IgA response is conflicting, with some identifying an elevation in serum IgA following prior exposure to the pathogen in the gut via natural exposure or vaccination[195] and, in some cases, without apparent previous exposure[196]. However, in these cases, there may be a cross-reactive component[299]. The only other iNTS vaccine candidate to enter human trials with published immunogenicity data is the *WT05* live attenuated *Salmonella* Typhimurium (with *aroC* and *ssaV* mutations)[114]. This initial study showed an increase in LPS-specific IgG and IgA at days 7-14 in the medium/higher doses given, similar to GMMA full-dose vaccine[114].

Baseline GMMA-specific IgG antibody levels were elevated in many samples, including placebo and active vaccine groups. This was either suggestive of cross-reactive or direct antibodies from prior *Salmonella* exposure. Given the availability of multiple potential immunogenic antigens within the GMMAs, a polyclonal response was initially considered likely. However, pre-adsorption assays revealed that the most immunodominant and cross-reactive antigen remained the O-antigen in both SEn and STmGMMA. However, GMMA-specific antibodies remained present despite the highest OAg pre-adsorption

concentration, suggesting the presence of alternative antibodies, particularly for STmGMMA. In addition, the *Shigella* GMMA vaccine studies investigated immunogenicity based on the OAg / lipopolysaccharide (LPS) and found that many participants demonstrated prior antibodies to LPS[129 131]. Therefore, this baseline antibody may not represent a non-specific response to the GMMAs but a direct or cross-reactive response to the OAg. The pending direct IgG OAg ELISAs (not included in this thesis) will investigate this further. The evidence for prior *Salmonella* exposure and a direct boosted antibody response after first vaccinations was seen in the early peak in antibodies in the GMMA-specific IgG at day 14. A similar response was seen in the *Shigella* GMMA vaccine trials, with almost all participants with a high baseline antibody level developing an early elevated response after vaccination and a dose-dependent response in those with a lower LPS IgG baseline[129 131].

### 8.2.5 Initial transient elevation in SEn GMMA mucosal/oral fluid IgA response to the full dose iNTS-GMMA Vaccine at day 14

A peak in oral fluid SEnGMMA-specific IgA at D14 was seen in many participants, with a decrease to baseline immediately after, suggesting that the intra-muscular full dose iNTS-GMMA vaccine stimulated a transient, non-sustained low-level rise in secreted SEnGMMA IgA antibodies. However, the lack of response in STmGMMA-specific IgA following oral fluid testing was consistent with the marginally lower results seen across the STmGMMA assays and presumed lower prior enteric exposure to STm in the UK[190]. The transient elevation in oral fluid IgA in response to an intramuscular vaccine following previous exposure was also seen in other vaccine studies, with oral fluid IgA seen only in

individuals with prior disease exposure[200 300 301]. Similar to a previous study of oral fluid post non-typhoid *Salmonella* infection, serum GMMA-specific IgA did not correlate with oral fluid GMMA-specific IgA and remained elevated up to the last timepoint tested (D84)[98]. Whilst this was the case for non-typhoid *Salmonella* in sub-Saharan Africa using the same collection method[98], other studies of oral fluid/serum IgA in other diseases did show a low correlation[300 302]. Oral fluid IgA production varies between multiple compartments, including the crevicular fluid, parotid and submandibular glands. This, together with the many methods of sampling, can affect the quantification of specific IgA, which may explain some of the differences seen[98].

The absence of a response after the second vaccine may be due to the short interval between the first and second vaccines. The planned third vaccine at month six would be well placed to determine if this absent response is sustained. Statistical testing was not performed due to sample numbers, with further testing required for confirmation. In addition, as discussed in *Chapter 4.4*, normalisation and testing at higher concentrations may have revealed more results.

The testing of oral fluid specific-IgA as a surrogate marker of intestinal specific-IgA and mucosal response at the site of infection has been investigated in a few studies[178 302] but has yet to be established. It remains to be determined if oral fluid correlates with faecal IgA following natural infection, let alone following intramuscular vaccination, where different sites of immune activation might occur. GMMA-specific stool IgA testing is planned to evaluate the mucosal response following intramuscular vaccination at the site of invasion of any *Salmonella* infection.

### 8.2.6 Elevation in SEn/STm GMMA and OAg antibody-dependant phagocytosis response to the full dose iNTS-GMMA vaccine from Day 0 – Day 84

The rise in IgG and IgA antibody levels correlated with the monocyte phagocytosis assay suggesting that the iNTS-GMMA vaccine stimulated a functional antibody response suggestive of a T cell-dependent B cell response sustained from D14-D84. The antibody dependant monocyte phagocytosis assay was based on using SEn/STm OAg coated beads and SEn/STm GMMAs as antigens. This is the first time an outer membrane vesicle has been used as the primary antigen in a functional antibody assay. The benefit of this approach was to fully assess the functional antibody response to the entire active vaccine component, assuming that a polyclonal functional response might be present. This response strongly suggests an immunogenic vaccine; however, whether this functional antibody level is protective remains to be determined.

### 8.2.7 SEn / STm GMMA CD3+CD4+ and CD3+CD8+ response to the full dose iNTS-GMMA Vaccine

SEnGMMA and STmGMMA-specific $CD3^+CD4^+$ and $CD3^+CD8^+$ cells were produced following full dose iNTS-GMMA vaccination. The proliferation of T helper $CD3^+CD4^+$ cells and antibody-dependent monocyte phagocytosis confirms that the iNTS-GMMA vaccine stimulates a T cell-dependent B cell response in adult participants. This T cell-dependent B cell response is of particular importance in children and the target demographic, where this response is particularly difficult to stimulate but is required for antibody maturation

and class switching[82]. A further study in children would be necessary to establish the presence of this response in the target demographic. Finally, the proliferation of T cytotoxic $CD3^{+}CD8^{+}$ cells suggests the vaccine might facilitate the clearance of *Salmonella* -infected cells.

## 8.2.8 Identification of active GMMA components

### The Immunodominant O-Antigen

The O-antigen has been extensively investigated as an antigen target for non-typhoid *Salmonella,* with significant antibodies identified in human convalescent sera[90 98] and animal models following NTS or O-antigen immunisation[65 282]. As the outermost component of the bacteria, it is the first point of contact with the immune system. The O-antigen component of the GMMA has been used to quantify the GMMAs for the iNTS-GMMA vaccination[7]. Sera following human iNTS-GMMA vaccination pre-adsorbed with O-antigen demonstrated a significant reduction (eliminating most activity) in GMMA-specific IgG and IgA levels suggesting that O-antigen was the most important component of the human B cell response to iNTS-GMMA vaccine. However, the IgG antibody levels were not completely eradicated, meaning that non-specific background responses or other antigenic components may contribute to the B cell/antibody response. This was particularly seen in the sustained antibody responses to STmGMMA despite 100µg/mL OAg pre-adsorption. A higher number of unique and abundant proteins was seen in the STmGMMA compared with the SEnGMMA, as seen in the proteomic analysis (*Chapter 5.3.1*), which could be an explanation. Significant OAg pre-adsorption of iNTS convalescent sera tested on IgG SEn / STm GMMA-specific ELISAs suggests that the OAg in

natural infection is the likely immunodominant surface antigen. As the surface immunodominant antigen and as the foremost point of contact with the immune system, the results from this thesis confirm that the OAg is the overall immunodominant antigen. Thus, the iNTS-GMMA vaccine preferentially stimulates an antibody response to the most likely immunodominant antigen in natural infection, suggesting a potentially efficacious vaccine necessitating further advancement.

### 8.2.9 GMMA Protein Components

#### Identification of shared immunogenic proteins between SEn / STm GMMA and corresponding origin bacteria

*Chapter 5* identifies the protein components of the SEn and STm GMMAs and their corresponding origin bacteria. The SEnGMMA and STmGMMA contained 294 (23%) and 458 (36%) of the SEn and STm bacterial lysates, respectively. In addition, whilst the GMMAs had a significant percentage of cytoplasmic proteins (SEnGMMA, 49.9%; STmGMMA, 28.2%), the proportion of outer membrane (SEnGMMA, 25.94%; STmGMMA, 11.4%), inner membrane (SEnGMMA, 16.39%; STmGMMA, 16.6%), periplasmic proteins (SEnGMMA, 11.79%; STmGMMA, 7.9%) of respective total GMMA proteins, were disproportionally larger compared with the corresponding bacteria. Whilst outer membrane vesicles contain a large proportion of membrane-based proteins as expected, many cytoplasmic proteins remained present, which was not seen in the *Shigella* GMMA[122]. STmGMMA contained more unique and abundant proteins than SEnGMMA. Computational immunogenicity analysis revealed multiple potential immunogenic

proteins across NTS strains homologous with *S.* Typhi and *S.* Paratyphi proteins. A limited number of proteins could be manufactured and tested; therefore, the most promising candidates were selected according to the criteria laid out in *Chapter 5.1.4*. As part of the predictive protein immunogenicity approach, 118 shared proteins were identified between GMMAs and bacteria. These were then filtered to 8 proteins which represented the most likely immunogenic proteins between non-typhoid *Salmonella* and typhoidal *Salmonella* . These proteins included OmpA, C, F, D, SlyB, FkpA, Flag, and lptA. A key choice was made at this step to include homologous proteins across *Salmonella* sp. This choice was based on the drive towards a pan-*Salmonella* vaccine with the possibility of the GMMAs being major components[137]. However, there was a risk of pre-existing antibodies in some of the selected proteins from either prior exposure to *Salmonella* sp. or, more significantly in this cohort, homologues proteins in Enterobacteriaceae[260 303].

## Elevated baseline and antibody response to GMMA Protein following iNTS-GMMA vaccine, iNTS, *S.* Typhi and *S.* Paratyphi infection

The immunogenicity analysis consisted of protein-based ELISAs and T cell proliferation assays tested with sera and PBMCS following the iNTS vaccine, iNTS (excluding PBMCs), *S.* Typhi[83] (CHIM) and *S.* Paratyphi[154 155] (CHIM) infection. The protein-based ELISA answered five key questions. Convalescent sera from all *Salmonella* sp. cross-reacted with the proteins. The iNTS convalescent sera, and particularly the iNTS-GMMA vaccine samples, had the highest antibody responses. The baseline antibody response in those tested varied from moderate to high, with sufficient numbers demonstrating high baseline

responses. This might explain the high baseline in the IgG and IgA GMMA assays, in particular in the STmGMMA. Finally, those with an initial low/mild baseline response showed increased protein antibody responses following iNTS vaccination. Whilst O-antigen may be the immunodominant antigen, the proteins do also contribute to the antibody responses which are stimulated by the iNTS-GMMA vaccine to a ceiling which, if already reached due to prior *Salmonella* exposure, demonstrates no further response to iNTS-GMMA vaccine in the short term. Whether the iNTS-GMMA vaccine stimulates antibody longevity requires further testing of later time points.

## No significant T cell response to individual GMMA Proteins following iNTS-GMMA vaccine, iNTS, *S.* Typhi and *S.* Paratyphi infection

Whilst some proteins demonstrated increased $CD3^{+}CD4^{+}$ protein-specific responses, no protein demonstrated a significant response contrary to the whole GMMAs at D28 post-infection, which may be due to the sample numbers, cells per well at day 14 or time points tested. Further testing of the proteins is required including pre-processed pooled peptides which might have demonstrated a better response. A cumulative response may also be responsible for the significant response seen in the whole GMMAs. It is also possible that proteins containing the most immunodominant T cell epitopes were not tested.

### 8.2.10 GMMA cross-reactivity and implications for Salmonella sp. Vaccines and immunity

Sera following *S.* Typhi and *S.* Paratyphi infection demonstrates cross-reactive antibodies that were functional in the opsonophagocytosis of GMMAs, and OAg-beads as measured by antibody-induced monocyte phagocytosis assays. This has a number of implications for the iNTS-GMMA vaccine and immunity to NTS in enteric fever endemic countries.

Higher baseline responses to the iNTS-GMMA vaccine may be seen in individuals who have been exposed to these Typhoidal *Salmonella* serovars. This is particularly relevant in subsequent iNTS-GMMA vaccine trials in areas of high incidence of these serovars. Whilst the use of GMMA-based assays may have a higher baseline response due to polyclonal pre-existing antibodies, the evidence presented suggests that cross-reactive responses are predominantly directed towards the O-antigen. Therefore, the planned OAg ELISAs may have similarly higher baseline responses. Furthermore, the second phase I study is required to establish whether prior exposure to these serovars may lead to interference or boosting of NTS, *S.* Typhi or *S.* Paratyphi immunity following iNTS-GMMA vaccination. As discussed in *Chapter 6.4*, it is unclear whether antigenic sin may play a role or an alternative pattern of interference. Whilst described in influenza, there is limited real-world evidence for this[304] and the most likely scenario is a GMMA boost of responses to both iNTS and other *Salmonella* serovars. Reassuringly, the meningitis B OMV-based vaccines not only stimulate a response to meningococcus B but also there is increasing evidence of a cross-species response to gonococcal disease[272].

In this thesis, I establish that the iNTS-GMMA vaccine stimulates functional cross-reactive antibodies to *S.*Paratyphi. Thus, GMMAs might themselves stimulate a degree of cross-protection to the other *Salmonella* serovars and specifically to the serovars causing enteric fever. Therefore, the SEn / STm GMMA could be a major component of a multivalent *Salmonella* vaccine[264]. However, further testing of the *S.* Typhi immune response following the iNTS-GMMA vaccine would be of greater benefit in sub-Saharan Africa, given the overlap of geographical burden[2 3 9].

If SEn / STm GMMAs can be considered surrogates of their origin bacteria, and considering the vaccine data presented in this thesis, this might suggest that functional antibodies might be generated to *S.* Paratyphi and *S.* Typhi following NTS infection and likewise to NTS following *S.* Typhi and *S.* Paratyphi infection. Whether these antibodies have a real-world benefit is yet to be determined. However, the rapid decline in iNTS incidence after five years of age (implying the acquisition of *Salmonella* antibodies), the lower incidence of *S.* Paratyphi where iNTS predominates and the higher incidence where fewer cases of iNTS are seen, might provide some circumstantial evidence[2 3 9 10 15]. This is far from conclusive, perhaps with further evidence coming from the epidemiology of *Salmonella* infections once *S.* Typhi and in particular, future *S.* Paratyphi and iNTS vaccines are deployed in sub-Saharan Africa and Southeast Asia.

## 8.3 Limitations of the Data

### 8.3.1 Determination of iNTS-GMMA vaccine reactogenicity

As is typical with a first-in-human phase I study, the sample size has been selected to identify significant safety concerns before the assessment of the vaccine in larger phase II and III studies. The blinded data I present in this thesis suggests that the vaccine is safe and well tolerated within the limited time points assessed and allows progression to third vaccinations but requires further assessment of unblinded safety data with longer term follow-up within the trial and including further participants in phase II and III studies to confirm safety prior to licensure.

### 8.3.2 GMMA and Protein-specific antibody assays

Further sample numbers, particularly of iNTS convalescent sera, to determine if there is a significant difference in GMMA-specific IgG and IgA response between iNTS infection and iNTS-GMMA vaccine/placebo samples would provide confirmation of the data presented in this thesis.

Cross-reactivity between SEn and STm-GMMA vaccine-generated antibodies was not tested. A greater response was seen across SEnGMMA-based assays, with a significant difference between SEn and STm GMMA only seen in some assays. However, it is unlikely the vaccine efficacy between serovars would be very different based on the immunogenicity seen in this thesis. Furthermore, it is unclear whether the second phase I

in a high iNTS endemic country would see a similar disparity in response given the higher incidence of STm ST313 in sub-Saharan Africa[305] compared with SEn in the UK[190].

### 8.3.3 GMMA and Protein-specific T-cell Assays

The GMMA-specific T cell response at D28 following the iNTS-GMMA vaccine was statistically significant, contrary to the responses seen at D14 compared with D0 (baseline).

Although there was an increase in protein-specific T cell proliferation in some D14 samples, a greater response might have been affected by the number of PBMC cells per well, which was reduced in the day 14 assay to accommodate the number of proteins tested. Further T cell testing is required using proteins or peptide pools at higher PBMC cell numbers to confirm the T cell response to specific GMMA proteins.

### 8.3.4 Determination of Active GMMA components

The most important GMMA components, including OAg and selected GMMA proteins, have been assessed in this thesis. Although these were identified at the peak antibody level, testing at further time points might have revealed further kinetics of the antibody response. Immunoassay testing of proteins which were unique to SEn or STm might have helped to further evaluate cross-reactivity, and further proteins with predictive immunogenicity evaluated in a protein microarray similar to Darton et al.(2017)[240]and Liang et al.(2013)[239], might have revealed further immunodominant protein antigens.

## 8.3.5 Determination of GMMA cross-reactivity

Further testing of sera following iNTS-GMMA vaccination in an *S.* Typhi functional antibody assay would be important to determine if the SEn/STm GMMA can confer a degree of protection. However, a lower antibody response to the GMMA for *S.* Typhi-induced antibodies than *S.* Paratyphi might suggest a reduced activity of GMMA-induced antibodies to *S.* Typhi. This could be explained by the presence of Vi antigen in *S.* Typhi compared with the availability of OAg in *S.* Paratyphi to initial immune response[306].

Whilst there may be cross-reactivity with Typhoidal *Salmonella*e, the primary aim of the vaccine is in the prevention of iNTS disease. Currently, SEn and STm remain the most common serovars[9 11], but with the deployment of a vaccine, serovars resistant to vaccine-induced immunity may emerge as with other vaccines, including pneumococcus[307 308].

With over 2500 serovars of non-typhoid *Salmonella*[18], cross-reactivity to these serovars will preserve the longevity of the vaccine. Cross-reactive antibodies generated following iNTS-GMMA vaccination to *S.* Paratyphi suggest that the coverage might extend to cover these NTS serovars, the extent of which remains to be determined.

It is clear from the evidence presented that OAg is immunodominant. However, STm ST313 serum resistance to OAg antibodies has already been reported due to elongation of the OAg chain length[96]. The presence of alternative antibodies to STmGMMA may prolong the longevity of the vaccine but this remains a concern.

## 8.4 Future Work and Direction

Overall, the safety and broad immunogenicity data presented in this thesis are consistent with the continued advancement of the iNTS-GMMA vaccine as a promising vaccine candidate. Further work has been partly discussed throughout this chapter. Ongoing work is required to establish safety and immunogenicity following the 3rd iNTS-GMMA vaccination and long-term follow-up to identify both safety and the duration of the immune response. The planned progression of a second phase I trial in a country of high endemicity would be important to determine the safety, immunogenicity, boosting or interference caused by prior high-level *Salmonella* exposure. Should the vaccine progress beyond this point, further testing in the demographic of the highest-burden and progression through clinical trials to confirm efficacy and safety would be required. Establishing a correlate of protection and standardisation of the assay assessing protection needs to be established in order to compare iNTS vaccine candidates.

The development of multivalent *Salmonella* candidate vaccines is already underway, and should these prove efficacious and safe in the highest burden settings, then it is likely that these are the future of iNTS vaccine research[137]. The co-localisation of iNTS and *S.* Typhi[13–15] in sub-Saharan Africa make this a particularly desirable, however further immunogenicity analysis on a combination pan-*Salmonella* vaccine would be required to confirm adequate immunogenicity across *Salmonella* serovars. Deployability of pan-*Salmonella* vaccine in an area of low NTS endemicity or higher *S.* Paratyphi will need to be considered, as alternative serovar combinations may be of more benefit in regions such as Southeast Asia[2 3].

Further work on outer membrane vesicles might include the inclusion of more immunogenic or consequential proteins, such as the type III secretion effector proteins. Modification of bacterial culture conditions has been shown to increase the presence of these proteins in STm OMVs[125], and further changes in OMV production could improve the immunogenicity of an OMV-based vaccine.

## 8.5 Conclusion

The iNTS-GMMA vaccine is provisionally safe and well tolerated up to D28 post-second vaccination within the limited blinded safety data assessed and supports the ongoing progression of the SALVO vaccine trial to include third (booster) vaccination. The final unblinded safety analysis following completion of the trial is required to confirm the initial safety assessment of the iNTS-GMMA vaccine within this phase I trial. The immunogenicity demonstrates both a significant functional antibody, CD3+CD4+ and CD3+CD8+ cell response confirming the broad immunogenicity of both SEn and STm GMMA vaccine components. The immunodominant antigen for the primary and cross-reactive response to the GMMA and natural infection is the O-antigen. However, proteins may contribute and provide greater longevity to the vaccine. The safety and immunogenicity data presented in this thesis demonstrate a vaccine of potential in the prevention of iNTS disease.

# References


1. Hanumunthadu B, Kanji N, Owino N, et al. Salmonella Vaccine Study in Oxford (SALVO) trial: protocol for an observer-participant blind randomised placebo-controlled trial of the iNTS-GMMA vaccine within a European cohort. *BMJ Open* 2023;13(11):e072938. doi: 10.1136/bmjopen-2023-072938 [published Online First: 20231114]
2. Stanaway JD, Parisi A, Sarkar K, et al. The global burden of non-typhoidal salmonella invasive disease: a systematic analysis for the Global Burden of Disease Study 2017. *The Lancet Infectious Diseases* 2019;19(12):1312-24. doi: 10.1016/s1473-3099(19)30418-9
3. Stanaway JD, Reiner RC, Blacker BF, et al. The global burden of typhoid and paratyphoid fevers: a systematic analysis for the Global Burden of Disease Study 2017. *The Lancet Infectious Diseases* 2019;19(4):369-81. doi: 10.1016/s1473-3099(18)30685-6
4. Feasey NA, Dougan G, Kingsley RA, et al. Invasive non-typhoidal salmonella disease: an emerging and neglected tropical disease in Africa. *The Lancet* 2012;379(9835):2489-99. doi: 10.1016/s0140-6736(11)61752-2
5. Feasey NA, Everett D, Faragher EB, et al. Modelling the Contributions of Malaria, HIV, Malnutrition and Rainfall to the Decline in Paediatric Invasive Non-typhoidal Salmonella Disease in Malawi. *PLoS Negl Trop Dis* 2015;9(7):e0003979. doi: 10.1371/journal.pntd.0003979 [published Online First: 2015/08/01]
6. Gilchrist JJ, MacLennan CA. Invasive Nontyphoidal Salmonella Disease in Africa. *EcoSal Plus* 2019;8(2) doi: 10.1128/ecosalplus.ESP-0007-2018 [published Online First: 2019/01/19]
7. De Benedetto G, Alfini R, Cescutti P, et al. Characterization of O-antigen delivered by Generalized Modules for Membrane Antigens (GMMA) vaccine candidates against nontyphoidal Salmonella. *Vaccine* 2017;35(3):419-26. doi: 10.1016/j.vaccine.2016.11.089 [published Online First: 2016/12/22]
8. Majowicz SE, Musto J, Scallan E, et al. The global burden of nontyphoidal Salmonella gastroenteritis. *Clin Infect Dis* 2010;50(6):882-9. doi: 10.1086/650733 [published Online First: 2010/02/18]
9. Marks F, von Kalckreuth V, Aaby P, et al. Incidence of invasive salmonella disease in sub-Saharan Africa: a multicentre population-based surveillance study. *Lancet Glob Health* 2017;5(3):e310-e23. doi: 10.1016/S2214-109X(17)30022-0 [published Online First: 2017/02/15]
10. Reddy EA, Shaw AV, Crump JA. Community-acquired bloodstream infections in Africa: a systematic review and meta-analysis. *The Lancet Infectious Diseases* 2010;10(6):417-32. doi: 10.1016/s1473-3099(10)70072-4
11. Gordon MA, Walsh AL, Chaponda M, et al. Bacteraemia and mortality among adult medical admissions in Malawi--predominance of non-typhi salmonellae and Streptococcus pneumoniae. *J Infect* 2001;42(1):44-9. doi: 10.1053/jinf.2000.0779 [published Online First: 2001/03/13]
12. Ley B, Le Hello S, Lunguya O, et al. Invasive Salmonella enterica serotype typhimurium infections, Democratic Republic of the Congo, 2007-2011. *Emerg Infect Dis*

2014;20(4):701-4. doi: 10.3201/eid2004.131488 [published Online First: 2014/03/25]
13. Msefula CL, Olgemoeller F, Jambo N, et al. Ascertaining the burden of invasive Salmonella disease in hospitalised febrile children aged under four years in Blantyre, Malawi. *PLoS Negl Trop Dis* 2019;13(7):e0007539. doi: 10.1371/journal.pntd.0007539 [published Online First: 2019/07/18]
14. Tapia MD, Tennant SM, Bornstein K, et al. Invasive Nontyphoidal Salmonella Infections Among Children in Mali, 2002-2014: Microbiological and Epidemiologic Features Guide Vaccine Development. *Clin Infect Dis* 2015;61 Suppl 4:S332-8. doi: 10.1093/cid/civ729 [published Online First: 2015/10/10]
15. Wilkens J, Newman MJ, Commey JO, et al. Salmonella bloodstream infection in Ghanaian children. *Clinical Microbiology and Infection* 1997;3(6):616-20. doi: 10.1111/j.1469-0691.1997.tb00467.x
16. Brown M, Eykyn SJ. Non-typhoidal Salmonella bacteraemia without gastroenteritis: a marker of underlying immunosuppression. Review Of cases at St. Thomas' Hospital 1970-1999. *J Infect* 2000;41(3):256-9. doi: 10.1053/jinf.2000.0750 [published Online First: 2000/12/20]
17. Ikumapayi UN, Antonio M, Sonne-Hansen J, et al. Molecular epidemiology of community-acquired invasive non-typhoidal Salmonella among children aged 2 29 months in rural Gambia and discovery of a new serovar, Salmonella enterica Dingiri. *J Med Microbiol* 2007;56(Pt 11):1479-84. doi: 10.1099/jmm.0.47416-0 [published Online First: 2007/10/30]
18. Park SE, Pham DT, Pak GD, et al. The genomic epidemiology of multi-drug resistant invasive non-typhoidal Salmonella in selected sub-Saharan African countries. *BMJ Glob Health* 2021;6(8) doi: 10.1136/bmjgh-2021-005659
19. Okoro CK, Kingsley RA, Connor TR, et al. Intracontinental spread of human invasive Salmonella Typhimurium pathovariants in sub-Saharan Africa. *Nat Genet* 2012;44(11):1215-21. doi: 10.1038/ng.2423 [published Online First: 2012/10/02]
20. Ashton PM, Owen SV, Kaindama L, et al. Public health surveillance in the UK revolutionises our understanding of the invasive Salmonella Typhimurium epidemic in Africa. *Genome Med* 2017;9(1):92. doi: 10.1186/s13073-017-0480-7 [published Online First: 2017/11/01]
21. Akullian A, Montgomery JM, John-Stewart G, et al. Multi-drug resistant non-typhoidal Salmonella associated with invasive disease in western Kenya. *PLoS Negl Trop Dis* 2018;12(1):e0006156. doi: 10.1371/journal.pntd.0006156 [published Online First: 2018/01/13]
22. Kariuki S, Gordon MA, Feasey N, et al. Antimicrobial resistance and management of invasive Salmonella disease. *Vaccine* 2015;33 Suppl 3:C21-9. doi: 10.1016/j.vaccine.2015.03.102 [published Online First: 2015/04/29]
23. Reis ROD, Souza MN, Cecconi MCP, et al. Increasing prevalence and dissemination of invasive nontyphoidal Salmonella serotype Typhimurium with multidrug resistance in hospitalized patients from southern Brazil. *Braz J Infect Dis* 2018;22(5):424-32. doi: 10.1016/j.bjid.2018.08.002 [published Online First: 2018/09/22]
24. Van Puyvelde S, Pickard D, Vandelannoote K, et al. An African Salmonella Typhimurium ST313 sublineage with extensive drug-resistance and signatures of

host adaptation. *Nat Commun* 2019;10(1):4280. doi: 10.1038/s41467-019-11844-z [published Online First: 2019/09/21]
25. Hiyoshi H, Tiffany CR, Bronner DN, et al. Typhoidal Salmonella serovars: ecological opportunity and the evolution of a new pathovar. *FEMS Microbiol Rev* 2018;42(4):527-41. doi: 10.1093/femsre/fuy024 [published Online First: 2018/05/24]
26. Owen SV, Wenner N, Canals R, et al. Characterization of the Prophage Repertoire of African Salmonella Typhimurium ST313 Reveals High Levels of Spontaneous Induction of Novel Phage BTP1. *Front Microbiol* 2017;8:235. doi: 10.3389/fmicb.2017.00235 [published Online First: 2017/03/11]
27. Pulford CV, Perez-Sepulveda BM, Canals R, et al. Stepwise evolution of Salmonella Typhimurium ST313 causing bloodstream infection in Africa. *Nat Microbiol* 2021;6(3):327-38. doi: 10.1038/s41564-020-00836-1 [published Online First: 2020/12/23]
28. Herrero-Fresno A, Wallrodt I, Leekitcharoenphon P, et al. The role of the st313-td gene in virulence of Salmonella Typhimurium ST313. *PLoS One* 2014;9(1):e84566. doi: 10.1371/journal.pone.0084566 [published Online First: 2014/01/10]
29. Keddy KH, Takuva S, Musekiwa A, et al. An association between decreasing incidence of invasive non-typhoidal salmonellosis and increased use of antiretroviral therapy, Gauteng Province, South Africa, 2003-2013. *PLoS One* 2017;12(3):e0173091. doi: 10.1371/journal.pone.0173091 [published Online First: 2017/03/07]
30. Kariuki S, Revathi G, Kariuki N, et al. Invasive multidrug-resistant non-typhoidal Salmonella infections in Africa: zoonotic or anthroponotic transmission? *J Med Microbiol* 2006;55(Pt 5):585-91. doi: 10.1099/jmm.0.46375-0 [published Online First: 2006/04/06]
31. Okoro CK, Barquist L, Connor TR, et al. Signatures of adaptation in human invasive Salmonella Typhimurium ST313 populations from sub-Saharan Africa. *PLoS Negl Trop Dis* 2015;9(3):e0003611. doi: 10.1371/journal.pntd.0003611 [published Online First: 2015/03/25]
32. Brent AJ, Oundo JO, Mwangi I, et al. Salmonella bacteremia in Kenyan children. *Pediatr Infect Dis J* 2006;25(3):230-6. doi: 10.1097/01.inf.0000202066.02212.ff
33. Nyirenda TS, Seeley AE, Mandala WL, et al. Early interferon-gamma production in human lymphocyte subsets in response to nontyphoidal Salmonella demonstrates inherent capacity in innate cells. *PLoS One* 2010;5(10):e13667. doi: 10.1371/journal.pone.0013667 [published Online First: 2010/11/05]
34. Le-Bury G, Niedergang F. Defective Phagocytic Properties of HIV-Infected Macrophages: How Might They Be Implicated in the Development of Invasive Salmonella Typhimurium? *Front Immunol* 2018;9:531. doi: 10.3389/fimmu.2018.00531 [published Online First: 2018/04/10]
35. Gordon MA, Kankwatira AM, Mwafulirwa G, et al. Invasive non-typhoid salmonellae establish systemic intracellular infection in HIV-infected adults: an emerging disease pathogenesis. *Clin Infect Dis* 2010;50(7):953-62. doi: 10.1086/651080 [published Online First: 2010/02/26]
36. Mackenzie G, Ceesay SJ, Hill PC, et al. A decline in the incidence of invasive non-typhoidal Salmonella infection in The Gambia temporally associated with a decline

in malaria infection. *PLoS One* 2010;5(5):e10568. doi: 10.1371/journal.pone.0010568 [published Online First: 2010/05/21]
37. Giglioli G. Paratyphoid C, an Endemic Disease in British Guiana. *J Hyg (Lond)* 1929;29(3):273-81. doi: 10.1017/s0022172400009992
38. Mabey DC, Brown A, Greenwood BM. Plasmodium falciparum malaria and Salmonella infections in Gambian children. *J Infect Dis* 1987;155(6):1319-21. doi: 10.1093/infdis/155.6.1319
39. Biggs HM, Lester R, Nadjm B, et al. Invasive Salmonella infections in areas of high and low malaria transmission intensity in Tanzania. *Clin Infect Dis* 2014;58(5):638-47. doi: 10.1093/cid/cit798 [published Online First: 2013/12/18]
40. Nyirenda TS, Nyirenda JT, Tembo DL, et al. Loss of Humoral and Cellular Immunity to Invasive Nontyphoidal Salmonella during Current or Convalescent Plasmodium falciparum Infection in Malawian Children. *Clin Vaccine Immunol* 2017;24(7) doi: 10.1128/CVI.00057-17 [published Online First: 2017/05/19]
41. MacLennan CA. Host defense against malaria favors Salmonella. *Nat Med* 2012;18(1):21-2. doi: 10.1038/nm.2636 [published Online First: 2012/01/10]
42. Lim SH, Methe BA, Knoll BM, et al. Invasive non-typhoidal Salmonella in sickle cell disease in Africa: is increased gut permeability the missing link? *J Transl Med* 2018;16(1):239. doi: 10.1186/s12967-018-1622-4 [published Online First: 2018/09/01]
43. Bourke CD, Berkley JA, Prendergast AJ. Immune Dysfunction as a Cause and Consequence of Malnutrition. *Trends Immunol* 2016;37(6):386-98. doi: 10.1016/j.it.2016.04.003 [published Online First: 20160526]
44. Hand WL, King NL. Serum opsonization of salmonella in sickle cell anemia. *Am J Med* 1978;64(3):388-95. doi: 10.1016/0002-9343(78)90217-6
45. Balandya E, Reynolds T, Obaro S, et al. Alteration of lymphocyte phenotype and function in sickle cell anemia: Implications for vaccine responses. *Am J Hematol* 2016;91(9):938-46. doi: 10.1002/ajh.24438 [published Online First: 20160714]
46. Nyirenda TS, Gilchrist JJ, Feasey NA, et al. Sequential acquisition of T cells and antibodies to nontyphoidal Salmonella in Malawian children. *J Infect Dis* 2014;210(1):56-64. doi: 10.1093/infdis/jiu045 [published Online First: 2014/01/21]
47. Gilchrist JJ, MacLennan CA, Hill AV. Genetic susceptibility to invasive Salmonella disease. *Nat Rev Immunol* 2015;15(7):452-63. doi: 10.1038/nri3858 [published Online First: 2015/06/26]
48. Santos RL, Raffatellu M, Bevins CL, et al. Life in the inflamed intestine, Salmonella style. *Trends Microbiol* 2009;17(11):498-506. doi: 10.1016/j.tim.2009.08.008 [published Online First: 2009/10/13]
49. Patel S, McCormick BA. Mucosal Inflammatory Response to Salmonella typhimurium Infection. *Front Immunol* 2014;5:311. doi: 10.3389/fimmu.2014.00311 [published Online First: 2014/07/30]
50. Griffin AJ, McSorley SJ. Development of protective immunity to Salmonella, a mucosal pathogen with a systemic agenda. *Mucosal Immunol* 2011;4(4):371-82. doi: 10.1038/mi.2011.2 [published Online First: 2011/02/11]
51. Raffatellu M, George MD, Akiyama Y, et al. Lipocalin-2 resistance confers an advantage to Salmonella enterica serotype Typhimurium for growth and survival

in the inflamed intestine. *Cell Host Microbe* 2009;5(5):476-86. doi: 10.1016/j.chom.2009.03.011
52. Thiennimitr P, Winter SE, Baumler AJ. Salmonella, the host and its microbiota. *Curr Opin Microbiol* 2012;15(1):108-14. doi: 10.1016/j.mib.2011.10.002 [published Online First: 2011/10/28]
53. Thiennimitr P, Winter SE, Winter MG, et al. Intestinal inflammation allows Salmonella to use ethanolamine to compete with the microbiota. *Proc Natl Acad Sci U S A* 2011;108(42):17480-5. doi: 10.1073/pnas.1107857108 [published Online First: 20111003]
54. Lou L, Zhang P, Piao R, et al. Salmonella Pathogenicity Island 1 (SPI-1) and Its Complex Regulatory Network. *Front Cell Infect Microbiol* 2019;9:270. doi: 10.3389/fcimb.2019.00270 [published Online First: 2019/08/21]
55. Jennings E, Thurston TLM, Holden DW. Salmonella SPI-2 Type III Secretion System Effectors: Molecular Mechanisms And Physiological Consequences. *Cell Host Microbe* 2017;22(2):217-31. doi: 10.1016/j.chom.2017.07.009 [published Online First: 2017/08/12]
56. Mastroeni P, Grant A, Restif O, et al. A dynamic view of the spread and intracellular distribution of Salmonella enterica. *Nat Rev Microbiol* 2009;7(1):73-80. doi: 10.1038/nrmicro2034 [published Online First: 2008/12/17]
57. Yrlid U, Svensson M, Kirby A, et al. Antigen-presenting cells and anti-Salmonella immunity. *Microbes Infect* 2001;3(14-15):1239-48. doi: 10.1016/s1286-4579(01)01484-8 [published Online First: 2002/01/05]
58. Gilchrist JJ, Heath JN, Msefula CL, et al. Cytokine Profiles during Invasive Nontyphoidal Salmonella Disease Predict Outcome in African Children. *Clin Vaccine Immunol* 2016;23(7):601-9. doi: 10.1128/CVI.00128-16 [published Online First: 2016/05/14]
59. Eckmann L, Kagnoff MF. Cytokines in host defense against Salmonella. *Microbes Infect* 2001;3(14-15):1191-200. doi: 10.1016/s1286-4579(01)01479-4 [published Online First: 2002/01/05]
60. MacLennan C, Fieschi C, Lammas DA, et al. Interleukin (IL)-12 and IL-23 are key cytokines for immunity against Salmonella in humans. *J Infect Dis* 2004;190(10):1755-7. doi: 10.1086/425021 [published Online First: 2004/10/23]
61. Fields PI, Swanson RV, Haidaris CG, et al. Mutants of Salmonella typhimurium that cannot survive within the macrophage are avirulent. *Proc Natl Acad Sci U S A* 1986;83(14):5189-93. doi: 10.1073/pnas.83.14.5189
62. Monack DM, Navarre WW, Falkow S. Salmonella-induced macrophage death: the role of caspase-1 in death and inflammation. *Microbes Infect* 2001;3(14-15):1201-12. doi: 10.1016/s1286-4579(01)01480-0 [published Online First: 2002/01/05]
63. Krishnakumar R, Kim B, Mollo EA, et al. Structural properties of periplasmic SodCI that correlate with virulence in Salmonella enterica serovar Typhimurium. *J Bacteriol* 2007;189(12):4343-52. doi: 10.1128/JB.00010-07 [published Online First: 20070406]
64. MacLennan CA, Gilchrist JJ, Gordon MA, et al. Dysregulated humoral immunity to nontyphoidal Salmonella in HIV-infected African adults. *Science* 2010;328(5977):508-12. doi: 10.1126/science.1180346 [published Online First: 2010/04/24]
65. Schager AE, Dominguez-Medina CC, Necchi F, et al. IgG Responses to Porins and Lipopolysaccharide within an Outer Membrane-Based Vaccine against

Nontyphoidal Salmonella Develop at Discordant Rates. *MBio* 2018;9(2) doi: 10.1128/mBio.02379-17 [published Online First: 2018/03/08]
66. Kupz A, Bedoui S, Strugnell RA. Cellular requirements for systemic control of Salmonella enterica serovar Typhimurium infections in mice. *Infect Immun* 2014;82(12):4997-5004. doi: 10.1128/IAI.02192-14 [published Online First: 2014/09/17]
67. MacLennan CA. Antibodies and protection against invasive salmonella disease. *Front Immunol* 2014;5:635. doi: 10.3389/fimmu.2014.00635 [published Online First: 2015/01/08]
68. Mastroeni P, Rossi O. Immunology, epidemiology and mathematical modelling towards a better understanding of invasive non-typhoidal Salmonella disease and rational vaccination approaches. *Expert Rev Vaccines* 2016;15(12):1545-55. doi: 10.1080/14760584.2016.1189330 [published Online First: 2016/05/14]
69. Gondwe EN, Molyneux ME, Goodall M, et al. Importance of antibody and complement for oxidative burst and killing of invasive nontyphoidal Salmonella by blood cells in Africans. *Proc Natl Acad Sci U S A* 2010;107(7):3070-5. doi: 10.1073/pnas.0910497107 [published Online First: 2010/02/06]
70. MacLennan CA, Tennant SM. Comparing the roles of antibodies to nontyphoidal Salmonella enterica in high- and low-income countries and implications for vaccine development. *Clin Vaccine Immunol* 2013;20(10):1487-90. doi: 10.1128/CVI.00465-13 [published Online First: 2013/08/02]
71. Hand TW, Reboldi A. Production and Function of Immunoglobulin A. *Annu Rev Immunol* 2021;39:695-718. doi: 10.1146/annurev-immunol-102119-074236 [published Online First: 20210301]
72. Woof JM, Kerr MA. The function of immunoglobulin A in immunity. *J Pathol* 2006;208(2):270-82. doi: 10.1002/path.1877 [published Online First: 2005/12/20]
73. Leong KW, Ding JL. The unexplored roles of human serum IgA. *DNA Cell Biol* 2014;33(12):823-9. doi: 10.1089/dna.2014.2639
74. Murphy BR. Mucosal Immunity to Viruses. Mucosal Immunology2005:799-813.
75. Dougan G, John V, Palmer S, et al. Immunity to salmonellosis. *Immunol Rev* 2011;240(1):196-210. doi: 10.1111/j.1600-065X.2010.00999.x [published Online First: 2011/02/26]
76. Behnsen J, Perez-Lopez A, Nuccio SP, et al. Exploiting host immunity: the Salmonella paradigm. *Trends Immunol* 2015;36(2):112-20. doi: 10.1016/j.it.2014.12.003 [published Online First: 2015/01/15]
77. Herve C, Laupeze B, Del Giudice G, et al. The how's and what's of vaccine reactogenicity. *NPJ Vaccines* 2019;4:39. doi: 10.1038/s41541-019-0132-6 [published Online First: 20190924]
78. Kitamura D, Roes J, Kuhn R, et al. A B cell-deficient mouse by targeted disruption of the membrane exon of the immunoglobulin mu chain gene. *Nature* 1991;350(6317):423-6. doi: 10.1038/350423a0
79. Chung AW, Alter G. Systems serology: profiling vaccine induced humoral immunity against HIV. *Retrovirology* 2017;14(1):57. doi: 10.1186/s12977-017-0380-3 [published Online First: 20171221]
80. Semmes EC, Chen JL, Goswami R, et al. Understanding Early-Life Adaptive Immunity to Guide Interventions for Pediatric Health. *Front Immunol* 2020;11:595297. doi: 10.3389/fimmu.2020.595297 [published Online First: 20210121]

81. Adderson EE. Antibody repertoires in infants and adults: effects of T-independent and T-dependent immunizations. *Springer Semin Immunopathol* 2001;23(4):387-403. doi: 10.1007/s281-001-8166-x [published Online First: 2002/02/06]
82. Cummings LA, Deatherage BL, Cookson BT. Adaptive Immune Responses during Salmonella Infection. *EcoSal Plus* 2009;3(2) doi: 10.1128/ecosalplus.8.8.11
83. Jin C, Gibani MM, Moore M, et al. Efficacy and immunogenicity of a Vi-tetanus toxoid conjugate vaccine in the prevention of typhoid fever using a controlled human infection model of Salmonella Typhi: a randomised controlled, phase 2b trial. *The Lancet* 2017;390(10111):2472-80. doi: 10.1016/s0140-6736(17)32149-9
84. Svensson T, Kattstrom M, Hammarlund Y, et al. Pneumococcal conjugate vaccine triggers a better immune response than pneumococcal polysaccharide vaccine in patients with chronic lymphocytic leukemia A randomized study by the Swedish CLL group. *Vaccine* 2018;36(25):3701-07. doi: 10.1016/j.vaccine.2018.05.012 [published Online First: 20180507]
85. Lee SJ, Dunmire S, McSorley SJ. MHC class-I-restricted CD8 T cells play a protective role during primary Salmonella infection. *Immunol Lett* 2012;148(2):138-43. doi: 10.1016/j.imlet.2012.10.009 [published Online First: 20121023]
86. Ravindran R, McSorley SJ. Tracking the dynamics of T-cell activation in response to Salmonella infection. *Immunology* 2005;114(4):450-8. doi: 10.1111/j.1365-2567.2005.02140.x [published Online First: 2005/04/05]
87. Preciado-Llanes L, Aulicino A, Canals R, et al. Evasion of MAIT cell recognition by the African Salmonella Typhimurium ST313 pathovar that causes invasive disease. *Proc Natl Acad Sci U S A* 2020;117(34):20717-28. doi: 10.1073/pnas.2007472117 [published Online First: 20200811]
88. Mayuzumi H, Inagaki-Ohara K, Uyttenhove C, et al. Interleukin-17A is required to suppress invasion of Salmonella enterica serovar Typhimurium to enteric mucosa. *Immunology* 2010;131(3):377-85. doi: 10.1111/j.1365-2567.2010.03310.x
89. Le Bourhis L, Dusseaux M, Bohineust A, et al. MAIT cells detect and efficiently lyse bacterially-infected epithelial cells. *PLoS Pathog* 2013;9(10):e1003681. doi: 10.1371/journal.ppat.1003681 [published Online First: 2013/10/17]
90. Stockdale L, Nalwoga A, Nash S, et al. Cross-sectional study of IgG antibody levels to invasive nontyphoidal Salmonella LPS O-antigen with age in Uganda. *Gates Open Research* 2019;3 doi: 10.12688/gatesopenres.13034.1
91. Gil-Cruz C, Bobat S, Marshall JL, et al. The porin OmpD from nontyphoidal Salmonella is a key target for a protective B1b cell antibody response. *Proc Natl Acad Sci U S A* 2009;106(24):9803-8. doi: 10.1073/pnas.0812431106 [published Online First: 2009/06/03]
92. Ramachandran G, Tennant SM, Boyd MA, et al. Functional Activity of Antibodies Directed towards Flagellin Proteins of Non-Typhoidal Salmonella. *PLoS One* 2016;11(3):e0151875. doi: 10.1371/journal.pone.0151875 [published Online First: 20160321]
93. Lee SJ, Benoun J, Sheridan BS, et al. Dual Immunization with SseB/Flagellin Provides Enhanced Protection against Salmonella Infection Mediated by Circulating Memory Cells. *J Immunol* 2017;199(4):1353-61. doi: 10.4049/jimmunol.1601357 [published Online First: 2017/07/16]
94. Reynolds CJ, Jones C, Blohmke CJ, et al. The serodominant secreted effector protein of Salmonella, SseB, is a strong CD4 antigen containing an immunodominant

epitope presented by diverse HLA class II alleles. *Immunology* 2014;143(3):438-46. doi: 10.1111/imm.12327
95. Bertani B, Ruiz N. Function and Biogenesis of Lipopolysaccharides. *EcoSal Plus* 2018;8(1) doi: 10.1128/ecosalplus.ESP-0001-2018
96. Murray GL, Attridge SR, Morona R. Inducible serum resistance in Salmonella typhimurium is dependent on wzz(fepE)-regulated very long O antigen chains. *Microbes Infect* 2005;7(13):1296-304. doi: 10.1016/j.micinf.2005.04.015 [published Online First: 20050609]
97. Ilg K, Endt K, Misselwitz B, et al. O-antigen-negative Salmonella enterica serovar Typhimurium is attenuated in intestinal colonization but elicits colitis in streptomycin-treated mice. *Infect Immun* 2009;77(6):2568-75. doi: 10.1128/IAI.01537-08 [published Online First: 2009/04/15]
98. Elias SC, Muthumbi E, Mwanzu A, et al. Complementary measurement of nontyphoidal Salmonella-specific IgG and IgA antibodies in oral fluid and serum. *Heliyon* 2023;9(1):e12071. doi: 10.1016/j.heliyon.2022.e12071 [published Online First: 20221215]
99. Micoli F, Rondini S, Alfini R, et al. Comparative immunogenicity and efficacy of equivalent outer membrane vesicle and glycoconjugate vaccines against nontyphoidal Salmonella. *Proc Natl Acad Sci U S A* 2018;115(41):10428-33. doi: 10.1073/pnas.1807655115 [published Online First: 2018/09/29]
100. Svenson SB, Lindberg AA. Artificial Salmonella vaccines: Salmonella typhimurium O-antigen-specific oligosaccharide-protein conjugates elicit protective antibodies in rabbits and mice. *Infect Immun* 1981;32(2):490-6. doi: 10.1128/iai.32.2.490-496.1981
101. Kurtz JR, Goggins JA, McLachlan JB. Salmonella infection: Interplay between the bacteria and host immune system. *Immunol Lett* 2017;190:42-50. doi: 10.1016/j.imlet.2017.07.006 [published Online First: 2017/07/20]
102. Marshall JM, Gunn JS. The O-Antigen Capsule of Salmonella enterica Serovar Typhimurium Facilitates Serum Resistance and Surface Expression of FliC. *Infect Immun* 2015;83(10):3946-59. doi: 10.1128/IAI.00634-15 [published Online First: 2015/07/22]
103. Perez-Toledo M, Valero-Pacheco N, Pastelin-Palacios R, et al. Salmonella Typhi Porins OmpC and OmpF Are Potent Adjuvants for T-Dependent and T-Independent Antigens. *Front Immunol* 2017;8:230. doi: 10.3389/fimmu.2017.00230 [published Online First: 2017/03/25]
104. Rondini S, Micoli F, Lanzilao L, et al. Design of glycoconjugate vaccines against invasive African Salmonella enterica serovar Typhimurium. *Infect Immun* 2015;83(3):996-1007. doi: 10.1128/IAI.03079-14 [published Online First: 2014/12/31]
105. Barat S, Willer Y, Rizos K, et al. Immunity to intracellular Salmonella depends on surface-associated antigens. *PLoS Pathog* 2012;8(10):e1002966. doi: 10.1371/journal.ppat.1002966 [published Online First: 2012/10/25]
106. Napolitani G, Kurupati P, Teng KWW, et al. Clonal analysis of Salmonella-specific effector T cells reveals serovar-specific and cross-reactive T cell responses. *Nat Immunol* 2018;19(7):742-54. doi: 10.1038/s41590-018-0133-z [published Online First: 2018/06/22]

107. Cummings KJ, Rodriguez-Rivera LD, Capel MB, et al. Short communication: Oral and intranasal administration of a modified-live Salmonella Dublin vaccine in dairy calves: Clinical efficacy and serologic response. *J Dairy Sci* 2019;102(4):3474-79. doi: 10.3168/jds.2018-14892 [published Online First: 20190207]
108. Farzan A, Friendship RM. A clinical field trial to evaluate the efficacy of vaccination in controlling Salmonella infection and the association of Salmonella-shedding and weight gain in pigs. *Can J Vet Res* 2010;74(4):258-63.
109. Jelinek PD, Robertson GM, Millar C. Vaccination of sheep with a live gal e mutant of Salmonella typhimurium. *Aust Vet J* 1982;59(1):31-2. doi: 10.1111/j.1751-0813.1982.tb02710.x
110. Methner U. Immunisation of chickens with live Salmonella vaccines - Role of booster vaccination. *Vaccine* 2018;36(21):2973-77. doi: 10.1016/j.vaccine.2018.04.041 [published Online First: 20180422]
111. O'Brien SJ. The "decline and fall" of nontyphoidal salmonella in the United kingdom. *Clin Infect Dis* 2013;56(5):705-10. doi: 10.1093/cid/cis967 [published Online First: 2012/11/21]
112. Veterinary Medicines Directorate. Product Information Database. vmd.defra.gov.uk, 2023.
113. Singh BR. Salmonella Vaccines for Animals and Birds and Their Future Perspective. *The Open Vaccine Journal* 2009:100-12. doi: 10.2174/1875035401002010100
114. Hindle Z, Chatfield SN, Phillimore J, et al. Characterization of Salmonella enterica derivatives harboring defined aroC and Salmonella pathogenicity island 2 type III secretion system (ssaV) mutations by immunization of healthy volunteers. *Infect Immun* 2002;70(7):3457-67. doi: 10.1128/iai.70.7.3457-3467.2002 [published Online First: 2002/06/18]
115. Chen W. NCT05525546 Salmonella Conjugates CVD 2000: Study of Responses to Vaccination With Trivalent Salmonella Conjugate Vaccine to Prevent Invasive Salmonella Disease. 27/02/2023 ed. ClinicalTrials.gov: Bethesda (MD): National Library of Medicine (US), 2023.
116. Tennant SM, MacLennan CA, Simon R, et al. Nontyphoidal salmonella disease: Current status of vaccine research and development. *Vaccine* 2016;34(26):2907-10. doi: 10.1016/j.vaccine.2016.03.072 [published Online First: 2016/04/02]
117. MacLennan CA, Martin LB, Micoli F. Vaccines against invasive Salmonella disease: current status and future directions. *Hum Vaccin Immunother* 2014;10(6):1478-93. doi: 10.4161/hv.29054 [published Online First: 2014/05/09]
118. Zhang F, Boerth EM, Gong J, et al. A Bivalent MAPS Vaccine Induces Protective Antibody Responses against Salmonella Typhi and Paratyphi A. *Vaccines (Basel)* 2022;11(1) doi: 10.3390/vaccines11010091 [published Online First: 20221230]
119. Acevedo R, Fernandez S, Zayas C, et al. Bacterial outer membrane vesicles and vaccine applications. *Front Immunol* 2014;5:121. doi: 10.3389/fimmu.2014.00121 [published Online First: 2014/04/10]
120. Valguarnera E, Feldman MF. Glycoengineered Outer Membrane Vesicles as a Platform for Vaccine Development. *Methods Enzymol* 2017;597:285-310. doi: 10.1016/bs.mie.2017.06.032 [published Online First: 2017/09/25]
121. Micoli F, MacLennan CA. Outer membrane vesicle vaccines. *Semin Immunol* 2020;50:101433. doi: 10.1016/j.smim.2020.101433 [published Online First: 20201209]

122. Maggiore L, Yu L, Omasits U, et al. Quantitative proteomic analysis of Shigella flexneri and Shigella sonnei Generalized Modules for Membrane Antigens (GMMA) reveals highly pure preparations. *Int J Med Microbiol* 2016;306(2):99-108. doi: 10.1016/j.ijmm.2015.12.003 [published Online First: 2016/01/10]
123. Kim SI, Kim S, Kim E, et al. Secretion of Salmonella Pathogenicity Island 1-Encoded Type III Secretion System Effectors by Outer Membrane Vesicles in Salmonella enterica Serovar Typhimurium. *Front Microbiol* 2018;9:2810. doi: 10.3389/fmicb.2018.02810 [published Online First: 2018/12/12]
124. Rappazzo CG, Watkins HC, Guarino CM, et al. Recombinant M2e outer membrane vesicle vaccines protect against lethal influenza A challenge in BALB/c mice. *Vaccine* 2016;34(10):1252-8. doi: 10.1016/j.vaccine.2016.01.028 [published Online First: 20160129]
125. Bai J, Kim SI, Ryu S, et al. Identification and characterization of outer membrane vesicle-associated proteins in Salmonella enterica serovar Typhimurium. *Infect Immun* 2014;82(10):4001-10. doi: 10.1128/IAI.01416-13 [published Online First: 2014/06/18]
126. Rossi O, Caboni M, Negrea A, et al. Toll-Like Receptor Activation by Generalized Modules for Membrane Antigens from Lipid A Mutants of Salmonella enterica Serovars Typhimurium and Enteritidis. *Clin Vaccine Immunol* 2016;23(4):304-14. doi: 10.1128/CVI.00023-16 [published Online First: 2016/02/13]
127. Balhuizen MD, Veldhuizen EJA, Haagsman HP. Outer Membrane Vesicle Induction and Isolation for Vaccine Development. *Front Microbiol* 2021;12:629090. doi: 10.3389/fmicb.2021.629090 [published Online First: 20210204]
128. Petousis-Harris H. Impact of meningococcal group B OMV vaccines, beyond their brief. *Hum Vaccin Immunother* 2018;14(5):1058-63. doi: 10.1080/21645515.2017.1381810 [published Online First: 2017/10/20]
129. Launay O, Lewis DJM, Anemona A, et al. Safety Profile and Immunologic Responses of a Novel Vaccine Against Shigella sonnei Administered Intramuscularly, Intradermally and Intranasally: Results From Two Parallel Randomized Phase 1 Clinical Studies in Healthy Adult Volunteers in Europe. *EBioMedicine* 2017;22:164-72. doi: 10.1016/j.ebiom.2017.07.013 [published Online First: 2017/07/25]
130. Launay O, Ndiaye AGW, Conti V, et al. Booster Vaccination With GVGH Shigella sonnei 1790GAHB GMMA Vaccine Compared to Single Vaccination in Unvaccinated Healthy European Adults: Results From a Phase 1 Clinical Trial. *Front Immunol* 2019;10:335. doi: 10.3389/fimmu.2019.00335 [published Online First: 2019/03/25]
131. Obiero CW, Ndiaye AGW, Scire AS, et al. A Phase 2a Randomized Study to Evaluate the Safety and Immunogenicity of the 1790GAHB Generalized Modules for Membrane Antigen Vaccine against Shigella sonnei Administered Intramuscularly to Adults from a Shigellosis-Endemic Country. *Front Immunol* 2017;8:1884. doi: 10.3389/fimmu.2017.01884 [published Online First: 2018/01/30]
132. Frenck RW, Jr., Conti V, Ferruzzi P, et al. Efficacy, safety, and immunogenicity of the Shigella sonnei 1790GAHB GMMA candidate vaccine: Results from a phase 2b randomized, placebo-controlled challenge study in adults. *EClinicalMedicine* 2021;39:101076. doi: 10.1016/j.eclinm.2021.101076 [published Online First: 20210813]

133. Semchenko EA, Seib KL. Outer membrane vesicle vaccines for Neisseria gonorrhoeae. *Nat Rev Urol* 2022;19(1):5-6. doi: 10.1038/s41585-021-00534-5
134. Jun SH, Lee JH, Kim BR, et al. Acinetobacter baumannii outer membrane vesicles elicit a potent innate immune response via membrane proteins. *PLoS One* 2013;8(8):e71751. doi: 10.1371/journal.pone.0071751 [published Online First: 20130814]
135. Zare Banadkoki E, Rasooli I, Ghazanfari T, et al. Pseudomonas aeruginosa PAO1 outer membrane vesicles-diphtheria toxoid conjugate as a vaccine candidate in a murine burn model. *Sci Rep* 2022;12(1):22324. doi: 10.1038/s41598-022-26846-z [published Online First: 20221224]
136. Mancini F, Alfini R, Caradonna V, et al. Exploring the Role of GMMA Components in the Immunogenicity of a 4-Valent Vaccine against Shigella. *Int J Mol Sci* 2023;24(3) doi: 10.3390/ijms24032742 [published Online First: 20230201]
137. MacLennan CA, Stanaway J, Grow S, et al. Salmonella Combination Vaccines: Moving Beyond Typhoid. *Open Forum Infect Dis* 2023;10(Suppl 1):S58-S66. doi: 10.1093/ofid/ofad041 [published Online First: 20230602]
138. Dale H, Chirwa E, Patel P, et al. Understanding the epidemiology of iNTS disease in Africa in preparation for future iNTS- vaccine studies in endemic countries: Seroepidemiology in Africa of iNTS (SAiNTS) Study Protocol [Version 9.0]. *Wellcome Open Research* 2023;8 doi: 10.12688/wellcomeopenres.18054.1
139. SCLAVO Vaccines Association. Vacc-iNTS 2023 [cited 2023 08/06/2023]. Available from: https://www.sclavo.org/projects/vacc-ints/#:~:text=Vacc%2DiNTS%20is%20a%20consortium,from%20academic%20and%20research%20institutions.
140. Vacc-iNTS Project. Vacc-iNTS Project 2023 [Available from: https://vacc-intsproject.eu accessed 09/11/2023 2023.
141. Pedvac-iNTS. The Pedvac-INTS Project 2023 [cited 2023 08/06/2023]. Available from: https://pedvac-ints.eu.
142. Shakya M, Colin-Jones R, Theiss-Nyland K, et al. Phase 3 Efficacy Analysis of a Typhoid Conjugate Vaccine Trial in Nepal. *N Engl J Med* 2019;381(23):2209-18. doi: 10.1056/NEJMoa1905047 [published Online First: 2019/12/05]
143. Darton TC, Jones C, Blohmke CJ, et al. Using a Human Challenge Model of Infection to Measure Vaccine Efficacy: A Randomised, Controlled Trial Comparing the Typhoid Vaccines M01ZH09 with Placebo and Ty21a. *PLoS Negl Trop Dis* 2016;10(8):e0004926. doi: 10.1371/journal.pntd.0004926 [published Online First: 2016/08/18]
144. Smith C, Smith E, Chiu C, et al. The Challenge Non-Typhoidal Salmonella (CHANTS) Consortium: Development of a non-typhoidal Salmonella controlled human infection model: Report from a consultation group workshop, 05 July 2022, London, UK [version 1; peer review: 1 approved]. *Wellcome Open Research* 2023;8(111) doi: 10.12688/wellcomeopenres.19012.1
145. Giersing BK, Porter CK, Kotloff K, et al. How can controlled human infection models accelerate clinical development and policy pathways for vaccines against Shigella? *Vaccine* 2019;37(34):4778-83. doi: 10.1016/j.vaccine.2019.03.036 [published Online First: 2019/07/31]
146. Gerke C, Colucci AM, Giannelli C, et al. Production of a Shigella sonnei Vaccine Based on Generalized Modules for Membrane Antigens (GMMA), 1790GAHB. *PLoS One*

2015;10(8):e0134478. doi: 10.1371/journal.pone.0134478 [published Online First: 2015/08/08]
147. Thermofisher-Scientific. User Guide: EasyPep Mini MS Sample Prep Kit. 2020
148. Cox J, Mann M. MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. *Nat Biotechnol* 2008;26(12):1367-72. doi: 10.1038/nbt.1511 [published Online First: 20081130]
149. Team RC. R: A language and environment for statistical computing. R Foundation for Statistical Computing,. Vienna, Austria., 2022.
150. RStudio: Integrated Development for R [program]: RStudio, PBC, Boston, MA 2020.
151. Park SE, Toy T, Cruz Espinoza LM, et al. The Severe Typhoid Fever in Africa Program: Study Design and Methodology to Assess Disease Severity, Host Immunity, and Carriage Associated With Invasive Salmonellosis. *Clin Infect Dis* 2019;69(Suppl 6):S422-S34. doi: 10.1093/cid/ciz715 [published Online First: 2019/10/31]
152. Feasey NA, Masesa C, Jassi C, et al. Three Epidemics of Invasive Multidrug-Resistant Salmonella Bloodstream Infection in Blantyre, Malawi, 1998-2014. *Clin Infect Dis* 2015;61 Suppl 4:S363-71. doi: 10.1093/cid/civ691 [published Online First: 2015/10/10]
153. Gordon MA, Graham SM. Invasive salmonellosis in Malawi. *J Infect Dev Ctries* 2008;2(6):438-42. doi: 10.3855/jidc.158 [published Online First: 2008/01/01]
154. Dobinson HC, Gibani MM, Jones C, et al. Evaluation of the Clinical and Microbiological Response to Salmonella Paratyphi A Infection in the First Paratyphoid Human Challenge Model. *Clin Infect Dis* 2017;64(8):1066-73. doi: 10.1093/cid/cix042 [published Online First: 2017/02/06]
155. Gibani MM, Jin C, Shrestha S, et al. Homologous and heterologous re-challenge with Salmonella Typhi and Salmonella Paratyphi A in a randomised controlled human infection model. *PLoS Negl Trop Dis* 2020;14(10):e0008783. doi: 10.1371/journal.pntd.0008783 [published Online First: 2020/10/21]
156. Schlottmann SA, Jain N, Chirmule N, et al. A novel chemistry for conjugating pneumococcal polysaccharides to Luminex microspheres. *J Immunol Methods* 2006;309(1-2):75-85. doi: 10.1016/j.jim.2005.11.019 [published Online First: 2006/02/02]
157. Ackerman ME, Moldt B, Wyatt RT, et al. A robust, high-throughput assay to determine the phagocytic activity of clinical antibody samples. *J Immunol Methods* 2011;366(1-2):8-19. doi: 10.1016/j.jim.2010.12.016 [published Online First: 20101227]
158. Barrett JR, Belij-Rammerstorfer S, Dold C, et al. Phase 1/2 trial of SARS-CoV-2 vaccine ChAdOx1 nCoV-19 with a booster dose induces multifunctional antibody responses. *Nat Med* 2021;27(2):279-88. doi: 10.1038/s41591-020-01179-4 [published Online First: 20201217]
159. Ao TT, Feasey NA, Gordon MA, et al. Global burden of invasive nontyphoidal Salmonella disease, 2010. *Emerg Infect Dis* 2015;21(6) doi: 10.3201/eid2106.140999 [published Online First: 2015/04/11]
160. Crump JA, Sjolund-Karlsson M, Gordon MA, et al. Epidemiology, Clinical Presentation, Laboratory Diagnosis, Antimicrobial Resistance, and Antimicrobial Management of Invasive Salmonella Infections. *Clin Microbiol Rev* 2015;28(4):901-37. doi: 10.1128/CMR.00002-15 [published Online First: 2015/07/17]

161. Mancini F, Rossi O, Necchi F, et al. OMV Vaccines and the Role of TLR Agonists in Immune Response. *Int J Mol Sci* 2020;21(12) doi: 10.3390/ijms21124416 [published Online First: 20200621]
162. McNeil MM, DeStefano F. Vaccine-associated hypersensitivity. *J Allergy Clin Immunol* 2018;141(2):463-72. doi: 10.1016/j.jaci.2017.12.971
163. Scott AJ, Oyler BL, Goodlett DR, et al. Lipid A structural modifications in extreme conditions and identification of unique modifying enzymes to define the Toll-like receptor 4 structure-activity relationship. *Biochim Biophys Acta Mol Cell Biol Lipids* 2017;1862(11):1439-50. doi: 10.1016/j.bbalip.2017.01.004 [published Online First: 2017/01/22]
164. Micoli F, Nakakana UN, Berlanda Scorza F. Towards a Four-Component GMMA-Based Vaccine against Shigella. *Vaccines (Basel)* 2022;10(2) doi: 10.3390/vaccines10020328 [published Online First: 20220218]
165. EMC. Bexsero Meningococcal Group B vaccine for injection in pre-filled syringe 2022 [Available from: https://www.medicines.org.uk/emc/product/5168/smpc.
166. UK Health Security Agency. Weekly national Influenza and COVID-19 surveillance report, Week 14 report (up to week 13 data) 6 April 2023 2023 [Available from: https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/1149331/Weekly_Flu_and_COVID-19_report_w14.pdf.
167. Group CTF. Recommendations related to contraception and pregnancy testing in clinical trials. 2014
168. Mold M, Shardlow E, Exley C. Insight into the cellular fate and toxicity of aluminium adjuvants used in clinically approved human vaccinations. *Sci Rep* 2016;6:31578. doi: 10.1038/srep31578 [published Online First: 20160812]
169. Petrovsky N. Comparative Safety of Vaccine Adjuvants: A Summary of Current Evidence and Future Needs. *Drug Saf* 2015;38(11):1059-74. doi: 10.1007/s40264-015-0350-4 [published Online First: 2015/10/09]
170. Deghmane AE, Taha MK. Product review on the IMD serogroup B vaccine Bexsero(R). *Hum Vaccin Immunother* 2022;18(1):2020043. doi: 10.1080/21645515.2021.2020043 [published Online First: 20220222]
171. Nokleby H, Aavitsland P, O'Hallahan J, et al. Safety review: two outer membrane vesicle (OMV) vaccines against systemic Neisseria meningitidis serogroup B disease. *Vaccine* 2007;25(16):3080-4. doi: 10.1016/j.vaccine.2007.01.022 [published Online First: 20070122]
172. Dowling DJ, Sanders H, Cheng WK, et al. A Meningococcal Outer Membrane Vesicle Vaccine Incorporating Genetically Attenuated Endotoxin Dissociates Inflammation from Immunogenicity. *Front Immunol* 2016;7:562. doi: 10.3389/fimmu.2016.00562 [published Online First: 20161208]
173. Bauernfeind S, Salzberger B, Hitzenbichler F, et al. Association between Reactogenicity and Immunogenicity after Vaccination with BNT162b2. *Vaccines (Basel)* 2021;9(10) doi: 10.3390/vaccines9101089 [published Online First: 20210927]
174. Martin CA, Nazareth J, Jarkhi A, et al. Ethnic differences in cellular and humoral immune responses to SARS-CoV-2 vaccination in UK healthcare workers: a cross-sectional analysis. *EClinicalMedicine* 2023;58:101926. doi: 10.1016/j.eclinm.2023.101926 [published Online First: 20230404]

175. Mastroeni P, Grant AJ. Spread of Salmonella enterica in the body during systemic infection: unravelling host and pathogen determinants. *Expert Rev Mol Med* 2011;13:e12. doi: 10.1017/S1462399411001840 [published Online First: 20110411]
176. Vossenkamper A, Blair PA, Safinia N, et al. A role for gut-associated lymphoid tissue in shaping the human B cell repertoire. *J Exp Med* 2013;210(9):1665-74. doi: 10.1084/jem.20122465 [published Online First: 20130812]
177. Richards AF, Doering JE, Lozito SA, et al. Inhibition of invasive salmonella by orally administered IgA and IgG monoclonal antibodies. *PLoS Negl Trop Dis* 2020;14(3):e0007803. doi: 10.1371/journal.pntd.0007803 [published Online First: 2020/03/24]
178. Aase A, Sommerfelt H, Petersen LB, et al. Salivary IgA from the sublingual compartment as a novel noninvasive proxy for intestinal immune induction. *Mucosal Immunol* 2016;9(4):884-93. doi: 10.1038/mi.2015.107 [published Online First: 20151028]
179. Wines BD, Billings H, McLean MR, et al. Antibody Functional Assays as Measures of Fc Receptor-Mediated Immunity to HIV - New Technologies and their Impact on the HIV Vaccine Field. *Curr HIV Res* 2017;15(3):202-15. doi: 10.2174/1570162X15666170320112247
180. Ndungo E, Pasetti MF. Functional antibodies as immunological endpoints to evaluate protective immunity against Shigella. *Hum Vaccin Immunother* 2020;16(1):197-205. doi: 10.1080/21645515.2019.1640427 [published Online First: 20190801]
181. Tay MZ, Wiehe K, Pollara J. Antibody-Dependent Cellular Phagocytosis in Antiviral Immune Responses. *Front Immunol* 2019;10:332. doi: 10.3389/fimmu.2019.00332 [published Online First: 20190228]
182. Dale DC, Boxer L, Liles WC. The phagocytes: neutrophils and monocytes. *Blood* 2008;112(4):935-45. doi: 10.1182/blood-2007-12-077917
183. Horowitz A, Stegmann KA, Riley EM. Activation of natural killer cells during microbial infections. *Front Immunol* 2011;2:88. doi: 10.3389/fimmu.2011.00088 [published Online First: 20120103]
184. Naess LM, Oftung F, Aase A, et al. Human T-cell responses after vaccination with the Norwegian group B meningococcal outer membrane vesicle vaccine. *Infect Immun* 1998;66(3):959-65. doi: 10.1128/IAI.66.3.959-965.1998
185. Cruz SC, Souza SL, Cruz AC, et al. Human antibody and memory B and T-cell responses after primary and booster immunisation against Neisseria meningitidis B. *Vaccine* 2011;29(43):7387-94. doi: 10.1016/j.vaccine.2011.07.072 [published Online First: 20110729]
186. Wei J, Stoesser N, Matthews PC, et al. Antibody responses to SARS-CoV-2 vaccines in 45,965 adults from the general population of the United Kingdom. *Nat Microbiol* 2021;6(9):1140-49. doi: 10.1038/s41564-021-00947-3 [published Online First: 20210721]
187. Molbak K, Simonsen J, Jorgensen CS, et al. Seroincidence of human infections with nontyphoid Salmonella compared with data from public health surveillance and food animals in 13 European countries. *Clin Infect Dis* 2014;59(11):1599-606. doi: 10.1093/cid/ciu627 [published Online First: 2014/08/08]

188. Vugia DJ, Samuel M, Farley MM, et al. Invasive Salmonella infections in the United States, FoodNet, 1996-1999: incidence, serotype distribution, and outcome. *Clin Infect Dis* 2004;38 Suppl 3:S149-56. doi: 10.1086/381581
189. Kuhn KG, Falkenhorst G, Ceper TH, et al. Detecting non-typhoid Salmonella in humans by ELISAs: a literature review. *J Med Microbiol* 2012;61(Pt 1):1-7. doi: 10.1099/jmm.0.034447-0 [published Online First: 20111027]
190. UK Health Security Agency. Non-typhoidal Salmonella data 2010 to 2019 2021 [Available from: https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/1026208/salmonella-annual-report-2019.pdf.
191. Troncoso G, Sanchez S, Moreda M, et al. Antigenic cross-reactivity between outer membrane proteins of Neisseria meningitidis and commensal Neisseria species. *FEMS Immunol Med Microbiol* 2000;27(2):103-9. doi: 10.1111/j.1574-695X.2000.tb01419.x
192. Simon AK, Hollander GA, McMichael A. Evolution of the immune system in humans from infancy to old age. *Proc Biol Sci* 2015;282(1821):20143085. doi: 10.1098/rspb.2014.3085 [published Online First: 2015/12/25]
193. Brandtzaeg P. Secretory immunity with special reference to the oral cavity. *J Oral Microbiol* 2013;5 doi: 10.3402/jom.v5i0.20401 [published Online First: 20130311]
194. Su F, Patel GB, Hu S, et al. Induction of mucosal immunity through systemic immunization: Phantom or reality? *Hum Vaccin Immunother* 2016;12(4):1070-9. doi: 10.1080/21645515.2015.1114195 [published Online First: 20160111]
195. Herremans TMPT, Reimerink JHJ, Buisman AM, et al. Induction of Mucosal Immunity by Inactivated Poliovirus Vaccine Is Dependent on Previous Mucosal Contact with Live Virus. *The Journal of Immunology* 1999;162(8):5011-18. doi: 10.4049/jimmunol.162.8.5011
196. Dahora LC, Jin C, Spreng RL, et al. IgA and IgG1 Specific to Vi Polysaccharide of Salmonella Typhi Correlate With Protection Status in a Typhoid Fever Controlled Human Infection Model. *Front Immunol* 2019;10:2582. doi: 10.3389/fimmu.2019.02582 [published Online First: 20191101]
197. Tanoue T, Umesaki Y, Honda K. Immune responses to gut microbiota-commensals and pathogens. *Gut Microbes* 2010;1(4):224-33. doi: 10.4161/gmic.1.4.12613 [published Online First: 20100607]
198. Tagliabue A, Villa L, De Magistris MT, et al. IgA-driven T cell-mediated anti-bacterial immunity in man after live oral Ty 21a vaccine. *J Immunol* 1986;137(5):1504-10.
199. Fiorino F, Pettini E, Koeberling O, et al. Long-Term Anti-Bacterial Immunity against Systemic Infection by Salmonella enterica Serovar Typhimurium Elicited by a GMMA-Based Vaccine. *Vaccines (Basel)* 2021;9(5) doi: 10.3390/vaccines9050495 [published Online First: 20210512]
200. Sheikh-Mohamed S, Isho B, Chao GYC, et al. Systemic and mucosal IgA responses are variably induced in response to SARS-CoV-2 mRNA vaccination and are associated with protection against subsequent infection. *Mucosal Immunol* 2022;15(5):799-808. doi: 10.1038/s41385-022-00511-0 [published Online First: 20220425]
201. Costa LF, Mol JP, Silva AP, et al. Iron acquisition pathways and colonization of the inflamed intestine by Salmonella enterica serovar Typhimurium. *Int J Med Microbiol* 2016;306(8):604-10. doi: 10.1016/j.ijmm.2016.10.004 [published Online First: 2016/10/21]

202. Bemark M, Hazanov H, Stromberg A, et al. Limited clonal relatedness between gut IgA plasma cells and memory B cells after oral immunization. *Nat Commun* 2016;7:12698. doi: 10.1038/ncomms12698 [published Online First: 20160906]
203. Lo Nigro C, Macagno M, Sangiolo D, et al. NK-mediated antibody-dependent cell-mediated cytotoxicity in solid tumors: biological evidence and clinical perspectives. *Ann Transl Med* 2019;7(5):105. doi: 10.21037/atm.2019.01.42
204. Pennock ND, White JT, Cross EW, et al. T cell responses: naive to memory and everything in between. *Adv Physiol Educ* 2013;37(4):273-83. doi: 10.1152/advan.00066.2013
205. Avci FY, Li X, Tsuji M, et al. Carbohydrates and T cells: a sweet twosome. *Semin Immunol* 2013;25(2):146-51. doi: 10.1016/j.smim.2013.05.005 [published Online First: 20130610]
206. Pollard AJ, Bijker EM. A guide to vaccinology: from basic principles to new developments. *Nat Rev Immunol* 2021;21(2):83-100. doi: 10.1038/s41577-020-00479-7 [published Online First: 20201222]
207. Parker DC. T cell-dependent B cell activation. *Annu Rev Immunol* 1993;11:331-60. doi: 10.1146/annurev.iy.11.040193.001555
208. de Roux A, Schmole-Thoma B, Siber GR, et al. Comparison of pneumococcal conjugate polysaccharide and free polysaccharide vaccines in elderly adults: conjugate vaccine elicits improved antibacterial immune responses and immunological memory. *Clin Infect Dis* 2008;46(7):1015-23. doi: 10.1086/529142
209. Falkenhorst G, Remschmidt C, Harder T, et al. Effectiveness of the 23-Valent Pneumococcal Polysaccharide Vaccine (PPV23) against Pneumococcal Disease in the Elderly: Systematic Review and Meta-Analysis. *PLoS One* 2017;12(1):e0169368. doi: 10.1371/journal.pone.0169368 [published Online First: 20170106]
210. Pollard AJ, Perrett, K.P., Beverley, P.C. Maintaining protection against invasive bacteria with protein–polysaccharide conjugate vaccines. *Nature Reviews Immunology* 2009;9:213–20 doi: https://doi.org/10.1038/nri2494
211. Schwechheimer C, Kuehn MJ. Outer-membrane vesicles from Gram-negative bacteria: biogenesis and functions. *Nat Rev Microbiol* 2015;13(10):605-19. doi: 10.1038/nrmicro3525
212. Hu R, Li J, Zhao Y, et al. Exploiting bacterial outer membrane vesicles as a cross-protective vaccine candidate against avian pathogenic Escherichia coli (APEC). *Microb Cell Fact* 2020;19(1):119. doi: 10.1186/s12934-020-01372-7 [published Online First: 20200603]
213. Reimer SL, Beniac DR, Hiebert SL, et al. Comparative Analysis of Outer Membrane Vesicle Isolation Methods With an Escherichia coli tolA Mutant Reveals a Hypervesiculating Phenotype With Outer-Inner Membrane Vesicle Content. *Front Microbiol* 2021;12:628801. doi: 10.3389/fmicb.2021.628801 [published Online First: 20210305]
214. Socorro Ruiz-Palma MD, Avila-Calderon ED, Aguilera-Arreola MG, et al. Comparative proteomic analysis of outer membrane vesicles from Brucella suis, Brucella ovis, Brucella canis and Brucella neotomae. *Arch Microbiol* 2021;203(4):1611-26. doi: 10.1007/s00203-020-02170-w [published Online First: 20210111]

215. Kwon SO, Gho YS, Lee JC, et al. Proteome analysis of outer membrane vesicles from a clinical Acinetobacter baumannii isolate. *FEMS Microbiol Lett* 2009;297(2):150-6. doi: 10.1111/j.1574-6968.2009.01669.x [published Online First: 20090603]
216. Choi DS, Kim DK, Choi SJ, et al. Proteomic analysis of outer membrane vesicles derived from Pseudomonas aeruginosa. *Proteomics* 2011;11(16):3424-9. doi: 10.1002/pmic.201000212
217. Alaniz RC, Deatherage BL, Lara JC, et al. Membrane vesicles are immunogenic facsimiles of Salmonella typhimurium that potently activate dendritic cells, prime B and T cell responses, and stimulate protective immunity in vivo. *J Immunol* 2007;179(11):7692-701. doi: 10.4049/jimmunol.179.11.7692 [published Online First: 2007/11/21]
218. Bruno VM, Hannemann S, Lara-Tejero M, et al. Salmonella Typhimurium type III secretion effectors stimulate innate immune responses in cultured epithelial cells. *PLoS Pathog* 2009;5(8):e1000538. doi: 10.1371/journal.ppat.1000538 [published Online First: 20090807]
219. Ramos-Morales F. Impact ofSalmonella entericaType III Secretion System Effectors on the Eukaryotic Host Cell. *ISRN Cell Biology* 2012;2012:1-36. doi: 10.5402/2012/787934
220. Hussain S, Ouyang P, Zhu Y, et al. Type 3 secretion system 1 of Salmonella typhimurium and its inhibitors: a novel strategy to combat salmonellosis. *Environ Sci Pollut Res Int* 2021;28(26):34154-66. doi: 10.1007/s11356-021-13986-4 [published Online First: 20210509]
221. Li S, Chen DQ, Ji L, et al. Development of Different Methods for Preparing Acinetobacter baumannii Outer Membrane Vesicles Vaccine: Impact of Preparation Method on Protective Efficacy. *Front Immunol* 2020;11:1069. doi: 10.3389/fimmu.2020.01069 [published Online First: 20200623]
222. Kim OY, Hong BS, Park KS, et al. Immunization with Escherichia coli outer membrane vesicles protects bacteria-induced lethality via Th1 and Th17 cell responses. *J Immunol* 2013;190(8):4092-102. doi: 10.4049/jimmunol.1200742 [published Online First: 20130320]
223. Enayatkhani M, Hasaniazad M, Faezi S, et al. Reverse vaccinology approach to design a novel multi-epitope vaccine candidate against COVID-19: an in silico study. *J Biomol Struct Dyn* 2021;39(8):2857-72. doi: 10.1080/07391102.2020.1756411 [published Online First: 20200502]
224. Esmailnia E, Amani J, Gargari SLM. Identification of novel vaccine candidate against Salmonella enterica serovar Typhi by reverse vaccinology method and evaluation of its immunization. *Genomics* 2020;112(5):3374-81. doi: 10.1016/j.ygeno.2020.06.022 [published Online First: 20200619]
225. Solanki V, Tiwari M, Tiwari V. Prioritization of potential vaccine targets using comparative proteomics and designing of the chimeric multi-epitope vaccine against Pseudomonas aeruginosa. *Sci Rep* 2019;9(1):5240. doi: 10.1038/s41598-019-41496-4 [published Online First: 20190327]
226. Yu NY, Wagner JR, Laird MR, et al. PSORTb 3.0: improved protein subcellular localization prediction with refined localization subcategories and predictive capabilities for all prokaryotes. *Bioinformatics* 2010;26(13):1608-15. doi: 10.1093/bioinformatics/btq249 [published Online First: 20100513]

227. He Y, Xiang Z, Mobley HL. Vaxign: the first web-based vaccine design program for reverse vaccinology and applications for vaccine development. *J Biomed Biotechnol* 2010;2010:297505. doi: 10.1155/2010/297505 [published Online First: 20100704]
228. Jespersen MC, Peters B, Nielsen M, et al. BepiPred-2.0: improving sequence-based B-cell epitope prediction using conformational epitopes. *Nucleic Acids Res* 2017;45(W1):W24-W29. doi: 10.1093/nar/gkx346
229. Dhanda SK, Karosiene E, Edwards L, et al. Predicting HLA CD4 Immunogenicity in Human Populations. *Front Immunol* 2018;9:1369. doi: 10.3389/fimmu.2018.01369 [published Online First: 20180614]
230. Segal Y, Shoenfeld Y. Vaccine-induced autoimmunity: the role of molecular mimicry and immune crossreaction. *Cell Mol Immunol* 2018;15(6):586-94. doi: 10.1038/cmi.2017.151 [published Online First: 20180305]
231. Soltan MA, Elbassiouny N, Gamal H, et al. In Silico Prediction of a Multitope Vaccine against Moraxella catarrhalis: Reverse Vaccinology and Immunoinformatics. *Vaccines (Basel)* 2021;9(6) doi: 10.3390/vaccines9060669 [published Online First: 20210618]
232. Jespersen MC, Mahajan S, Peters B, et al. Antibody Specific B-Cell Epitope Predictions: Leveraging Information From Antibody-Antigen Protein Complexes. *Front Immunol* 2019;10:298. doi: 10.3389/fimmu.2019.00298 [published Online First: 20190226]
233. Birch J, Quigley A. The high-throughput production of membrane proteins. *Emerg Top Life Sci* 2021;5(5):655-63. doi: 10.1042/ETLS20210196
234. Ong E, Wong MU, Huffman A, et al. COVID-19 Coronavirus Vaccine Design Using Reverse Vaccinology and Machine Learning. *Front Immunol* 2020;11:1581. doi: 10.3389/fimmu.2020.01581 [published Online First: 20200703]
235. Sachdeva G, Kumar K, Jain P, et al. SPAAN: a software program for prediction of adhesins and adhesin-like proteins using neural networks. *Bioinformatics* 2005;21(4):483-91. doi: 10.1093/bioinformatics/bti028 [published Online First: 20040916]
236. Billeskov R, Beikzadeh B, Berzofsky JA. The effect of antigen dose on T cell-targeting vaccine outcome. *Hum Vaccin Immunother* 2019;15(2):407-11. doi: 10.1080/21645515.2018.1527496 [published Online First: 20181025]
237. UniProt C. UniProt: the universal protein knowledgebase in 2021. *Nucleic Acids Res* 2021;49(D1):D480-D89. doi: 10.1093/nar/gkaa1100
238. Sztein MB. Cell-mediated immunity and antibody responses elicited by attenuated Salmonella enterica Serovar Typhi strains used as live oral vaccines in humans. *Clin Infect Dis* 2007;45 Suppl 1:S15-9. doi: 10.1086/518140
239. Liang L, Juarez S, Nga TV, et al. Immune profiling with a Salmonella Typhi antigen microarray identifies new diagnostic biomarkers of human typhoid. *Sci Rep* 2013;3:1043. doi: 10.1038/srep01043 [published Online First: 2013/01/11]
240. Darton TC, Baker S, Randall A, et al. Identification of Novel Serodiagnostic Signatures of Typhoid Fever Using a Salmonella Proteome Array. *Front Microbiol* 2017;8:1794. doi: 10.3389/fmicb.2017.01794 [published Online First: 2017/10/04]
241. Szklarczyk D, Gable AL, Nastou KC, et al. The STRING database in 2021: customizable protein-protein networks, and functional characterization of user-uploaded

gene/measurement sets. *Nucleic Acids Res* 2021;49(D1):D605-D12. doi: 10.1093/nar/gkaa1074
242. Jensen LJ, Kuhn M, Stark M, et al. STRING 8--a global view on proteins and their functional interactions in 630 organisms. *Nucleic Acids Res* 2009;37(Database issue):D412-6. doi: 10.1093/nar/gkn760 [published Online First: 20081021]
243. Ashburner M, Ball CA, Blake JA, et al. Gene ontology: tool for the unification of biology. The Gene Ontology Consortium. *Nat Genet* 2000;25(1):25-9. doi: 10.1038/75556
244. Kanehisa M, Sato Y, Kawashima M, et al. KEGG as a reference resource for gene and protein annotation. *Nucleic Acids Res* 2016;44(D1):D457-62. doi: 10.1093/nar/gkv1070 [published Online First: 20151017]
245. Ahlmann-Eltze C, Anders S. proDA: Probabilistic Dropout Analysis for Identifying Differentially Abundant Proteins in Label-Free Mass Spectrometry. 2020 doi: 10.1101/661496
246. Ritchie ME, Phipson B, Wu D, et al. limma powers differential expression analyses for RNA-sequencing and microarray studies. *Nucleic Acids Res* 2015;43(7):e47. doi: 10.1093/nar/gkv007 [published Online First: 20150120]
247. Amanakis G, Murphy E. Cyclophilin D: An Integrator of Mitochondrial Function. *Front Physiol* 2020;11:595. doi: 10.3389/fphys.2020.00595 [published Online First: 20200617]
248. Onesti S, Miler, A.D., Brick, P. The crystal structure of the lysyl-tRNA synthetase (LysU) from Escherichia coli. *Structure* 1995;3:163-76.
249. Brohee S, van Helden J. Evaluation of clustering algorithms for protein-protein interaction networks. *BMC Bioinformatics* 2006;7:488. doi: 10.1186/1471-2105-7-488 [published Online First: 20061106]
250. Saleh S, Van Puyvelde S, Staes A, et al. Salmonella Typhi, Paratyphi A, Enteritidis and Typhimurium core proteomes reveal differentially expressed proteins linked to the cell surface and pathogenicity. *PLoS Negl Trop Dis* 2019;13(5):e0007416. doi: 10.1371/journal.pntd.0007416 [published Online First: 20190524]
251. Li M, Gu B, Bomjan R, et al. YggG is a Novel SPI-1 Effector Essential for Salmonella Virulence. *bioRxiv* 2018 doi: 10.1101/300152
252. Fasciano AC, Shaban L, Mecsas J. Promises and Challenges of the Type Three Secretion System Injectisome as an Antivirulence Target. *EcoSal Plus* 2019;8(2) doi: 10.1128/ecosalplus.ESP-0032-2018
253. Kurtz JR, Petersen HE, Frederick DR, et al. Vaccination with a single CD4 T cell peptide epitope from a Salmonella type III-secreted effector protein provides protection against lethal infection. *Infect Immun* 2014;82(6):2424-33. doi: 10.1128/IAI.00052-14 [published Online First: 20140331]
254. Grund ME, Soo JC, Cote CK, et al. Thinking Outside the Bug: Targeting Outer Membrane Proteins for Burkholderia Vaccines. *Cells* 2021;10(3) doi: 10.3390/cells10030495 [published Online First: 20210225]
255. Nie D, Hu Y, Chen Z, et al. Outer membrane protein A (OmpA) as a potential therapeutic target for Acinetobacter baumannii infection. *J Biomed Sci* 2020;27(1):26. doi: 10.1186/s12929-020-0617-7 [published Online First: 20200118]
256. Guan Q, Wang X, Wang X, et al. Recombinant outer membrane protein A induces a protective immune response against Escherichia coli infection in mice. *Appl*

*Microbiol Biotechnol* 2015;99(13):5451-60. doi: 10.1007/s00253-014-6339-6 [published Online First: 20150108]
257. Ansari H, Tahmasebi-Birgani M, Bijanzadeh M, et al. Study of the immunogenicity of outer membrane protein A (ompA) gene from Acinetobacter baumannii as DNA vaccine candidate in vivo. *Iran J Basic Med Sci* 2019;22(6):669-75. doi: 10.22038/ijbms.2019.30799.7427
258. Roy Chowdhury A, Sah S, Varshney U, et al. Salmonella Typhimurium outer membrane protein A (OmpA) renders protection from nitrosative stress of macrophages by maintaining the stability of bacterial outer membrane. *PLoS Pathog* 2022;18(8):e1010708. doi: 10.1371/journal.ppat.1010708 [published Online First: 20220815]
259. Gu H, Liao Y, Zhang J, et al. Rational Design and Evaluation of an Artificial Escherichia coli K1 Protein Vaccine Candidate Based on the Structure of OmpA. *Front Cell Infect Microbiol* 2018;8:172. doi: 10.3389/fcimb.2018.00172 [published Online First: 20180523]
260. Pore D, Chakrabarti MK. Outer membrane protein A (OmpA) from Shigella flexneri 2a: a promising subunit vaccine candidate. *Vaccine* 2013;31(36):3644-50. doi: 10.1016/j.vaccine.2013.05.100 [published Online First: 20130610]
261. Valero-Pacheco N, Blight J, Aldapa-Vega G, et al. Conservation of the OmpC Porin Among Typhoidal and Non-Typhoidal Salmonella Serovars. *Front Immunol* 2019;10:2966. doi: 10.3389/fimmu.2019.02966 [published Online First: 20200109]
262. Ontiveros-Padilla L, Garcia-Lozano A, Tepale-Segura A, et al. CD4+ and CD8+ Circulating Memory T Cells Are Crucial in the Protection Induced by Vaccination with Salmonella Typhi Porins. *Microorganisms* 2021;9(4) doi: 10.3390/microorganisms9040770 [published Online First: 20210407]
263. Hajam IA, Dar PA, Shahnawaz I, et al. Bacterial flagellin-a potent immunomodulatory agent. *Exp Mol Med* 2017;49(9):e373. doi: 10.1038/emm.2017.172 [published Online First: 20170901]
264. Baliban SM, Allen JC, Curtis B, et al. Immunogenicity and Induction of Functional Antibodies in Rabbits Immunized with a Trivalent Typhoid-Invasive Nontyphoidal Salmonella Glycoconjugate Formulation. *Molecules* 2018;23(7) doi: 10.3390/molecules23071749 [published Online First: 2018/07/19]
265. Kenyon WJ, Humphreys S, Roberts M, et al. Periplasmic peptidyl-prolyl isomerases SurA and FkpA play an important role in the starvation-stress response (SSR) of Salmonella enterica serovar Typhimurium. *Antonie Van Leeuwenhoek* 2010;98(1):51-63. doi: 10.1007/s10482-010-9428-2 [published Online First: 20100317]
266. Humphreys S, Rowley G, Stevenson A, et al. Role of periplasmic peptidylprolyl isomerases in Salmonella enterica serovar Typhimurium virulence. *Infect Immun* 2003;71(9):5386-8. doi: 10.1128/IAI.71.9.5386-5388.2003
267. Rojas M, Restrepo-Jimenez P, Monsalve DM, et al. Molecular mimicry and autoimmunity. *J Autoimmun* 2018;95:100-23. doi: 10.1016/j.jaut.2018.10.012 [published Online First: 20181026]
268. Torres VVL, Coggon CF, Wells TJ. Antibody-Dependent Enhancement of Bacterial Disease: Prevalence, Mechanisms, and Treatment. *Infect Immun* 2021;89(4) doi: 10.1128/IAI.00054-21 [published Online First: 20210317]

269. Rijkers GT, van Overveld FJ. The “original antigenic sin” and its relevance for SARS-CoV-2 (COVID-19) vaccination. *Clinical Immunology Communications* 2021;1:13-16. doi: 10.1016/j.clicom.2021.10.001
270. Vojtek I, Buchy P, Doherty TM, et al. Would immunization be the same without cross-reactivity? *Vaccine* 2019;37(4):539-49. doi: 10.1016/j.vaccine.2018.12.005 [published Online First: 2018/12/29]
271. Harari A, Chen Z, Rodriguez AC, et al. Cross-protection of the Bivalent Human Papillomavirus (HPV) Vaccine Against Variants of Genetically Related High-Risk HPV Infections. *J Infect Dis* 2016;213(6):939-47. doi: 10.1093/infdis/jiv519 [published Online First: 20151030]
272. Bruxvoort KJ, Lewnard JA, Chen LH, et al. Prevention of Neisseria gonorrhoeae With Meningococcal B Vaccine: A Matched Cohort Study in Southern California. *Clin Infect Dis* 2023;76(3):e1341-e49. doi: 10.1093/cid/ciac436
273. Chessa D, Spiga L, De Riu N, et al. Lipopolysaccharides belonging to different Salmonella serovars are differentially capable of activating Toll-like receptor 4. *Infect Immun* 2014;82(11):4553-62. doi: 10.1128/IAI.02297-14 [published Online First: 20140818]
274. Raetz CR, Whitfield C. Lipopolysaccharide endotoxins. *Annu Rev Biochem* 2002;71:635-700. doi: 10.1146/annurev.biochem.71.110601.135414 [published Online First: 20011109]
275. Li P, Liu Q, Luo H, et al. O-Serotype Conversion in Salmonella Typhimurium Induces Protective Immune Responses against Invasive Non-Typhoidal Salmonella Infections. *Front Immunol* 2017;8:1647. doi: 10.3389/fimmu.2017.01647 [published Online First: 2017/12/20]
276. Juel HB, Thomaides-Brears HB, Darton TC, et al. Salmonella Typhi Bactericidal Antibodies Reduce Disease Severity but Do Not Protect against Typhoid Fever in a Controlled Human Infection Model. *Front Immunol* 2017;8:1916. doi: 10.3389/fimmu.2017.01916 [published Online First: 20180117]
277. Muller-Loennies S, Brade L, Brade H. Neutralizing and cross-reactive antibodies against enterobacterial lipopolysaccharide. *Int J Med Microbiol* 2007;297(5):321-40. doi: 10.1016/j.ijmm.2007.04.002 [published Online First: 20070601]
278. Olopoenia LA, King AL. Widal agglutination test - 100 years later: still plagued by controversy. *Postgrad Med J* 2000;76(892):80-4. doi: 10.1136/pmj.76.892.80
279. Chattaway MA, Langridge GC, Wain J. Salmonella nomenclature in the genomic era: a time for change. *Sci Rep* 2021;11(1):7494. doi: 10.1038/s41598-021-86243-w [published Online First: 20210405]
280. Pakkanen SH, Kantele JM, Savolainen LE, et al. Specific and cross-reactive immune response to oral Salmonella Typhi Ty21a and parenteral Vi capsular polysaccharide typhoid vaccines administered concomitantly. *Vaccine* 2015;33(3):451-8. doi: 10.1016/j.vaccine.2014.11.030 [published Online First: 2014/11/30]
281. Wahid R, Zafar SJ, McArthur MA, et al. Live oral Salmonella enterica serovar Typhi vaccines Ty21a and CVD 909 induce opsonophagocytic functional antibodies in humans that cross-react with S. Paratyphi A and S. Paratyphi B. *Clin Vaccine Immunol* 2014;21(3):427-34. doi: 10.1128/CVI.00786-13 [published Online First: 20140115]

282. Ahmed A, Akhade AS, Qadri A. Accessibility of O Antigens Shared between Salmonella Serovars Determines Antibody-Mediated Cross-Protection. *J Immunol* 2020;205(2):438-46. doi: 10.4049/jimmunol.1900624 [published Online First: 20200615]
283. Hiyoshi H, Wangdi T, Lock G, et al. Mechanisms to Evade the Phagocyte Respiratory Burst Arose by Convergent Evolution in Typhoidal Salmonella Serovars. *Cell Rep* 2018;22(7):1787-97. doi: 10.1016/j.celrep.2018.01.016
284. Mohamed OE, Williams L, Wong GK, et al. Isolating polysaccharide IgG pneumococcal antibody responses by pre-adsorption of conjugate vaccine serotypes: A modified approach for the conjugate vaccine era. *J Immunol Methods* 2020;486:112846. doi: 10.1016/j.jim.2020.112846 [published Online First: 20200831]
285. Collaborators GBDN-TSID. The global burden of non-typhoidal salmonella invasive disease: a systematic analysis for the Global Burden of Disease Study 2017. *Lancet Infect Dis* 2019;19(12):1312-24. doi: 10.1016/S1473-3099(19)30418-9 [published Online First: 20190924]
286. Typhoid GBD, Paratyphoid C. The global burden of typhoid and paratyphoid fevers: a systematic analysis for the Global Burden of Disease Study 2017. *Lancet Infect Dis* 2019;19(4):369-81. doi: 10.1016/S1473-3099(18)30685-6 [published Online First: 20190218]
287. Rapaka RR, Wahid R, Fresnay S, et al. Human Salmonella Typhi exposure generates differential multifunctional cross-reactive T-cell memory responses against Salmonella Paratyphi and invasive nontyphoidal Salmonella. *Clin Transl Immunology* 2020;9(9):e1178. doi: 10.1002/cti2.1178 [published Online First: 2020/10/03]
288. Kintz E, Heiss C, Black I, et al. Salmonella enterica Serovar Typhi Lipopolysaccharide O-Antigen Modification Impact on Serum Resistance and Antibody Recognition. *Infect Immun* 2017;85(4) doi: 10.1128/IAI.01021-16 [published Online First: 2017/02/09]
289. Mousset CM, Hobo W, Woestenenk R, et al. Comprehensive Phenotyping of T Cells Using Flow Cytometry. *Cytometry A* 2019;95(6):647-54. doi: 10.1002/cyto.a.23724 [published Online First: 20190204]
290. Swain SL, McKinstry KK, Strutt TM. Expanding roles for CD4(+) T cells in immunity to viruses. *Nat Rev Immunol* 2012;12(2):136-48. doi: 10.1038/nri3152 [published Online First: 20120120]
291. LaRock DL, Chaudhary A, Miller SI. Salmonellae interactions with host processes. *Nat Rev Microbiol* 2015;13(4):191-205. doi: 10.1038/nrmicro3420 [published Online First: 2015/03/10]
292. Wahid R, Fresnay S, Levine MM, et al. Cross-reactive multifunctional CD4+ T cell responses against Salmonella enterica serovars Typhi, Paratyphi A and Paratyphi B in humans following immunization with live oral typhoid vaccine Ty21a. *Clin Immunol* 2016;173:87-95. doi: 10.1016/j.clim.2016.09.006 [published Online First: 20160912]
293. Prior JT, Davitt C, Kurtz J, et al. Bacterial-Derived Outer Membrane Vesicles are Potent Adjuvants that Drive Humoral and Cellular Immune Responses. *Pharmaceutics* 2021;13(2) doi: 10.3390/pharmaceutics13020131 [published Online First: 20210120]

294. Oftung F, Korsvold GE, Aase A, et al. Cellular Immune Responses in Humans Induced by Two Serogroup B Meningococcal Outer Membrane Vesicle Vaccines Given Separately and in Combination. *Clin Vaccine Immunol* 2016;23(4):353-62. doi: 10.1128/CVI.00666-15 [published Online First: 20160404]
295. Wick MJ. Innate immune control of Salmonella enterica serovar Typhimurium: mechanisms contributing to combating systemic Salmonella infection. *J Innate Immun* 2011;3(6):543-9. doi: 10.1159/000330771 [published Online First: 2011/09/14]
296. Lundin BS, Johansson C, Svennerholm AM. Oral immunization with a Salmonella enterica serovar typhi vaccine induces specific circulating mucosa-homing CD4(+) and CD8(+) T cells in humans. *Infect Immun* 2002;70(10):5622-7. doi: 10.1128/IAI.70.10.5622-5627.2002
297. Jeannin P, Magistrelli G, Goetsch L, et al. Outer membrane protein A (OmpA): a new pathogen-associated molecular pattern that interacts with antigen presenting cells-impact on vaccine strategies. *Vaccine* 2002;20 Suppl 4:A23-7. doi: 10.1016/s0264-410x(02)00383-3
298. W C. Salmonella Conjugates CVD 1000: Study of Responses to Vaccination With Trivalent Invasive Salmonella Disease Vaccine: ClinicalTrials.gov; 2021 [Available from: https://clinicaltrials.gov/ct2/show/NCT03981952.
299. Snellings NJ, Johnson EM, Kopecko DJ, et al. Genetic regulation of variable Vi antigen expression in a strain of Citrobacter freundii. *J Bacteriol* 1981;145(2):1010-7. doi: 10.1128/jb.145.2.1010-1017.1981
300. Denis J, Garnier A, Cheutin L, et al. Long-term systemic and mucosal SARS-CoV-2 IgA response and its association with persistent smell and taste disorders. *Front Immunol* 2023;14:1140714. doi: 10.3389/fimmu.2023.1140714 [published Online First: 20230308]
301. Sheikh-Mohamed S, Sanders EC, Gommerman JL, et al. Guardians of the oral and nasopharyngeal galaxy: IgA and protection against SARS-CoV-2 infection. *Immunol Rev* 2022;309(1):75-85. doi: 10.1111/imr.13118 [published Online First: 20220711]
302. Paul A, Babji S, Sarkar R, et al. Rotavirus specific Salivary and Fecal IgA in Indian Children and Adults. *Indian Pediatr* 2016;53(7):601-6. doi: **10.1007/s13312-016-0895-z**
303. Wang X, Teng D, Guan Q, et al. Escherichia coli outer membrane protein F (OmpF): an immunogenic protein induces cross-reactive antibodies against Escherichia coli and Shigella. *AMB Express* 2017;7(1):155. doi: 10.1186/s13568-017-0452-8 [published Online First: 20170719]
304. Vatti A, Monsalve DM, Pacheco Y, et al. Original antigenic sin: A comprehensive review. *J Autoimmun* 2017;83:12-21. doi: 10.1016/j.jaut.2017.04.008 [published Online First: 2017/05/10]
305. Kariuki S, Onsare RS. Epidemiology and Genomics of Invasive Nontyphoidal Salmonella Infections in Kenya. *Clin Infect Dis* 2015;61 Suppl 4:S317-24. doi: 10.1093/cid/civ711 [published Online First: 2015/10/10]
306. Simon R, Levine MM. Glycoconjugate vaccine strategies for protection against invasive Salmonella infections. *Hum Vaccin Immunother* 2012;8(4):494-8. doi: 10.4161/hv.19158 [published Online First: 2012/03/01]

307. Wijayasri S, Hillier K, Lim GH, et al. The shifting epidemiology and serotype distribution of invasive pneumococcal disease in Ontario, Canada, 2007-2017. *PLoS One* 2019;14(12):e0226353. doi: 10.1371/journal.pone.0226353 [published Online First: 20191213]
308. Galanis I, Lindstrand A, Darenberg J, et al. Effects of PCV7 and PCV13 on invasive pneumococcal disease and carriage in Stockholm, Sweden. *Eur Respir J* 2016;47(4):1208-18. doi: 10.1183/13993003.01451-2015 [published Online First: 20160121]
309. Tran Vu Thieu N, Trinh Van T, Tran Tuan A, et al. An evaluation of purified Salmonella Typhi protein antigens for the serological diagnosis of acute typhoid fever. *J Infect* 2017;75(2):104-14. doi: 10.1016/j.jinf.2017.05.007 [published Online First: 20170525]

# Appendix A

Protein production was performed by collaborators at the University of Oxford. The method courtesy of *Dr Young Kim* is provided below:

**Transgene Design, Gene Expression and Protein Purification of Selected Proteins:**

OmpA, OmpC, OmpF, OmpD, SlyB, FkpA, LptA, YnCE, CdtB, HlyE and Flagellin.

The expression plasmids encoding YnCE, CdtB and HlyE proteins were obtained from Prof Steve Baker, University of Cambridge. The other selected genes were synthesised by GeneArt and cloned into the 5' NcoI and 3' NotI restriction sites of pET28b vector for further His-Tag purification as previous described[309].

Successfully cloned expression plasmids were verified by DNA sequencing using flanking primers. For protein expression, the *E. coli* BL21(DE3) pLysS strains with unique plasmid constructs containing the genes of interest were inoculated into LB broth containing 100 mg/L kanamycin and 34 μg/mL chloramphenicol and incubated at 37°C overnight. Overnight cultures were diluted (1:100) into LB broth and incubated at 37°C with agitation until optical density $OD_{600}$ ~ 0.4. Expression of the exogenous proteins was induced by the addition of IPTG, to a final concentration of 0.5 mM and cultured for an additional 3 hours (or overnight). Bacterial cells were harvested (5000g at 4°C for 20 min) and the pellets were lysed and sonicated on ice. After sonication, cell debris and the membrane fragment were pelleted by centrifugation at 25000g for 30 minutes at 4°C. For soluble proteins, bacterial pellets were resuspended in 50 mM Tris HCl buffer (pH 8) containing 150 mM NaCl and 10 mM imidazole. Supernatants were filtered through a 0.45 μm membrane and

was purified by Ni Sepharose affinity chromatography, using the Äkta Pure chromatography system and eluted with 500mM imidazole. For insoluble proteins a denaturing protocol was performed as previously described[309] using solubilisation buffer (pH 8) containing 50mM Tris-HCl and 8M urea to isolate the proteins from the inclusion bodies. The same protein purification method was performed once the proteins were soluble.

# Appendix B

*Table 37 SEnGMMA protein String Analysis including GO process, GO function, GO component, String Clusters, KEGG, and UniProt keywords (output from STRING [String Consortium 2023, Switzerland[241])*

| Category | ID | Description | Observed Gene Count | Background Gene Count | Strength | False Discovery Rate |
|---|---|---|---|---|---|---|
| GO Process | GO:0006412 | Translation | 17 | 111 | 0.69 | 0.00036 |
| GO Process | GO:0033036 | Macromolecule localization | 16 | 116 | 0.64 | 0.00049 |
| GO Process | GO:0043165 | Gram-negative-bacterium-type cell outer membrane assembly | 7 | 12 | 1.27 | 0.00049 |
| GO Process | GO:0046034 | ATP metabolic process | 10 | 40 | 0.9 | 0.00049 |
| GO Process | GO:0061024 | Membrane organization | 8 | 18 | 1.15 | 0.00049 |
| GO Process | GO:0045229 | External encapsulating structure organization | 14 | 105 | 0.63 | 0.0018 |
| GO Process | GO:0034613 | Cellular protein localization | 7 | 22 | 1.01 | 0.0025 |
| GO Process | GO:0072657 | Protein localization to membrane | 6 | 14 | 1.14 | 0.0025 |
| GO Process | GO:0006096 | Glycolytic process | 6 | 18 | 1.03 | 0.0062 |
| GO Process | GO:0043603 | Cellular amide metabolic process | 18 | 194 | 0.47 | 0.0062 |
| GO Process | GO:0051179 | Localization | 43 | 761 | 0.26 | 0.0062 |
| GO Process | GO:0051234 | Establishment of localization | 41 | 723 | 0.26 | 0.0062 |
| GO Process | GO:0090150 | Establishment of protein localization to membrane | 5 | 10 | 1.2 | 0.0062 |
| GO Process | GO:0019538 | Protein metabolic process | 26 | 372 | 0.35 | 0.0072 |
| GO Process | GO:0051205 | Protein insertion into membrane | 4 | 6 | 1.33 | 0.0087 |
| GO Process | GO:0044267 | Cellular protein metabolic process | 20 | 252 | 0.4 | 0.0093 |
| GO Process | GO:0009150 | Purine ribonucleotide metabolic process | 10 | 77 | 0.62 | 0.0139 |
| GO Process | GO:0071840 | Cellular component organization or biogenesis | 23 | 332 | 0.35 | 0.0182 |
| GO Process | GO:0034645 | Cellular macromolecule biosynthetic process | 25 | 381 | 0.32 | 0.0208 |
| GO Process | GO:0010876 | Lipid localization | 5 | 19 | 0.92 | 0.0288 |
| GO Process | GO:0006091 | Generation of precursor metabolites and energy | 14 | 164 | 0.44 | 0.0382 |
| GO Process | GO:1901564 | Organonitrogen compound metabolic process | 46 | 943 | 0.19 | 0.04 |
| GO Process | GO:0009060 | Aerobic respiration | 6 | 34 | 0.75 | 0.047 |

| | | | | | | |
|---|---|---|---|---|---|---|
| GO Function | GO:0015267 | Channel activity | 18 | 62 | 0.97 | 1.16E-08 |
| GO Function | GO:0015288 | Porin activity | 14 | 37 | 1.08 | 4.93E-08 |
| GO Function | GO:0003735 | Structural constituent of ribosome | 14 | 56 | 0.9 | 2.70E-06 |
| GO Function | GO:0005198 | Structural molecule activity | 15 | 86 | 0.75 | 4.37E-05 |
| GO Function | GO:0019843 | rRNA binding | 10 | 45 | 0.85 | 0.00064 |
| GO Function | GO:0042834 | Peptidoglycan binding | 4 | 8 | 1.2 | 0.0428 |
| GO Component | GO:0019867 | Outer membrane | 37 | 121 | 0.99 | 8.49E-22 |
| GO Component | GO:0009279 | Cell outer membrane | 34 | 109 | 1 | 2.37E-20 |
| GO Component | GO:0030313 | Cell envelope | 38 | 191 | 0.8 | 2.10E-17 |
| GO Component | GO:0016020 | Membrane | 85 | 1373 | 0.3 | 1.56E-11 |
| GO Component | GO:0110165 | Cellular anatomical entity | 135 | 3496 | 0.09 | 1.27E-08 |
| GO Component | GO:0071944 | Cell periphery | 75 | 1287 | 0.27 | 2.90E-08 |
| GO Component | GO:0005840 | Ribosome | 14 | 61 | 0.87 | 7.05E-07 |
| GO Component | GO:0043232 | Intracellular non-membrane-bounded organelle | 15 | 106 | 0.66 | 5.22E-05 |
| GO Component | GO:0046930 | Pore complex | 7 | 16 | 1.15 | 5.73E-05 |
| GO Component | GO:0043228 | Non-membrane-bounded organelle | 16 | 138 | 0.57 | 0.00021 |
| GO Component | GO:0098796 | Membrane protein complex | 12 | 87 | 0.64 | 0.00053 |
| GO Component | GO:0032991 | Protein-containing complex | 23 | 301 | 0.39 | 0.0012 |
| GO Component | GO:0042597 | Periplasmic space | 18 | 210 | 0.44 | 0.0019 |
| GO Component | GO:0044391 | Ribosomal subunit | 5 | 17 | 0.97 | 0.0046 |
| GO Component | GO:0031230 | Intrinsic component of cell outer membrane | 3 | 7 | 1.14 | 0.0266 |
| GO Component | GO:0015934 | Large ribosomal subunit | 3 | 8 | 1.08 | 0.0341 |
| GO Component | GO:0045252 | Oxoglutarate dehydrogenase complex | 2 | 2 | 1.5 | 0.0425 |
| GO Component | GO:0045254 | Pyruvate dehydrogenase complex | 2 | 2 | 1.5 | 0.0425 |
| GO Component | GO:0045259 | Proton-transporting atp synthase complex | 3 | 9 | 1.03 | 0.0425 |
| STRING clusters | CL:870 | Mixed, incl. lipopolysaccharide biosynthesis, and membrane organization | 24 | 93 | 0.92 | 1.44E-11 |
| STRING clusters | CL:871 | Mixed, incl. lipopolysaccharide biosynthesis, and membrane organization | 23 | 80 | 0.96 | 1.44E-11 |
| STRING clusters | CL:959 | Mixed, incl. porin domain superfamily, and protein insertion into membrane | 15 | 36 | 1.12 | 3.49E-09 |
| STRING clusters | CL:83 | Ribosome | 17 | 66 | 0.92 | 4.20E-08 |

| | | | | | | |
|---|---|---|---|---|---|---|
| STRING clusters | CL:869 | Mixed, incl. external encapsulating structure organization, and lipopolysaccharide biosynthesis | 26 | 176 | 0.67 | 4.20E-08 |
| STRING clusters | CL:90 | Ribosome | 16 | 55 | 0.97 | 4.20E-08 |
| STRING clusters | CL:960 | Mixed, incl. protein insertion into membrane, and porin domain superfamily | 12 | 26 | 1.17 | 7.45E-08 |
| STRING clusters | CL:92 | Ribosome | 14 | 48 | 0.97 | 2.55E-07 |
| STRING clusters | CL:2040 | Pyruvate metabolism, and Oxidative phosphorylation | 21 | 134 | 0.7 | 4.28E-07 |
| STRING clusters | CL:79 | Translation, and rna polymerase | 19 | 109 | 0.75 | 4.90E-07 |
| STRING clusters | CL:71 | Translation, and protein export | 23 | 176 | 0.62 | 1.32E-06 |
| STRING clusters | CL:962 | Protein insertion into membrane, and porin domain superfamily | 9 | 16 | 1.25 | 1.59E-06 |
| STRING clusters | CL:72 | Translation, and ribosome biogenesis | 20 | 151 | 0.63 | 7.20E-06 |
| STRING clusters | CL:964 | Protein insertion into membrane, and porin domain superfamily | 7 | 11 | 1.31 | 2.23E-05 |
| STRING clusters | CL:93 | Ribosome | 9 | 36 | 0.9 | 0.00026 |
| STRING clusters | CL:2041 | Pyruvate metabolism, and Valine, leucine and isoleucine biosynthesis | 13 | 94 | 0.65 | 0.00067 |
| STRING clusters | CL:2131 | Tricarboxylic acid cycle enzyme complex, and pyruvate dehydrogenase complex | 5 | 7 | 1.36 | 0.00067 |
| STRING clusters | CL:2042 | Citrate cycle (TCA cycle), and Propanoate metabolism | 10 | 54 | 0.77 | 0.00069 |
| STRING clusters | CL:166 | Ribosome | 5 | 9 | 1.25 | 0.0014 |
| STRING clusters | CL:2229 | Oxidative phosphorylation | 8 | 36 | 0.85 | 0.0015 |
| STRING clusters | CL:2098 | Citrate cycle (TCA cycle) | 7 | 26 | 0.93 | 0.0016 |
| STRING clusters | CL:966 | Protein insertion into membrane | 4 | 5 | 1.41 | 0.0031 |
| STRING clusters | CL:3029 | Mixed, incl. lipoprotein, and dodecin-like | 10 | 71 | 0.65 | 0.0047 |
| STRING clusters | CL:3034 | Mostly uncharacterized, incl. cell wall, and dodecin-like | 6 | 23 | 0.92 | 0.0059 |
| STRING clusters | CL:3030 | Mixed, incl. lipoprotein, and ltxxq motif family protein | 8 | 47 | 0.74 | 0.0066 |
| STRING clusters | CL:126 | rRNA-binding | 5 | 15 | 1.03 | 0.0077 |
| STRING clusters | CL:3023 | Mostly uncharacterized, incl. lipoprotein, and dodecin-like | 11 | 93 | 0.58 | 0.0078 |
| STRING clusters | CL:3021 | Mixed, incl. lipoprotein, and response to chemical | 12 | 112 | 0.53 | 0.009 |
| STRING clusters | CL:94 | Ribosome | 6 | 31 | 0.79 | 0.0192 |
| STRING clusters | CL:913 | Lipid transport, and lipoprotein localization | 5 | 20 | 0.9 | 0.0194 |
| STRING clusters | CL:155 | Mixed, incl. ribosomal protein l9, and ribosomal protein l17 | 3 | 5 | 1.28 | 0.0334 |
| STRING clusters | CL:976 | Porin domain superfamily, and Conjugation | 3 | 5 | 1.28 | 0.0334 |
| STRING clusters | CL:2230 | Oxidative phosphorylation | 5 | 25 | 0.81 | 0.0431 |

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|---|---|---|---|---|---|---|
| STRING clusters | CL:144 | Large ribosomal subunit, and ribosomal protein s8 | 3 | 6 | 1.2 | 0.0458 |
| STRING clusters | CL:997 | Mixed, incl. protein import, and ybgf, trimerisation domain | 3 | 6 | 1.2 | 0.0458 |
| STRING clusters | CL:914 | Lipid transport, and stas domain | 4 | 15 | 0.93 | 0.0482 |
| KEGG | seni03010 | Ribosome | 14 | 54 | 0.92 | 1.00E-06 |
| KEGG | seni00190 | Oxidative phosphorylation | 10 | 43 | 0.87 | 0.00017 |
| KEGG | seni00020 | Citrate cycle (TCA cycle) | 8 | 28 | 0.96 | 0.00033 |
| KEGG | seni00010 | Glycolysis / Gluconeogenesis | 8 | 44 | 0.76 | 0.0041 |
| KEGG | seni01200 | Carbon metabolism | 12 | 114 | 0.53 | 0.0086 |
| KEGG | seni01503 | Cationic antimicrobial peptide (CAMP) resistance | 6 | 36 | 0.73 | 0.0283 |
| UniProt Keywords | KW-0732 | Signal | 67 | 437 | 0.69 | 8.61E-27 |
| UniProt Keywords | KW-0998 | Cell outer membrane | 26 | 56 | 1.17 | 1.89E-18 |
| UniProt Keywords | KW-0449 | Lipoprotein | 22 | 81 | 0.94 | 1.44E-11 |
| UniProt Keywords | KW-1134 | Transmembrane beta strand | 12 | 26 | 1.17 | 3.84E-08 |
| UniProt Keywords | KW-0689 | Ribosomal protein | 14 | 55 | 0.91 | 6.35E-07 |
| UniProt Keywords | KW-0564 | Palmitate | 10 | 32 | 1 | 1.29E-05 |
| UniProt Keywords | KW-0626 | Porin | 7 | 13 | 1.24 | 4.18E-05 |
| UniProt Keywords | KW-0699 | rRNA-binding | 10 | 40 | 0.9 | 5.70E-05 |
| UniProt Keywords | KW-0472 | Membrane | 58 | 1073 | 0.24 | 0.00016 |
| UniProt Keywords | KW-0406 | Ion transport | 11 | 73 | 0.68 | 0.001 |
| UniProt Keywords | KW-0813 | Transport | 30 | 453 | 0.33 | 0.002 |
| UniProt Keywords | KW-1015 | Disulfide bond | 8 | 50 | 0.71 | 0.0071 |
| UniProt Keywords | KW-0675 | Receptor | 4 | 9 | 1.15 | 0.0099 |
| UniProt Keywords | KW-0574 | Periplasm | 9 | 78 | 0.57 | 0.0213 |
| UniProt Keywords | KW-0324 | Glycolysis | 4 | 14 | 0.96 | 0.0309 |
| UniProt Keywords | KW-0798 | TonB box | 3 | 7 | 1.14 | 0.0459 |

*Table 38 STmGMMA protein String Analysis including GO process, GO function, GO component, String Clusters, KEGG, and UniProt keywords (output from STRING [String Consortium 2023, Switzerland[241])*

| Category | ID | Description | Observed Gene Count | Background Gene Count | Strength | False Discovery Rate |
|---|---|---|---|---|---|---|
| GO Process | GO:0006412 | Translation | 46 | 111 | 0.7 | 1.93E-13 |
| GO Process | GO:0006518 | Peptide metabolic process | 47 | 131 | 0.64 | 2.60E-12 |
| GO Process | GO:0043604 | Amide biosynthetic process | 50 | 157 | 0.59 | 1.17E-11 |
| GO Process | GO:0009059 | Macromolecule biosynthetic process | 80 | 390 | 0.4 | 1.16E-10 |
| GO Process | GO:0034645 | Cellular macromolecule biosynthetic process | 79 | 381 | 0.4 | 1.16E-10 |
| GO Process | GO:0043603 | Cellular amide metabolic process | 53 | 194 | 0.52 | 1.86E-10 |
| GO Process | GO:0009987 | Cellular process | 296 | 2853 | 0.1 | 8.22E-10 |
| GO Process | GO:1901576 | Organic substance biosynthetic process | 128 | 870 | 0.25 | 4.48E-09 |
| GO Process | GO:0009058 | Biosynthetic process | 129 | 888 | 0.25 | 7.29E-09 |
| GO Process | GO:1901564 | Organonitrogen compound metabolic process | 134 | 943 | 0.24 | 8.96E-09 |
| GO Process | GO:1901566 | Organonitrogen compound biosynthetic process | 87 | 496 | 0.33 | 8.96E-09 |
| GO Process | GO:0044249 | Cellular biosynthetic process | 124 | 854 | 0.25 | 1.76E-08 |
| GO Process | GO:0044271 | Cellular nitrogen compound biosynthetic process | 78 | 437 | 0.34 | 4.51E-08 |
| GO Process | GO:0019538 | Protein metabolic process | 70 | 372 | 0.36 | 5.65E-08 |
| GO Process | GO:0044267 | Cellular protein metabolic process | 53 | 252 | 0.41 | 3.59E-07 |
| GO Process | GO:0019693 | Ribose phosphate metabolic process | 29 | 96 | 0.57 | 2.77E-06 |
| GO Process | GO:0009259 | Ribonucleotide metabolic process | 28 | 91 | 0.57 | 3.31E-06 |
| GO Process | GO:0010467 | Gene expression | 57 | 307 | 0.36 | 3.75E-06 |
| GO Process | GO:0044238 | Primary metabolic process | 179 | 1580 | 0.14 | 1.97E-05 |
| GO Process | GO:1901135 | Carbohydrate derivative metabolic process | 62 | 369 | 0.31 | 2.02E-05 |
| GO Process | GO:0008152 | Metabolic process | 237 | 2286 | 0.1 | 2.20E-05 |
| GO Process | GO:0009150 | Purine ribonucleotide metabolic process | 24 | 77 | 0.58 | 2.20E-05 |
| GO Process | GO:0046034 | ATP metabolic process | 17 | 40 | 0.71 | 3.76E-05 |
| GO Process | GO:0061024 | Membrane organization | 12 | 18 | 0.91 | 5.18E-05 |
| GO Process | GO:0072521 | Purine-containing compound metabolic process | 25 | 96 | 0.5 | 0.00017 |
| GO Process | GO:0033036 | Macromolecule localization | 27 | 116 | 0.45 | 0.00039 |
| GO Process | GO:0044237 | Cellular metabolic process | 199 | 1899 | 0.11 | 0.00044 |

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|---|---|---|---|---|---|---|
| GO Process | GO:0071704 | Organic substance metabolic process | 199 | 1900 | 0.11 | 0.00044 |
| GO Process | GO:0009117 | Nucleotide metabolic process | 29 | 138 | 0.41 | 0.00088 |
| GO Process | GO:0006091 | Generation of precursor metabolites and energy | 32 | 164 | 0.38 | 0.0011 |
| GO Process | GO:0006096 | Glycolytic process | 10 | 18 | 0.83 | 0.0011 |
| GO Process | GO:0006090 | Pyruvate metabolic process | 12 | 29 | 0.7 | 0.0014 |
| GO Process | GO:0006418 | tRNA aminoacylation for protein translation | 11 | 24 | 0.75 | 0.0014 |
| GO Process | GO:0046390 | Ribose phosphate biosynthetic process | 18 | 65 | 0.53 | 0.0014 |
| GO Process | GO:0019752 | Carboxylic acid metabolic process | 61 | 432 | 0.24 | 0.0018 |
| GO Process | GO:0043165 | Gram-negative-bacterium-type cell outer membrane assembly | 8 | 12 | 0.91 | 0.002 |
| GO Process | GO:0043170 | Macromolecule metabolic process | 113 | 968 | 0.15 | 0.002 |
| GO Process | GO:0006807 | Nitrogen compound metabolic process | 151 | 1395 | 0.12 | 0.0021 |
| GO Process | GO:0009260 | Ribonucleotide biosynthetic process | 17 | 63 | 0.52 | 0.0024 |
| GO Process | GO:0019637 | Organophosphate metabolic process | 42 | 264 | 0.29 | 0.0025 |
| GO Process | GO:0034613 | Cellular protein localization | 10 | 22 | 0.74 | 0.0025 |
| GO Process | GO:0044260 | Cellular macromolecule metabolic process | 92 | 755 | 0.17 | 0.0025 |
| GO Process | GO:0009060 | Aerobic respiration | 12 | 34 | 0.63 | 0.0032 |
| GO Process | GO:0055086 | Nucleobase-containing small molecule metabolic process | 34 | 202 | 0.31 | 0.0045 |
| GO Process | GO:1901137 | Carbohydrate derivative biosynthetic process | 38 | 241 | 0.28 | 0.006 |
| GO Process | GO:0006099 | Tricarboxylic acid cycle | 10 | 26 | 0.67 | 0.0064 |
| GO Process | GO:0006457 | Protein folding | 14 | 53 | 0.51 | 0.0102 |
| GO Process | GO:0034641 | Cellular nitrogen compound metabolic process | 102 | 910 | 0.14 | 0.016 |
| GO Process | GO:0072657 | Protein localization to membrane | 7 | 14 | 0.79 | 0.016 |
| GO Process | GO:0009152 | Purine ribonucleotide biosynthetic process | 13 | 50 | 0.5 | 0.0175 |
| GO Process | GO:0051205 | Protein insertion into membrane | 5 | 6 | 1.01 | 0.0187 |
| GO Process | GO:0090150 | Establishment of protein localization to membrane | 6 | 10 | 0.86 | 0.0187 |
| GO Process | GO:0045229 | External encapsulating structure organization | 20 | 105 | 0.37 | 0.0233 |
| GO Process | GO:0045184 | Establishment of protein localization | 18 | 92 | 0.38 | 0.0304 |
| GO Process | GO:0009165 | Nucleotide biosynthetic process | 18 | 94 | 0.37 | 0.0367 |
| GO Process | GO:0015986 | ATP synthesis coupled proton transport | 5 | 8 | 0.88 | 0.0402 |
| GO Process | GO:0006869 | Lipid transport | 7 | 18 | 0.68 | 0.041 |

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|---|---|---|---|---|---|---|
| GO Process | GO:0010608 | Posttranscriptional regulation of gene expression | 11 | 43 | 0.49 | 0.0411 |
| GO Process | GO:0008610 | Lipid biosynthetic process | 23 | 138 | 0.31 | 0.0425 |
| GO Function | GO:0003735 | Structural constituent of ribosome | 29 | 56 | 0.8 | 9.74E-10 |
| GO Function | GO:0003723 | RNA binding | 44 | 156 | 0.54 | 1.74E-08 |
| GO Function | GO:0005198 | Structural molecule activity | 31 | 86 | 0.64 | 8.64E-08 |
| GO Function | GO:0019843 | rRNA binding | 23 | 45 | 0.8 | 8.64E-08 |
| GO Function | GO:0015267 | Channel activity | 21 | 62 | 0.62 | 0.00011 |
| GO Function | GO:0016874 | Ligase activity | 27 | 104 | 0.5 | 0.00016 |
| GO Function | GO:0000166 | Nucleotide binding | 88 | 663 | 0.21 | 0.00082 |
| GO Function | GO:0036094 | Small molecule binding | 96 | 749 | 0.19 | 0.00082 |
| GO Function | GO:0097367 | Carbohydrate derivative binding | 78 | 564 | 0.23 | 0.00082 |
| GO Function | GO:0000049 | tRNA binding | 13 | 33 | 0.68 | 0.0019 |
| GO Function | GO:0035639 | Purine ribonucleoside triphosphate binding | 68 | 496 | 0.22 | 0.003 |
| GO Function | GO:0004812 | aminoacyl-tRNA ligase activity | 11 | 25 | 0.73 | 0.0032 |
| GO Function | GO:0015288 | Porin activity | 13 | 37 | 0.63 | 0.0038 |
| GO Function | GO:0032555 | Purine ribonucleotide binding | 68 | 505 | 0.22 | 0.0038 |
| GO Function | GO:0043168 | Anion binding | 84 | 666 | 0.19 | 0.0038 |
| GO Function | GO:0032553 | Ribonucleotide binding | 70 | 531 | 0.21 | 0.0044 |
| GO Function | GO:0005524 | ATP binding | 62 | 458 | 0.22 | 0.0058 |
| GO Function | GO:0003755 | Peptidyl-prolyl cis-trans isomerase activity | 6 | 10 | 0.86 | 0.031 |
| GO Function | GO:0003824 | Catalytic activity | 205 | 2114 | 0.07 | 0.0319 |
| GO Function | GO:0004175 | Endopeptidase activity | 12 | 45 | 0.51 | 0.0352 |
| GO Function | GO:0097159 | Organic cyclic compound binding | 142 | 1374 | 0.1 | 0.0372 |
| GO Function | GO:1901363 | Heterocyclic compound binding | 142 | 1374 | 0.1 | 0.0372 |
| GO Function | GO:0051082 | Unfolded protein binding | 8 | 22 | 0.65 | 0.0457 |
| GO Component | GO:0110165 | Cellular anatomical entity | 354 | 3496 | 0.09 | 1.03E-21 |
| GO Component | GO:0019867 | Outer membrane | 42 | 121 | 0.63 | 3.44E-11 |
| GO Component | GO:0009279 | Cell outer membrane | 39 | 109 | 0.64 | 7.88E-11 |
| GO Component | GO:0005840 | Ribosome | 29 | 61 | 0.76 | 2.33E-10 |
| GO Component | GO:0030312 | External encapsulating structure | 40 | 138 | 0.55 | 4.69E-09 |

| | | | | | | |
|---|---|---|---|---|---|---|
| GO Component | GO:0030313 | Cell envelope | 48 | 191 | 0.49 | 4.69E-09 |
| GO Component | GO:0043232 | Intracellular non-membrane-bounded organelle | 31 | 106 | 0.55 | 3.88E-07 |
| GO Component | GO:0043228 | Non-membrane-bounded organelle | 33 | 138 | 0.47 | 6.91E-06 |
| GO Component | GO:0016020 | Membrane | 154 | 1373 | 0.14 | 0.00013 |
| GO Component | GO:0044391 | Ribosomal subunit | 10 | 17 | 0.86 | 0.00025 |
| GO Component | GO:0005622 | Intracellular | 194 | 1873 | 0.1 | 0.00037 |
| GO Component | GO:0032991 | Protein-containing complex | 48 | 301 | 0.29 | 0.00037 |
| GO Component | GO:0098796 | Membrane protein complex | 21 | 87 | 0.47 | 0.00053 |
| GO Component | GO:0071944 | Cell periphery | 138 | 1287 | 0.12 | 0.0028 |
| GO Component | GO:0015934 | Large ribosomal subunit | 6 | 8 | 0.96 | 0.0032 |
| GO Component | GO:0042597 | Periplasmic space | 32 | 210 | 0.27 | 0.0108 |
| GO Component | GO:0046930 | Pore complex | 7 | 16 | 0.73 | 0.0108 |
| GO Component | GO:0098552 | Side of membrane | 8 | 23 | 0.63 | 0.0145 |
| GO Component | GO:0045259 | Proton-transporting atp synthase complex | 5 | 9 | 0.83 | 0.0226 |
| GO Component | GO:0045261 | Proton-transporting atp synthase complex, catalytic core f(1) | 4 | 5 | 0.99 | 0.0228 |
| GO Component | GO:0045239 | Tricarboxylic acid cycle enzyme complex | 4 | 6 | 0.91 | 0.0331 |
| STRING clusters | CL:70 | Translation, and protein export | 65 | 182 | 0.64 | 1.38E-17 |
| STRING clusters | CL:71 | Translation, and protein export | 63 | 176 | 0.64 | 2.70E-17 |
| STRING clusters | CL:79 | Translation, and rna polymerase | 48 | 109 | 0.73 | 1.10E-15 |
| STRING clusters | CL:72 | Translation, and ribosome biogenesis | 55 | 151 | 0.65 | 2.30E-15 |
| STRING clusters | CL:74 | Translation, and ribosome biogenesis | 53 | 141 | 0.66 | 2.34E-15 |
| STRING clusters | CL:76 | Translation, and ribosome biogenesis | 52 | 135 | 0.67 | 2.34E-15 |
| STRING clusters | CL:78 | Translation, and ribosome biogenesis | 50 | 127 | 0.68 | 3.67E-15 |
| STRING clusters | CL:80 | Translation | 41 | 91 | 0.74 | 6.92E-14 |
| STRING clusters | CL:90 | Ribosome | 30 | 55 | 0.82 | 1.40E-11 |
| STRING clusters | CL:87 | Ribosome | 31 | 60 | 0.8 | 1.49E-11 |
| STRING clusters | CL:83 | Ribosome | 32 | 66 | 0.77 | 2.09E-11 |
| STRING clusters | CL:82 | Ribosome, and translation factor activity, rna binding | 33 | 72 | 0.75 | 2.75E-11 |
| STRING clusters | CL:2039 | Mixed, incl. pyruvate metabolism, and oxidative phosphorylation | 47 | 162 | 0.55 | 2.17E-10 |
| STRING clusters | CL:2040 | Pyruvate metabolism, and Oxidative phosphorylation | 40 | 134 | 0.56 | 4.78E-09 |

| | | | | | | |
|---|---|---|---|---|---|---|
| STRING clusters | CL:92 | Ribosome | 24 | 48 | 0.79 | 1.07E-08 |
| STRING clusters | CL:869 | Mixed, incl. external encapsulating structure organization, and lipopolysaccharide biosynthesis | 42 | 176 | 0.46 | 4.40E-07 |
| STRING clusters | CL:93 | Ribosome | 19 | 36 | 0.81 | 4.40E-07 |
| STRING clusters | CL:871 | Mixed, incl. lipopolysaccharide biosynthesis, and membrane organization | 26 | 80 | 0.6 | 2.37E-06 |
| STRING clusters | CL:870 | Mixed, incl. lipopolysaccharide biosynthesis, and membrane organization | 28 | 93 | 0.57 | 2.62E-06 |
| STRING clusters | CL:2041 | Pyruvate metabolism, and Valine, leucine and isoleucine biosynthesis | 26 | 94 | 0.53 | 3.05E-05 |
| STRING clusters | CL:444 | Mixed, incl. peptidase complex, and unfolded protein binding | 10 | 12 | 1.01 | 7.42E-05 |
| STRING clusters | CL:94 | Ribosome | 14 | 31 | 0.74 | 0.00014 |
| STRING clusters | CL:6727 | Pentose phosphate pathway, and Phosphotransferase system | 18 | 58 | 0.58 | 0.00041 |
| STRING clusters | CL:962 | Protein insertion into membrane, and porin domain superfamily | 10 | 16 | 0.88 | 0.00041 |
| STRING clusters | CL:2229 | Oxidative phosphorylation | 14 | 36 | 0.68 | 0.00048 |
| STRING clusters | CL:959 | Mixed, incl. porin domain superfamily, and protein insertion into membrane | 14 | 36 | 0.68 | 0.00048 |
| STRING clusters | CL:960 | Mixed, incl. protein insertion into membrane, and porin domain superfamily | 12 | 26 | 0.75 | 0.00049 |
| STRING clusters | CL:2131 | Tricarboxylic acid cycle enzyme complex, and pyruvate dehydrogenase complex | 7 | 7 | 1.09 | 0.001 |
| STRING clusters | CL:438 | Protein folding, and protein catabolic process | 13 | 34 | 0.67 | 0.001 |
| STRING clusters | CL:439 | Protein folding, and small heat shock protein hsp20 | 12 | 29 | 0.7 | 0.001 |
| STRING clusters | CL:964 | Protein insertion into membrane, and porin domain superfamily | 8 | 11 | 0.95 | 0.0011 |
| STRING clusters | CL:2230 | Oxidative phosphorylation | 11 | 25 | 0.73 | 0.0013 |
| STRING clusters | CL:2042 | Citrate cycle (TCA cycle), and Propanoate metabolism | 16 | 54 | 0.56 | 0.0014 |
| STRING clusters | CL:446 | Mixed, incl. protein refolding, and hsp90 protein | 6 | 6 | 1.09 | 0.0032 |
| STRING clusters | CL:6732 | Pentose-phosphate shunt, and glycolytic process | 8 | 14 | 0.84 | 0.0034 |
| STRING clusters | CL:126 | rRNA-binding | 8 | 15 | 0.81 | 0.0047 |
| STRING clusters | CL:2098 | Citrate cycle (TCA cycle) | 10 | 26 | 0.67 | 0.0058 |
| STRING clusters | CL:229 | Aminoacyl-tRNA synthetase | 8 | 16 | 0.79 | 0.0061 |
| STRING clusters | CL:155 | Mixed, incl. ribosomal protein l9, and ribosomal protein l17 | 5 | 5 | 1.09 | 0.0106 |
| STRING clusters | CL:6729 | Pentose phosphate pathway, and Glycolysis / Gluconeogenesis | 11 | 35 | 0.58 | 0.0112 |
| STRING clusters | CL:374 | Protein export | 8 | 19 | 0.71 | 0.0141 |

| | | | | | | |
|---|---|---|---|---|---|---|
| STRING clusters | CL:4297 | Mixed, incl. efflux pump complex, and rnd efflux system, outer membrane lipoprotein, nodt | 5 | 6 | 1.01 | 0.017 |
| STRING clusters | CL:913 | Lipid transport, and lipoprotein localization | 8 | 20 | 0.69 | 0.0176 |
| STRING clusters | CL:914 | Lipid transport, and stas domain | 7 | 15 | 0.76 | 0.0178 |
| STRING clusters | CL:6730 | Pentose phosphate pathway, and Glyceraldehyde-3-phosphate dehydrogenase, type I | 9 | 27 | 0.61 | 0.0228 |
| STRING clusters | CL:6724 | Phosphoenolpyruvate-dependent sugar phosphotransferase system, and pentose phosphate pathway | 19 | 99 | 0.37 | 0.0246 |
| STRING clusters | CL:2263 | ATP synthesis coupled proton transport | 5 | 8 | 0.88 | 0.0371 |
| KEGG | seni03010 | Ribosome | 29 | 54 | 0.82 | 5.36E-11 |
| KEGG | seni01200 | Carbon metabolism | 37 | 114 | 0.6 | 2.93E-09 |
| KEGG | seni01110 | Biosynthesis of secondary metabolites | 64 | 346 | 0.35 | 2.18E-07 |
| KEGG | seni00190 | Oxidative phosphorylation | 17 | 43 | 0.68 | 3.28E-05 |
| KEGG | seni00010 | Glycolysis / Gluconeogenesis | 17 | 44 | 0.67 | 3.39E-05 |
| KEGG | seni01100 | Metabolic pathways | 113 | 899 | 0.19 | 3.99E-05 |
| KEGG | seni00020 | Citrate cycle (TCA cycle) | 13 | 28 | 0.75 | 8.85E-05 |
| KEGG | seni01120 | Microbial metabolism in diverse environments | 47 | 277 | 0.32 | 9.56E-05 |
| KEGG | seni01230 | Biosynthesis of amino acids | 27 | 128 | 0.41 | 0.00037 |
| KEGG | seni00970 | Aminoacyl-tRNA biosynthesis | 11 | 26 | 0.71 | 0.00065 |
| KEGG | seni00680 | Methane metabolism | 12 | 32 | 0.66 | 0.0007 |
| KEGG | seni03018 | RNA degradation | 8 | 16 | 0.79 | 0.0024 |
| KEGG | seni03060 | Protein export | 8 | 18 | 0.73 | 0.0041 |
| KEGG | seni00620 | Pyruvate metabolism | 14 | 58 | 0.47 | 0.0067 |
| KEGG | seni01503 | Cationic antimicrobial peptide (CAMP) resistance | 10 | 36 | 0.53 | 0.0144 |
| KEGG | seni00061 | Fatty acid biosynthesis | 6 | 13 | 0.75 | 0.015 |
| KEGG | seni01210 | 2-Oxocarboxylic acid metabolism | 8 | 29 | 0.53 | 0.0369 |
| KEGG | seni00520 | Amino sugar and nucleotide sugar metabolism | 11 | 51 | 0.42 | 0.0387 |
| UniProt Keywords | KW-0732 | Signal | 93 | 437 | 0.41 | 8.79E-14 |
| UniProt Keywords | KW-0689 | Ribosomal protein | 29 | 55 | 0.81 | 9.86E-11 |
| UniProt Keywords | KW-0998 | Cell outer membrane | 28 | 56 | 0.79 | 3.37E-10 |
| UniProt Keywords | KW-0694 | RNA-binding | 34 | 93 | 0.65 | 1.39E-09 |
| UniProt Keywords | KW-0699 | rRNA-binding | 22 | 40 | 0.83 | 1.20E-08 |

| | | | | | | |
|---|---|---|---|---|---|---|
| UniProt Keywords | KW-0449 | Lipoprotein | 29 | 81 | 0.64 | 4.71E-08 |
| UniProt Keywords | KW-0564 | Palmitate | 15 | 32 | 0.76 | 3.41E-05 |
| UniProt Keywords | KW-0547 | Nucleotide-binding | 66 | 441 | 0.26 | 0.00013 |
| UniProt Keywords | KW-1134 | Transmembrane beta strand | 12 | 26 | 0.75 | 0.00039 |
| UniProt Keywords | KW-0067 | ATP-binding | 57 | 378 | 0.26 | 0.00043 |
| UniProt Keywords | KW-0648 | Protein biosynthesis | 15 | 43 | 0.63 | 0.00043 |
| UniProt Keywords | KW-0436 | Ligase | 21 | 82 | 0.49 | 0.00044 |
| UniProt Keywords | KW-0963 | Cytoplasm | 70 | 504 | 0.23 | 0.00044 |
| UniProt Keywords | KW-0030 | Aminoacyl-tRNA synthetase | 11 | 24 | 0.75 | 0.00063 |
| UniProt Keywords | KW-0677 | Repeat | 19 | 80 | 0.46 | 0.0022 |
| UniProt Keywords | KW-0816 | Tricarboxylic acid cycle | 9 | 19 | 0.76 | 0.0024 |
| UniProt Keywords | KW-0143 | Chaperone | 14 | 54 | 0.5 | 0.0072 |
| UniProt Keywords | KW-0346 | Stress response | 11 | 35 | 0.58 | 0.0072 |
| UniProt Keywords | KW-0626 | Porin | 7 | 13 | 0.82 | 0.0072 |
| UniProt Keywords | KW-1278 | Translocase | 14 | 55 | 0.49 | 0.0074 |
| UniProt Keywords | KW-0324 | Glycolysis | 7 | 14 | 0.79 | 0.0083 |
| UniProt Keywords | KW-0444 | Lipid biosynthesis | 12 | 43 | 0.53 | 0.0083 |
| UniProt Keywords | KW-0574 | Periplasm | 17 | 78 | 0.42 | 0.0083 |
| UniProt Keywords | KW-1015 | Disulfide bond | 13 | 50 | 0.5 | 0.0083 |
| UniProt Keywords | KW-0560 | Oxidoreductase | 39 | 268 | 0.25 | 0.0085 |
| UniProt Keywords | KW-0697 | Rotamase | 6 | 10 | 0.86 | 0.0085 |
| UniProt Keywords | KW-0443 | Lipid metabolism | 13 | 54 | 0.47 | 0.0135 |
| UniProt Keywords | KW-0820 | tRNA-binding | 8 | 22 | 0.65 | 0.0135 |
| UniProt Keywords | KW-0645 | Protease | 15 | 72 | 0.41 | 0.0193 |
| UniProt Keywords | KW-0811 | Translocation | 6 | 13 | 0.75 | 0.02 |
| UniProt Keywords | KW-0275 | Fatty acid biosynthesis | 6 | 14 | 0.72 | 0.0259 |
| UniProt Keywords | KW-0139 | CF(1) | 4 | 5 | 0.99 | 0.0268 |
| UniProt Keywords | KW-0046 | Antibiotic resistance | 7 | 21 | 0.61 | 0.0334 |
| UniProt Keywords | KW-0830 | Ubiquinone | 5 | 10 | 0.79 | 0.0334 |
| UniProt Keywords | KW-0813 | Transport | 54 | 453 | 0.16 | 0.0407 |

| | | | | | | |
|---|---|---|---|---|---|---|
| UniProt Keywords | KW-0066 | ATP synthesis | 5 | 11 | 0.74 | 0.0419 |
| UniProt Keywords | KW-0406 | Ion transport | 14 | 73 | 0.37 | 0.0419 |
| UniProt Keywords | KW-0479 | Metal-binding | 57 | 490 | 0.15 | 0.0473 |

*Table 39 SEn protein String Analysis including GO process, GO function, GO component, String Clusters, KEGG, and UniProt keywords (output from STRING [String Consortium 2023, Switzerland[241])*

| Category | ID | Description | Observed Gene Count | Background Gene Count | Strength | False Discovery Rate |
|---|---|---|---|---|---|---|
| GO Process | GO:0008152 | Metabolic process | 696 | 2305 | 0.14 | 3.44E-25 |
| GO Process | GO:1901576 | Organic substance biosynthetic process | 335 | 881 | 0.24 | 4.63E-18 |
| GO Process | GO:0009058 | Biosynthetic process | 338 | 899 | 0.23 | 8.71E-18 |
| GO Process | GO:1901564 | Organonitrogen compound metabolic process | 353 | 957 | 0.22 | 8.71E-18 |
| GO Process | GO:0044237 | Cellular metabolic process | 579 | 1910 | 0.14 | 1.81E-17 |
| GO Process | GO:1901566 | Organonitrogen compound biosynthetic process | 226 | 505 | 0.31 | 1.81E-17 |
| GO Process | GO:0044249 | Cellular biosynthetic process | 325 | 866 | 0.23 | 4.31E-17 |
| GO Process | GO:0006518 | Peptide metabolic process | 99 | 135 | 0.52 | 2.05E-16 |
| GO Process | GO:0044271 | Cellular nitrogen compound biosynthetic process | 203 | 443 | 0.32 | 2.05E-16 |
| GO Process | GO:0006412 | Translation | 90 | 115 | 0.55 | 3.91E-16 |
| GO Process | GO:0043043 | Peptide biosynthetic process | 92 | 124 | 0.53 | 1.68E-15 |
| GO Process | GO:0071704 | Organic substance metabolic process | 571 | 1920 | 0.13 | 1.73E-15 |
| GO Process | GO:0043604 | Amide biosynthetic process | 105 | 159 | 0.48 | 2.34E-15 |
| GO Process | GO:0044238 | Primary metabolic process | 496 | 1599 | 0.15 | 4.12E-15 |
| GO Process | GO:0043603 | Cellular amide metabolic process | 117 | 196 | 0.43 | 7.12E-15 |
| GO Process | GO:0044281 | Small molecule metabolic process | 289 | 811 | 0.21 | 1.87E-12 |
| GO Process | GO:0009987 | Cellular process | 756 | 2884 | 0.07 | 4.57E-12 |
| GO Process | GO:0044267 | Cellular protein metabolic process | 127 | 256 | 0.35 | 1.18E-11 |
| GO Process | GO:0006807 | Nitrogen compound metabolic process | 430 | 1403 | 0.14 | 2.89E-11 |
| GO Process | GO:0019538 | Protein metabolic process | 160 | 378 | 0.28 | 1.85E-10 |
| GO Process | GO:0034641 | Cellular nitrogen compound metabolic process | 299 | 908 | 0.17 | 1.60E-09 |
| GO Process | GO:0034645 | Cellular macromolecule biosynthetic process | 157 | 386 | 0.27 | 3.41E-09 |

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|---|---|---|---|---|---|---|
| GO Process | GO:0055114 | Oxidation-reduction process | 188 | 498 | 0.23 | 4.87E-09 |
| GO Process | GO:0019752 | Carboxylic acid metabolic process | 171 | 441 | 0.24 | 7.89E-09 |
| GO Process | GO:0043436 | Oxoacid metabolic process | 174 | 460 | 0.23 | 2.37E-08 |
| GO Process | GO:0006082 | Organic acid metabolic process | 175 | 469 | 0.23 | 4.79E-08 |
| GO Process | GO:0010467 | Gene expression | 127 | 311 | 0.27 | 2.19E-07 |
| GO Process | GO:0055086 | Nucleobase-containing small molecule metabolic process | 94 | 208 | 0.31 | 6.51E-07 |
| GO Process | GO:0009117 | Nucleotide metabolic process | 72 | 140 | 0.37 | 6.98E-07 |
| GO Process | GO:0019637 | Organophosphate metabolic process | 110 | 266 | 0.27 | 1.49E-06 |
| GO Process | GO:0019693 | Ribose phosphate metabolic process | 55 | 97 | 0.41 | 3.20E-06 |
| GO Process | GO:0009259 | Ribonucleotide metabolic process | 53 | 92 | 0.42 | 3.69E-06 |
| GO Process | GO:0006520 | Cellular amino acid metabolic process | 100 | 241 | 0.27 | 5.31E-06 |
| GO Process | GO:0006163 | Purine nucleotide metabolic process | 48 | 84 | 0.41 | 1.74E-05 |
| GO Process | GO:0006091 | Generation of precursor metabolites and energy | 74 | 164 | 0.31 | 2.16E-05 |
| GO Process | GO:1901135 | Carbohydrate derivative metabolic process | 134 | 372 | 0.21 | 2.50E-05 |
| GO Process | GO:0009150 | Purine ribonucleotide metabolic process | 45 | 78 | 0.42 | 3.08E-05 |
| GO Process | GO:0072521 | Purine-containing compound metabolic process | 51 | 100 | 0.36 | 9.10E-05 |
| GO Process | GO:0044283 | Small molecule biosynthetic process | 115 | 315 | 0.22 | 9.77E-05 |
| GO Process | GO:0006793 | Phosphorus metabolic process | 160 | 485 | 0.17 | 0.00013 |
| GO Process | GO:0006418 | tRNA aminoacylation for protein translation | 22 | 24 | 0.62 | 0.00014 |
| GO Process | GO:0006796 | Phosphate-containing compound metabolic process | 155 | 468 | 0.18 | 0.00016 |
| GO Process | GO:1901362 | Organic cyclic compound biosynthetic process | 126 | 361 | 0.2 | 0.00018 |
| GO Process | GO:0044260 | Cellular macromolecule metabolic process | 224 | 753 | 0.13 | 0.00049 |
| GO Process | GO:0018130 | Heterocycle biosynthetic process | 119 | 345 | 0.19 | 0.0005 |
| GO Process | GO:0009165 | Nucleotide biosynthetic process | 46 | 94 | 0.35 | 0.00053 |
| GO Process | GO:0006090 | Pyruvate metabolic process | 23 | 30 | 0.54 | 0.00055 |
| GO Process | GO:0046390 | Ribose phosphate biosynthetic process | 36 | 65 | 0.4 | 0.00059 |
| GO Process | GO:0006165 | Nucleoside diphosphate phosphorylation | 18 | 19 | 0.63 | 0.00066 |
| GO Process | GO:0009179 | Purine ribonucleoside diphosphate metabolic process | 18 | 19 | 0.63 | 0.00066 |
| GO Process | GO:0009132 | Nucleoside diphosphate metabolic process | 19 | 22 | 0.59 | 0.00085 |
| GO Process | GO:0006096 | Glycolytic process | 17 | 18 | 0.63 | 0.001 |

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|---|---|---|---|---|---|---|
| GO Process | GO:0019438 | Aromatic compound biosynthetic process | 110 | 321 | 0.19 | 0.0011 |
| GO Process | GO:0043170 | Macromolecule metabolic process | 271 | 961 | 0.11 | 0.0011 |
| GO Process | GO:0009260 | Ribonucleotide biosynthetic process | 34 | 63 | 0.39 | 0.0012 |
| GO Process | GO:1901605 | Alpha-amino acid metabolic process | 71 | 184 | 0.24 | 0.0015 |
| GO Process | GO:0032787 | Monocarboxylic acid metabolic process | 64 | 161 | 0.26 | 0.0017 |
| GO Process | GO:0046034 | ATP metabolic process | 26 | 42 | 0.45 | 0.0017 |
| GO Process | GO:0034654 | Nucleobase-containing compound biosynthetic process | 79 | 214 | 0.22 | 0.0018 |
| GO Process | GO:1901360 | Organic cyclic compound metabolic process | 230 | 802 | 0.11 | 0.002 |
| GO Process | GO:0046394 | Carboxylic acid biosynthetic process | 73 | 196 | 0.23 | 0.0027 |
| GO Process | GO:0006935 | Chemotaxis | 20 | 28 | 0.51 | 0.0028 |
| GO Process | GO:0006979 | Response to oxidative stress | 31 | 59 | 0.38 | 0.0033 |
| GO Process | GO:0046483 | Heterocycle metabolic process | 219 | 768 | 0.11 | 0.0039 |
| GO Process | GO:0009123 | Nucleoside monophosphate metabolic process | 25 | 44 | 0.41 | 0.0057 |
| GO Process | GO:0010608 | Posttranscriptional regulation of gene expression | 25 | 44 | 0.41 | 0.0057 |
| GO Process | GO:0006164 | Purine nucleotide biosynthetic process | 28 | 53 | 0.38 | 0.006 |
| GO Process | GO:0006417 | Regulation of translation | 23 | 40 | 0.42 | 0.0083 |
| GO Process | GO:0042221 | Response to chemical | 70 | 196 | 0.21 | 0.0086 |
| GO Process | GO:0090407 | Organophosphate biosynthetic process | 68 | 189 | 0.21 | 0.0087 |
| GO Process | GO:0009060 | Aerobic respiration | 21 | 35 | 0.43 | 0.0093 |
| GO Process | GO:0006099 | Tricarboxylic acid cycle | 18 | 27 | 0.48 | 0.0094 |
| GO Process | GO:0009152 | Purine ribonucleotide biosynthetic process | 26 | 50 | 0.37 | 0.0107 |
| GO Process | GO:0006725 | Cellular aromatic compound metabolic process | 215 | 771 | 0.1 | 0.0114 |
| GO Process | GO:1901137 | Carbohydrate derivative biosynthetic process | 82 | 243 | 0.18 | 0.0114 |
| GO Process | GO:0009161 | Ribonucleoside monophosphate metabolic process | 21 | 36 | 0.42 | 0.0116 |
| GO Process | GO:0006006 | Glucose metabolic process | 17 | 26 | 0.47 | 0.0149 |
| GO Process | GO:0006457 | Protein folding | 26 | 52 | 0.35 | 0.0158 |
| GO Process | GO:0009156 | Ribonucleoside monophosphate biosynthetic process | 20 | 35 | 0.41 | 0.018 |
| GO Process | GO:0070887 | Cellular response to chemical stimulus | 29 | 62 | 0.33 | 0.0181 |
| GO Process | GO:0040011 | Locomotion | 27 | 56 | 0.34 | 0.0187 |
| GO Process | GO:0006139 | Nucleobase-containing compound metabolic process | 178 | 631 | 0.11 | 0.0222 |

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|---|---|---|---|---|---|---|
| GO Process | GO:0009064 | Glutamine family amino acid metabolic process | 26 | 54 | 0.34 | 0.0228 |
| GO Process | GO:0009124 | Nucleoside monophosphate biosynthetic process | 21 | 39 | 0.39 | 0.0228 |
| GO Process | GO:0034599 | Cellular response to oxidative stress | 15 | 23 | 0.47 | 0.0279 |
| GO Process | GO:0072330 | Monocarboxylic acid biosynthetic process | 23 | 46 | 0.35 | 0.0284 |
| GO Process | GO:0000302 | Response to reactive oxygen species | 16 | 26 | 0.45 | 0.03 |
| GO Process | GO:0006740 | NADPH regeneration | 10 | 11 | 0.61 | 0.0313 |
| GO Process | GO:0051156 | Glucose 6-phosphate metabolic process | 10 | 11 | 0.61 | 0.0313 |
| GO Process | GO:0051246 | Regulation of protein metabolic process | 25 | 54 | 0.32 | 0.0391 |
| GO Process | GO:0006739 | NADP metabolic process | 11 | 14 | 0.55 | 0.0392 |
| GO Function | GO:0003824 | Catalytic activity | 621 | 2137 | 0.12 | 3.40E-15 |
| GO Function | GO:0005488 | Binding | 587 | 2091 | 0.1 | 1.38E-10 |
| GO Function | GO:0003723 | RNA binding | 91 | 159 | 0.41 | 4.99E-10 |
| GO Function | GO:0003735 | Structural constituent of ribosome | 50 | 58 | 0.59 | 2.47E-09 |
| GO Function | GO:0043167 | Ion binding | 381 | 1288 | 0.13 | 3.42E-07 |
| GO Function | GO:0005198 | Structural molecule activity | 55 | 90 | 0.44 | 1.51E-06 |
| GO Function | GO:0019843 | rRNA binding | 37 | 46 | 0.56 | 2.66E-06 |
| GO Function | GO:0016491 | Oxidoreductase activity | 152 | 424 | 0.21 | 1.33E-05 |
| GO Function | GO:0016874 | Ligase activity | 58 | 108 | 0.39 | 1.33E-05 |
| GO Function | GO:0043169 | Cation binding | 242 | 773 | 0.15 | 1.74E-05 |
| GO Function | GO:0036094 | Small molecule binding | 238 | 759 | 0.15 | 1.88E-05 |
| GO Function | GO:0000166 | Nucleotide binding | 215 | 670 | 0.16 | 1.96E-05 |
| GO Function | GO:0046872 | Metal ion binding | 238 | 763 | 0.15 | 2.12E-05 |
| GO Function | GO:0097159 | Organic cyclic compound binding | 380 | 1366 | 0.1 | 6.89E-05 |
| GO Function | GO:1901363 | Heterocyclic compound binding | 380 | 1366 | 0.1 | 6.89E-05 |
| GO Function | GO:0004812 | aminoacyl-tRNA ligase activity | 22 | 25 | 0.6 | 0.00033 |
| GO Function | GO:0097367 | Carbohydrate derivative binding | 178 | 568 | 0.15 | 0.00062 |
| GO Function | GO:0043168 | Anion binding | 204 | 672 | 0.14 | 0.00065 |
| GO Function | GO:0032553 | Ribonucleotide binding | 165 | 534 | 0.15 | 0.0024 |
| GO Function | GO:0017076 | Purine nucleotide binding | 158 | 509 | 0.15 | 0.0028 |
| GO Function | GO:0032555 | Purine ribonucleotide binding | 157 | 507 | 0.15 | 0.0031 |

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|---|---|---|---|---|---|---|
| GO Function | GO:0035639 | Purine ribonucleoside triphosphate binding | 154 | 498 | 0.15 | 0.0038 |
| GO Function | GO:0000287 | Magnesium ion binding | 52 | 133 | 0.25 | 0.0177 |
| GO Function | GO:0030554 | Adenyl nucleotide binding | 140 | 465 | 0.13 | 0.0202 |
| GO Function | GO:0032559 | Adenyl ribonucleotide binding | 139 | 463 | 0.13 | 0.0226 |
| GO Function | GO:0016209 | Antioxidant activity | 17 | 26 | 0.47 | 0.0282 |
| GO Function | GO:0005524 | ATP binding | 137 | 460 | 0.13 | 0.0303 |
| GO Component | GO:0005622 | Intracellular | 676 | 1889 | 0.21 | 3.90E-49 |
| GO Component | GO:0005737 | Cytoplasm | 612 | 1760 | 0.2 | 1.41E-36 |
| GO Component | GO:0110165 | Cellular anatomical entity | 933 | 3544 | 0.08 | 2.22E-35 |
| GO Component | GO:0005829 | Cytosol | 263 | 653 | 0.26 | 8.29E-17 |
| GO Component | GO:0005840 | Ribosome | 54 | 63 | 0.59 | 6.15E-11 |
| GO Component | GO:0043232 | Intracellular non-membrane-bounded organelle | 62 | 108 | 0.41 | 1.87E-07 |
| GO Component | GO:0043228 | Non-membrane-bounded organelle | 69 | 140 | 0.35 | 2.38E-06 |
| GO Component | GO:0043229 | Intracellular organelle | 65 | 161 | 0.26 | 0.00094 |
| GO Component | GO:0043226 | Organelle | 72 | 194 | 0.23 | 0.0028 |
| GO Component | GO:0042597 | Periplasmic space | 77 | 214 | 0.21 | 0.0032 |
| GO Component | GO:0044391 | Ribosomal subunit | 15 | 17 | 0.6 | 0.0032 |
| GO Component | GO:1990904 | Ribonucleoprotein complex | 16 | 20 | 0.56 | 0.0036 |
| STRING clusters | CL:79 | Translation, and protein export | 121 | 162 | 0.53 | 2.73E-20 |
| STRING clusters | CL:78 | Translation, and protein export | 125 | 175 | 0.51 | 3.85E-20 |
| STRING clusters | CL:80 | Translation, and proton channel activity | 112 | 145 | 0.54 | 7.09E-20 |
| STRING clusters | CL:81 | Translation, and ribosome biogenesis | 102 | 132 | 0.54 | 4.75E-18 |
| STRING clusters | CL:83 | Ribosome, and Protein biosynthesis | 79 | 97 | 0.57 | 1.29E-14 |
| STRING clusters | CL:82 | Ribosome, and Protein biosynthesis | 82 | 110 | 0.53 | 9.97E-14 |
| STRING clusters | CL:84 | Ribosomal protein, and translation factor activity, RNA binding | 68 | 84 | 0.56 | 1.86E-12 |
| STRING clusters | CL:89 | Ribosomal protein | 58 | 68 | 0.59 | 3.45E-11 |
| STRING clusters | CL:86 | Ribosomal protein, and translation factor activity, RNA binding | 60 | 73 | 0.57 | 3.55E-11 |
| STRING clusters | CL:93 | Ribosomal protein | 55 | 62 | 0.6 | 3.95E-11 |
| STRING clusters | CL:95 | Ribosomal protein | 51 | 57 | 0.61 | 2.13E-10 |
| STRING clusters | CL:96 | Ribosomal protein | 46 | 52 | 0.6 | 3.12E-09 |

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|---|---|---|---|---|---|---|
| STRING clusters | CL:98 | Ribosomal protein | 42 | 46 | 0.62 | 1.07E-08 |
| STRING clusters | CL:99 | Ribosomal protein | 31 | 35 | 0.6 | 4.34E-06 |
| STRING clusters | CL:100 | Ribosomal protein | 24 | 28 | 0.59 | 0.00019 |
| STRING clusters | CL:2585 | Pyruvate metabolism, and Oxidative phosphorylation | 60 | 131 | 0.32 | 0.00025 |
| STRING clusters | CL:2028 | Antioxidant activity, and protein disulfide oxidoreductase activity | 26 | 34 | 0.54 | 0.00028 |
| STRING clusters | CL:4302 | Pentose phosphate pathway, and Glycolysis | 33 | 52 | 0.46 | 0.00028 |
| STRING clusters | CL:4304 | Pentose phosphate pathway, and Glycolysis | 28 | 40 | 0.5 | 0.00036 |
| STRING clusters | CL:1291 | Cellular amino acid biosynthetic process, and ribonucleoside monophosphate biosynthetic process | 63 | 149 | 0.28 | 0.00083 |
| STRING clusters | CL:2026 | Mixed, incl. antioxidant activity, and iron-sulfur cluster assembly | 38 | 72 | 0.38 | 0.0011 |
| STRING clusters | CL:4305 | Pentose phosphate pathway, and Glycolysis | 22 | 29 | 0.54 | 0.0012 |
| STRING clusters | CL:302 | Aminoacyl-tRNA synthetase | 18 | 20 | 0.61 | 0.0013 |
| STRING clusters | CL:4300 | Pentose phosphate pathway, and phosphoenolpyruvate-dependent sugar phosphotransferase system | 49 | 107 | 0.32 | 0.0013 |
| STRING clusters | CL:2027 | Mixed, incl. antioxidant activity, and iron-sulfur cluster assembly | 35 | 65 | 0.39 | 0.0014 |
| STRING clusters | CL:2583 | Mixed, incl. pyruvate metabolism, and oxidative phosphorylation | 66 | 165 | 0.26 | 0.0016 |
| STRING clusters | CL:4308 | Glycolysis, and fructose 1,6-bisphosphate 1-phosphatase activity | 16 | 17 | 0.63 | 0.0023 |
| STRING clusters | CL:2582 | Mixed, incl. pyruvate metabolism, and oxidative phosphorylation | 68 | 175 | 0.25 | 0.0024 |
| STRING clusters | CL:4306 | Glycolysis, and pentose-phosphate shunt | 18 | 22 | 0.57 | 0.0026 |
| STRING clusters | CL:4724 | Mixed, incl. glycogen metabolic process, and trehalose metabolic process | 20 | 27 | 0.53 | 0.0026 |
| STRING clusters | CL:1292 | Cellular amino acid biosynthetic process, and ribonucleoside monophosphate biosynthetic process | 56 | 136 | 0.27 | 0.0028 |
| STRING clusters | CL:2029 | Reactive oxygen species metabolic process, and protein disulfide oxidoreductase activity | 17 | 20 | 0.59 | 0.0028 |
| STRING clusters | CL:2648 | Tricarboxylic acid cycle, and pyruvate dehydrogenase complex | 15 | 16 | 0.63 | 0.0033 |
| STRING clusters | CL:305 | Aminoacyl-tRNA synthetase | 14 | 14 | 0.66 | 0.0034 |
| STRING clusters | CL:2587 | Propanoate metabolism, and tricarboxylic acid cycle | 28 | 50 | 0.4 | 0.0037 |
| STRING clusters | CL:6488 | Taxis | 14 | 15 | 0.63 | 0.0053 |
| STRING clusters | CL:1293 | Amino-acid biosynthesis, and ribonucleoside monophosphate biosynthetic process | 42 | 95 | 0.3 | 0.0055 |
| STRING clusters | CL:4723 | Starch and sucrose metabolism, and galactose metabolic process | 22 | 36 | 0.44 | 0.0071 |
| STRING clusters | CL:4299 | Mixed, incl. pentose phosphate pathway, and phosphoenolpyruvate-dependent sugar phosphotransferase system | 52 | 131 | 0.25 | 0.0083 |

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|---|---|---|---|---|---|---|
| STRING clusters | CL:906 | Mixed, incl. external encapsulating structure organization, and cell cycle | 69 | 192 | 0.21 | 0.0097 |
| STRING clusters | CL:1295 | Ribonucleoside monophosphate biosynthetic process, and histidine biosynthesis | 25 | 46 | 0.39 | 0.01 |
| STRING clusters | CL:2586 | Pyruvate metabolism, and Valine, leucine and isoleucine biosynthesis | 37 | 83 | 0.31 | 0.0102 |
| STRING clusters | CL:4725 | Glycogen metabolic process, and trehalose metabolic process | 16 | 22 | 0.52 | 0.0111 |
| STRING clusters | CL:1294 | Aromatic amino acid family biosynthetic process, and ribonucleoside monophosphate biosynthetic process | 32 | 68 | 0.33 | 0.0114 |
| STRING clusters | CL:1069 | Mixed, incl. membrane organization, and lipoprotein localization | 25 | 47 | 0.38 | 0.0117 |
| STRING clusters | CL:5612 | Mostly uncharacterized, incl. ribonucleoprotein complex binding, and bacterial-type flagellum-dependent swarming motility | 28 | 56 | 0.35 | 0.0117 |
| STRING clusters | CL:168 | Ribosomal protein | 11 | 11 | 0.66 | 0.0137 |
| STRING clusters | CL:101 | rRNA-binding | 13 | 16 | 0.57 | 0.0165 |
| STRING clusters | CL:1068 | Mixed, incl. membrane organization, and lipoprotein localization | 28 | 58 | 0.34 | 0.0165 |
| STRING clusters | CL:907 | Mixed, incl. aminoglycan metabolic process, and membrane organization | 53 | 141 | 0.23 | 0.0165 |
| STRING clusters | CL:132 | Ribosomal protein | 11 | 12 | 0.62 | 0.02 |
| STRING clusters | CL:252 | Mixed, incl. rna polymerase, and transcription termination | 11 | 12 | 0.62 | 0.02 |
| STRING clusters | CL:4309 | Glycolysis, and transketolase activity | 11 | 12 | 0.62 | 0.02 |
| STRING clusters | CL:6493 | Chemotaxis | 10 | 10 | 0.66 | 0.0209 |
| STRING clusters | CL:1070 | Mixed, incl. membrane organization, and lipoprotein localization | 22 | 42 | 0.38 | 0.0233 |
| STRING clusters | CL:5615 | Mostly uncharacterized, incl. bacterial-type flagellum-dependent swarming motility, and response to oxygen-containing compound | 24 | 48 | 0.35 | 0.0233 |
| STRING clusters | CL:437 | Mixed, incl. peptidase complex, and unfolded protein binding | 11 | 13 | 0.58 | 0.0275 |
| STRING clusters | CL:4722 | Polysaccharide metabolic process, and disaccharide metabolic process | 31 | 71 | 0.3 | 0.0275 |
| STRING clusters | CL:434 | Stress response, and unfolded protein binding | 13 | 18 | 0.51 | 0.0282 |
| STRING clusters | CL:4721 | Cellular polysaccharide metabolic process, and oligosaccharide metabolic process | 43 | 112 | 0.24 | 0.0289 |
| STRING clusters | CL:1589 | Nicotinate and nicotinamide metabolism, and Pyrimidine metabolism | 29 | 67 | 0.29 | 0.0398 |
| STRING clusters | CL:2751 | Oxidative phosphorylation, and fumarate hydratase activity | 23 | 48 | 0.34 | 0.0398 |
| STRING clusters | CL:2030 | Redox-active center, and ferredoxin-NADP+ reductase activity | 10 | 12 | 0.58 | 0.0444 |
| STRING clusters | CL:1072 | Mixed, incl. protein insertion into membrane, and protein import | 12 | 17 | 0.5 | 0.0447 |
| STRING clusters | CL:4726 | Glycogen metabolic process, and glucose-1-phosphatase activity | 12 | 17 | 0.5 | 0.0447 |
| STRING clusters | CL:1837 | Fatty acid synthase activity, and nadph binding | 9 | 10 | 0.61 | 0.0489 |

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|---|---|---|---|---|---|---|
| KEGG | stm01110 | Biosynthesis of secondary metabolites | 206 | 520 | 0.25 | 1.64E-11 |
| KEGG | stm01100 | Metabolic pathways | 439 | 1448 | 0.14 | 4.08E-11 |
| KEGG | stm03010 | Ribosome | 50 | 58 | 0.59 | 4.15E-10 |
| KEGG | stm01200 | Carbon metabolism | 80 | 146 | 0.39 | 5.80E-09 |
| KEGG | stm00010 | Glycolysis / Gluconeogenesis | 38 | 57 | 0.48 | 9.81E-06 |
| KEGG | stm00970 | Aminoacyl-tRNA biosynthesis | 26 | 34 | 0.54 | 0.0001 |
| KEGG | stm02030 | Bacterial chemotaxis | 25 | 32 | 0.55 | 0.0001 |
| KEGG | stm00620 | Pyruvate metabolism | 36 | 67 | 0.39 | 0.00046 |
| KEGG | stm01230 | Biosynthesis of amino acids | 67 | 169 | 0.25 | 0.00068 |
| KEGG | stm00020 | Citrate cycle (TCA cycle) | 22 | 34 | 0.47 | 0.0019 |
| KEGG | stm01120 | Microbial metabolism in diverse environments | 127 | 409 | 0.15 | 0.0041 |
| KEGG | stm00480 | Glutathione metabolism | 22 | 39 | 0.41 | 0.0067 |
| KEGG | stm00260 | Glycine, serine and threonine metabolism | 24 | 46 | 0.37 | 0.0083 |
| KEGG | stm00030 | Pentose phosphate pathway | 25 | 50 | 0.35 | 0.0096 |
| KEGG | stm00240 | Pyrimidine metabolism | 26 | 56 | 0.32 | 0.0164 |
| KEGG | stm00061 | Fatty acid biosynthesis | 13 | 20 | 0.47 | 0.0257 |
| KEGG | stm00230 | Purine metabolism | 38 | 99 | 0.24 | 0.0257 |
| KEGG | stm00250 | Alanine, aspartate and glutamate metabolism | 21 | 43 | 0.34 | 0.0257 |
| KEGG | stm00630 | Glyoxylate and dicarboxylate metabolism | 19 | 37 | 0.37 | 0.0257 |
| KEGG | stm00220 | Arginine biosynthesis | 14 | 24 | 0.42 | 0.0306 |
| KEGG | stm00520 | Amino sugar and nucleotide sugar metabolism | 34 | 88 | 0.24 | 0.0306 |
| KEGG | stm00190 | Oxidative phosphorylation | 23 | 52 | 0.3 | 0.0318 |
| UniProt Keywords | KW-0963 | Cytoplasm | 229 | 505 | 0.31 | 2.09E-18 |
| UniProt Keywords | KW-0479 | Metal-binding | 189 | 492 | 0.24 | 2.18E-09 |
| UniProt Keywords | KW-0687 | Ribonucleoprotein | 50 | 59 | 0.58 | 2.18E-09 |
| UniProt Keywords | KW-0689 | Ribosomal protein | 49 | 57 | 0.59 | 2.18E-09 |
| UniProt Keywords | KW-0694 | RNA-binding | 60 | 92 | 0.47 | 1.69E-08 |
| UniProt Keywords | KW-0699 | rRNA-binding | 36 | 40 | 0.61 | 1.87E-07 |

| | | | | | | |
|---|---|---|---|---|---|---|
| UniProt Keywords | KW-0560 | Oxidoreductase | 112 | 273 | 0.27 | 1.15E-06 |
| UniProt Keywords | KW-0648 | Protein biosynthesis | 36 | 45 | 0.56 | 1.15E-06 |
| UniProt Keywords | KW-0436 | Ligase | 48 | 84 | 0.41 | 1.18E-05 |
| UniProt Keywords | KW-0547 | Nucleotide-binding | 155 | 448 | 0.2 | 1.39E-05 |
| UniProt Keywords | KW-0460 | Magnesium | 81 | 191 | 0.28 | 2.55E-05 |
| UniProt Keywords | KW-0030 | Aminoacyl-tRNA synthetase | 22 | 24 | 0.62 | 8.63E-05 |
| UniProt Keywords | KW-0574 | Periplasm | 36 | 75 | 0.34 | 0.0039 |
| UniProt Keywords | KW-0520 | NAD | 42 | 97 | 0.29 | 0.0061 |
| UniProt Keywords | KW-0862 | Zinc | 54 | 137 | 0.25 | 0.0061 |
| UniProt Keywords | KW-0324 | Glycolysis | 13 | 14 | 0.62 | 0.0062 |
| UniProt Keywords | KW-0521 | NADP | 29 | 60 | 0.34 | 0.0114 |
| UniProt Keywords | KW-0067 | ATP-binding | 117 | 383 | 0.14 | 0.0163 |
| UniProt Keywords | KW-0021 | Allosteric enzyme | 14 | 20 | 0.5 | 0.0218 |
| UniProt Keywords | KW-0143 | Chaperone | 26 | 55 | 0.33 | 0.0232 |
| UniProt Keywords | KW-0816 | Tricarboxylic acid cycle | 13 | 18 | 0.51 | 0.0238 |
| UniProt Keywords | KW-0276 | Fatty acid metabolism | 14 | 21 | 0.48 | 0.0256 |
| UniProt Keywords | KW-0732 | Signal | 131 | 448 | 0.12 | 0.0256 |
| UniProt Keywords | KW-0998 | Cell outer membrane | 26 | 56 | 0.32 | 0.0256 |
| UniProt Keywords | KW-0275 | Fatty acid biosynthesis | 11 | 14 | 0.55 | 0.0286 |
| UniProt Keywords | KW-0132 | Cell division | 25 | 55 | 0.31 | 0.032 |
| UniProt Keywords | KW-0145 | Chemotaxis | 12 | 17 | 0.5 | 0.032 |
| UniProt Keywords | KW-0413 | Isomerase | 42 | 112 | 0.23 | 0.032 |

*Table 40 STm protein String Analysis including GO process, GO function, GO component, String Clusters, KEGG, and UniProt keywords (output from STRING [String Consortium 2023, Switzerland[241])*

| Category | ID | Description | Observed Gene Count | Background Gene Count | Strength | False Discovery Rate |
|---|---|---|---|---|---|---|
| GO Process | GO:0008152 | Metabolic process | 787 | 2305 | 0.13 | 1.49E-27 |
| GO Process | GO:0044237 | Cellular metabolic process | 660 | 1910 | 0.14 | 8.23E-20 |
| GO Process | GO:1901564 | Organonitrogen compound metabolic process | 400 | 957 | 0.22 | 1.15E-19 |
| GO Process | GO:0044238 | Primary metabolic process | 574 | 1599 | 0.16 | 9.32E-19 |
| GO Process | GO:1901576 | Organic substance biosynthetic process | 371 | 881 | 0.23 | 2.36E-18 |
| GO Process | GO:0009058 | Biosynthetic process | 375 | 899 | 0.22 | 3.76E-18 |
| GO Process | GO:0071704 | Organic substance metabolic process | 653 | 1920 | 0.13 | 3.76E-18 |
| GO Process | GO:1901566 | Organonitrogen compound biosynthetic process | 251 | 505 | 0.3 | 3.90E-18 |
| GO Process | GO:0044249 | Cellular biosynthetic process | 361 | 866 | 0.22 | 2.57E-17 |
| GO Process | GO:0044271 | Cellular nitrogen compound biosynthetic process | 222 | 443 | 0.3 | 4.78E-16 |
| GO Process | GO:0006518 | Peptide metabolic process | 103 | 135 | 0.48 | 9.10E-15 |
| GO Process | GO:0043603 | Cellular amide metabolic process | 128 | 196 | 0.42 | 9.11E-15 |
| GO Process | GO:0006412 | Translation | 93 | 115 | 0.51 | 2.20E-14 |
| GO Process | GO:0006807 | Nitrogen compound metabolic process | 498 | 1403 | 0.15 | 2.29E-14 |
| GO Process | GO:0043604 | Amide biosynthetic process | 111 | 159 | 0.45 | 3.61E-14 |
| GO Process | GO:0043043 | Peptide biosynthetic process | 95 | 124 | 0.49 | 9.30E-14 |
| GO Process | GO:0044281 | Small molecule metabolic process | 327 | 811 | 0.21 | 1.06E-13 |
| GO Process | GO:0009987 | Cellular process | 855 | 2884 | 0.07 | 4.59E-13 |
| GO Process | GO:0034641 | Cellular nitrogen compound metabolic process | 341 | 908 | 0.18 | 6.74E-11 |
| GO Process | GO:0044267 | Cellular protein metabolic process | 131 | 256 | 0.31 | 2.09E-09 |
| GO Process | GO:0034645 | Cellular macromolecule biosynthetic process | 174 | 386 | 0.26 | 2.97E-09 |
| GO Process | GO:0019538 | Protein metabolic process | 171 | 378 | 0.26 | 3.47E-09 |
| GO Process | GO:0055114 | Oxidation-reduction process | 207 | 498 | 0.22 | 9.10E-09 |
| GO Process | GO:0019752 | Carboxylic acid metabolic process | 188 | 441 | 0.23 | 1.49E-08 |
| GO Process | GO:0043436 | Oxoacid metabolic process | 193 | 460 | 0.22 | 2.26E-08 |

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|---|---|---|---|---|---|---|
| GO Process | GO:0006082 | Organic acid metabolic process | 194 | 469 | 0.22 | 5.00E-08 |
| GO Process | GO:0055086 | Nucleobase-containing small molecule metabolic process | 107 | 208 | 0.31 | 9.74E-08 |
| GO Process | GO:0009117 | Nucleotide metabolic process | 81 | 140 | 0.36 | 2.08E-07 |
| GO Process | GO:0019693 | Ribose phosphate metabolic process | 64 | 97 | 0.42 | 2.44E-07 |
| GO Process | GO:0006753 | Nucleoside phosphate metabolic process | 82 | 144 | 0.36 | 2.69E-07 |
| GO Process | GO:0019637 | Organophosphate metabolic process | 124 | 266 | 0.27 | 4.18E-07 |
| GO Process | GO:0009259 | Ribonucleotide metabolic process | 61 | 92 | 0.42 | 4.53E-07 |
| GO Process | GO:0006520 | Cellular amino acid metabolic process | 115 | 241 | 0.28 | 5.34E-07 |
| GO Process | GO:0010467 | Gene expression | 137 | 311 | 0.25 | 9.48E-07 |
| GO Process | GO:1901135 | Carbohydrate derivative metabolic process | 155 | 372 | 0.22 | 1.83E-06 |
| GO Process | GO:0006163 | Purine nucleotide metabolic process | 54 | 84 | 0.41 | 6.19E-06 |
| GO Process | GO:0009150 | Purine ribonucleotide metabolic process | 50 | 78 | 0.41 | 1.89E-05 |
| GO Process | GO:0006091 | Generation of precursor metabolites and energy | 81 | 164 | 0.3 | 2.80E-05 |
| GO Process | GO:0044283 | Small molecule biosynthetic process | 130 | 315 | 0.22 | 3.65E-05 |
| GO Process | GO:1901362 | Organic cyclic compound biosynthetic process | 144 | 361 | 0.2 | 4.11E-05 |
| GO Process | GO:0072521 | Purine-containing compound metabolic process | 57 | 100 | 0.36 | 4.65E-05 |
| GO Process | GO:0006793 | Phosphorus metabolic process | 181 | 485 | 0.17 | 4.82E-05 |
| GO Process | GO:0006796 | Phosphate-containing compound metabolic process | 174 | 468 | 0.17 | 9.59E-05 |
| GO Process | GO:0044260 | Cellular macromolecule metabolic process | 256 | 753 | 0.13 | 0.0001 |
| GO Process | GO:1901605 | Alpha-amino acid metabolic process | 85 | 184 | 0.27 | 0.0001 |
| GO Process | GO:0018130 | Heterocycle biosynthetic process | 136 | 345 | 0.2 | 0.00012 |
| GO Process | GO:0009165 | Nucleotide biosynthetic process | 52 | 94 | 0.34 | 0.00023 |
| GO Process | GO:0043170 | Macromolecule metabolic process | 309 | 961 | 0.11 | 0.00035 |
| GO Process | GO:0046390 | Ribose phosphate biosynthetic process | 40 | 65 | 0.39 | 0.00042 |
| GO Process | GO:1901137 | Carbohydrate derivative biosynthetic process | 101 | 243 | 0.22 | 0.00042 |
| GO Process | GO:0019438 | Aromatic compound biosynthetic process | 125 | 321 | 0.19 | 0.00046 |
| GO Process | GO:1901360 | Organic cyclic compound metabolic process | 264 | 802 | 0.12 | 0.00046 |
| GO Process | GO:0006418 | tRNA aminoacylation for protein translation | 22 | 24 | 0.56 | 0.00062 |
| GO Process | GO:0034654 | Nucleobase-containing compound biosynthetic process | 90 | 214 | 0.23 | 0.00083 |
| GO Process | GO:0009260 | Ribonucleotide biosynthetic process | 38 | 63 | 0.38 | 0.00085 |

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|---|---|---|---|---|---|---|
| GO Process | GO:0046483 | Heterocycle metabolic process | 252 | 768 | 0.12 | 0.00087 |
| GO Process | GO:0009123 | Nucleoside monophosphate metabolic process | 30 | 44 | 0.44 | 0.0011 |
| GO Process | GO:0009132 | Nucleoside diphosphate metabolic process | 20 | 22 | 0.56 | 0.0014 |
| GO Process | GO:0046394 | Carboxylic acid biosynthetic process | 82 | 196 | 0.22 | 0.0019 |
| GO Process | GO:0006165 | Nucleoside diphosphate phosphorylation | 18 | 19 | 0.58 | 0.0023 |
| GO Process | GO:0009179 | Purine ribonucleoside diphosphate metabolic process | 18 | 19 | 0.58 | 0.0023 |
| GO Process | GO:0006725 | Cellular aromatic compound metabolic process | 248 | 771 | 0.11 | 0.0026 |
| GO Process | GO:0006096 | Glycolytic process | 17 | 18 | 0.58 | 0.0034 |
| GO Process | GO:0090407 | Organophosphate biosynthetic process | 78 | 189 | 0.22 | 0.0035 |
| GO Process | GO:0009064 | Glutamine family amino acid metabolic process | 31 | 54 | 0.36 | 0.0066 |
| GO Process | GO:0009161 | Ribonucleoside monophosphate metabolic process | 24 | 36 | 0.43 | 0.0066 |
| GO Process | GO:0005975 | Carbohydrate metabolic process | 133 | 377 | 0.15 | 0.0067 |
| GO Process | GO:0006139 | Nucleobase-containing compound metabolic process | 205 | 631 | 0.11 | 0.0071 |
| GO Process | GO:0009124 | Nucleoside monophosphate biosynthetic process | 25 | 39 | 0.41 | 0.0073 |
| GO Process | GO:0046034 | ATP metabolic process | 26 | 42 | 0.39 | 0.0082 |
| GO Process | GO:0006090 | Pyruvate metabolic process | 21 | 30 | 0.45 | 0.0092 |
| GO Process | GO:0006164 | Purine nucleotide biosynthetic process | 30 | 53 | 0.35 | 0.0092 |
| GO Process | GO:0009156 | Ribonucleoside monophosphate biosynthetic process | 23 | 35 | 0.42 | 0.0093 |
| GO Process | GO:0006935 | Chemotaxis | 20 | 28 | 0.46 | 0.01 |
| GO Process | GO:0006006 | Glucose metabolic process | 19 | 26 | 0.47 | 0.011 |
| GO Process | GO:0008652 | Cellular amino acid biosynthetic process | 55 | 128 | 0.23 | 0.013 |
| GO Process | GO:0006099 | Tricarboxylic acid cycle | 19 | 27 | 0.45 | 0.0146 |
| GO Process | GO:0009152 | Purine ribonucleotide biosynthetic process | 28 | 50 | 0.35 | 0.0146 |
| GO Process | GO:0009060 | Aerobic respiration | 22 | 35 | 0.4 | 0.0171 |
| GO Process | GO:1901607 | Alpha-amino acid biosynthetic process | 49 | 112 | 0.24 | 0.0178 |
| GO Process | GO:0009084 | Glutamine family amino acid biosynthetic process | 17 | 23 | 0.47 | 0.018 |
| GO Process | GO:0042221 | Response to chemical | 75 | 196 | 0.18 | 0.0214 |
| GO Process | GO:0010608 | Posttranscriptional regulation of gene expression | 25 | 44 | 0.36 | 0.0222 |
| GO Process | GO:0040011 | Locomotion | 29 | 56 | 0.32 | 0.0272 |
| GO Process | GO:0006417 | Regulation of translation | 23 | 40 | 0.36 | 0.0294 |

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|---|---|---|---|---|---|---|
| GO Process | GO:0015980 | Energy derivation by oxidation of organic compounds | 40 | 89 | 0.25 | 0.0316 |
| GO Process | GO:0009056 | Catabolic process | 124 | 368 | 0.13 | 0.0333 |
| GO Process | GO:1901575 | Organic substance catabolic process | 119 | 351 | 0.13 | 0.0342 |
| GO Process | GO:0046364 | Monosaccharide biosynthetic process | 13 | 16 | 0.51 | 0.0355 |
| GO Process | GO:0009218 | Pyrimidine ribonucleotide metabolic process | 12 | 14 | 0.53 | 0.0384 |
| GO Process | GO:0006220 | Pyrimidine nucleotide metabolic process | 15 | 21 | 0.46 | 0.0395 |
| GO Process | GO:0032787 | Monocarboxylic acid metabolic process | 62 | 161 | 0.19 | 0.0438 |
| GO Function | GO:0003824 | Catalytic activity | 708 | 2137 | 0.12 | 6.81E-18 |
| GO Function | GO:0005488 | Binding | 659 | 2091 | 0.1 | 1.01E-10 |
| GO Function | GO:0043167 | Ion binding | 437 | 1288 | 0.13 | 1.27E-08 |
| GO Function | GO:0003723 | RNA binding | 93 | 159 | 0.37 | 3.66E-08 |
| GO Function | GO:0003735 | Structural constituent of ribosome | 51 | 58 | 0.55 | 3.85E-08 |
| GO Function | GO:0036094 | Small molecule binding | 280 | 759 | 0.17 | 1.87E-07 |
| GO Function | GO:0005198 | Structural molecule activity | 62 | 90 | 0.44 | 3.24E-07 |
| GO Function | GO:0016491 | Oxidoreductase activity | 171 | 424 | 0.21 | 5.27E-06 |
| GO Function | GO:0019843 | rRNA binding | 39 | 46 | 0.53 | 5.98E-06 |
| GO Function | GO:0043169 | Cation binding | 273 | 773 | 0.15 | 6.48E-06 |
| GO Function | GO:0000166 | Nucleotide binding | 243 | 670 | 0.16 | 6.74E-06 |
| GO Function | GO:0016874 | Ligase activity | 63 | 108 | 0.37 | 1.28E-05 |
| GO Function | GO:0043168 | Anion binding | 240 | 672 | 0.15 | 1.88E-05 |
| GO Function | GO:0046872 | Metal ion binding | 266 | 763 | 0.14 | 1.88E-05 |
| GO Function | GO:0097159 | Organic cyclic compound binding | 428 | 1366 | 0.1 | 3.88E-05 |
| GO Function | GO:1901363 | Heterocyclic compound binding | 428 | 1366 | 0.1 | 3.88E-05 |
| GO Function | GO:0097367 | Carbohydrate derivative binding | 199 | 568 | 0.15 | 0.00058 |
| GO Function | GO:0017076 | Purine nucleotide binding | 180 | 509 | 0.15 | 0.001 |
| GO Function | GO:0032553 | Ribonucleotide binding | 187 | 534 | 0.15 | 0.0011 |
| GO Function | GO:0032555 | Purine ribonucleotide binding | 179 | 507 | 0.15 | 0.0011 |
| GO Function | GO:0035639 | Purine ribonucleoside triphosphate binding | 176 | 498 | 0.15 | 0.0012 |
| GO Function | GO:0004812 | aminoacyl-tRNA ligase activity | 22 | 25 | 0.55 | 0.0013 |
| GO Function | GO:0030554 | Adenyl nucleotide binding | 160 | 465 | 0.14 | 0.0076 |

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|---|---|---|---|---|---|---|
| GO Function | GO:0032559 | Adenyl ribonucleotide binding | 159 | 463 | 0.14 | 0.0083 |
| GO Function | GO:0005524 | ATP binding | 157 | 460 | 0.13 | 0.0112 |
| GO Function | GO:0000287 | Magnesium ion binding | 58 | 133 | 0.24 | 0.013 |
| GO Function | GO:0016209 | Antioxidant activity | 19 | 26 | 0.47 | 0.0191 |
| GO Function | GO:0016879 | Ligase activity, forming carbon-nitrogen bonds | 26 | 44 | 0.37 | 0.0216 |
| GO Function | GO:0051287 | NAD binding | 25 | 42 | 0.38 | 0.0244 |
| GO Function | GO:0016740 | Transferase activity | 217 | 689 | 0.1 | 0.0272 |
| GO Component | GO:0005622 | Intracellular | 760 | 1889 | 0.21 | 4.00E-52 |
| GO Component | GO:0005737 | Cytoplasm | 690 | 1760 | 0.2 | 2.43E-39 |
| GO Component | GO:0110165 | Cellular anatomical entity | 1052 | 3544 | 0.07 | 2.46E-36 |
| GO Component | GO:0005829 | Cytosol | 299 | 653 | 0.26 | 9.03E-19 |
| GO Component | GO:0005840 | Ribosome | 55 | 63 | 0.54 | 1.63E-09 |
| GO Component | GO:0043232 | Intracellular non-membrane-bounded organelle | 67 | 108 | 0.39 | 3.49E-07 |
| GO Component | GO:0043228 | Non-membrane-bounded organelle | 76 | 140 | 0.34 | 2.19E-06 |
| GO Component | GO:0042597 | Periplasmic space | 87 | 214 | 0.21 | 0.0022 |
| GO Component | GO:0043229 | Intracellular organelle | 70 | 161 | 0.24 | 0.0022 |
| GO Component | GO:0043226 | Organelle | 79 | 194 | 0.21 | 0.0036 |
| GO Component | GO:0044391 | Ribosomal subunit | 16 | 17 | 0.58 | 0.0038 |
| GO Component | GO:1990904 | Ribonucleoprotein complex | 17 | 20 | 0.53 | 0.005 |
| STRING clusters | CL:79 | Translation, and protein export | 126 | 162 | 0.49 | 4.09E-18 |
| STRING clusters | CL:78 | Translation, and protein export | 130 | 175 | 0.47 | 7.54E-18 |
| STRING clusters | CL:80 | Translation, and proton channel activity | 115 | 145 | 0.5 | 2.99E-17 |
| STRING clusters | CL:81 | Translation, and ribosome biogenesis | 105 | 132 | 0.5 | 8.91E-16 |
| STRING clusters | CL:83 | Ribosome, and Protein biosynthesis | 81 | 97 | 0.52 | 9.84E-13 |
| STRING clusters | CL:82 | Ribosome, and Protein biosynthesis | 85 | 110 | 0.49 | 3.73E-12 |
| STRING clusters | CL:84 | Ribosomal protein, and translation factor activity, RNA binding | 70 | 84 | 0.52 | 5.89E-11 |
| STRING clusters | CL:86 | Ribosomal protein, and translation factor activity, RNA binding | 62 | 73 | 0.53 | 6.83E-10 |
| STRING clusters | CL:89 | Ribosomal protein | 59 | 68 | 0.54 | 1.11E-09 |
| STRING clusters | CL:93 | Ribosomal protein | 56 | 62 | 0.56 | 1.18E-09 |
| STRING clusters | CL:95 | Ribosomal protein | 52 | 57 | 0.56 | 4.63E-09 |

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|---|---|---|---|---|---|---|
| STRING clusters | CL:96 | Ribosomal protein | 47 | 52 | 0.56 | 4.53E-08 |
| STRING clusters | CL:98 | Ribosomal protein | 42 | 46 | 0.56 | 2.88E-07 |
| STRING clusters | CL:99 | Ribosomal protein | 31 | 35 | 0.55 | 4.76E-05 |
| STRING clusters | CL:1291 | Cellular amino acid biosynthetic process, and ribonucleoside monophosphate biosynthetic process | 74 | 149 | 0.3 | 0.00011 |
| STRING clusters | CL:2027 | Mixed, incl. antioxidant activity, and iron-sulfur cluster assembly | 41 | 65 | 0.4 | 0.00039 |
| STRING clusters | CL:100 | Ribosomal protein | 25 | 28 | 0.55 | 0.00043 |
| STRING clusters | CL:2026 | Mixed, incl. antioxidant activity, and iron-sulfur cluster assembly | 43 | 72 | 0.38 | 0.00056 |
| STRING clusters | CL:1292 | Cellular amino acid biosynthetic process, and ribonucleoside monophosphate biosynthetic process | 66 | 136 | 0.29 | 0.00059 |
| STRING clusters | CL:2028 | Antioxidant activity, and protein disulfide oxidoreductase activity | 27 | 34 | 0.5 | 0.0007 |
| STRING clusters | CL:4302 | Pentose phosphate pathway, and Glycolysis | 34 | 52 | 0.42 | 0.0011 |
| STRING clusters | CL:4724 | Mixed, incl. glycogen metabolic process, and trehalose metabolic process | 23 | 27 | 0.53 | 0.0013 |
| STRING clusters | CL:2585 | Pyruvate metabolism, and Oxidative phosphorylation | 62 | 131 | 0.28 | 0.0016 |
| STRING clusters | CL:4304 | Pentose phosphate pathway, and Glycolysis | 28 | 40 | 0.45 | 0.0021 |
| STRING clusters | CL:4300 | Pentose phosphate pathway, and phosphoenolpyruvate-dependent sugar phosphotransferase system | 53 | 107 | 0.3 | 0.0022 |
| STRING clusters | CL:4305 | Pentose phosphate pathway, and Glycolysis | 23 | 29 | 0.5 | 0.0024 |
| STRING clusters | CL:1293 | Amino-acid biosynthesis, and ribonucleoside monophosphate biosynthetic process | 47 | 95 | 0.3 | 0.0045 |
| STRING clusters | CL:2029 | Reactive oxygen species metabolic process, and protein disulfide oxidoreductase activity | 18 | 20 | 0.56 | 0.0045 |
| STRING clusters | CL:302 | Aminoacyl-tRNA synthetase | 18 | 20 | 0.56 | 0.0045 |
| STRING clusters | CL:4306 | Glycolysis, and pentose-phosphate shunt | 19 | 22 | 0.54 | 0.0045 |
| STRING clusters | CL:4723 | Starch and sucrose metabolism, and galactose metabolic process | 25 | 36 | 0.44 | 0.0045 |
| STRING clusters | CL:4725 | Glycogen metabolic process, and trehalose metabolic process | 19 | 22 | 0.54 | 0.0045 |
| STRING clusters | CL:1294 | Aromatic amino acid family biosynthetic process, and ribonucleoside monophosphate biosynthetic process | 37 | 68 | 0.34 | 0.0052 |
| STRING clusters | CL:4308 | Glycolysis, and fructose 1,6-bisphosphate 1-phosphatase activity | 16 | 17 | 0.58 | 0.0064 |
| STRING clusters | CL:4726 | Glycogen metabolic process, and glucose-1-phosphatase activity | 16 | 17 | 0.58 | 0.0064 |
| STRING clusters | CL:1295 | Ribonucleoside monophosphate biosynthetic process, and histidine biosynthesis | 28 | 46 | 0.39 | 0.0076 |
| STRING clusters | CL:2648 | Tricarboxylic acid cycle, and pyruvate dehydrogenase complex | 15 | 16 | 0.57 | 0.0095 |
| STRING clusters | CL:305 | Aminoacyl-tRNA synthetase | 14 | 14 | 0.6 | 0.0095 |

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|---|---|---|---|---|---|---|
| STRING clusters | CL:2587 | Propanoate metabolism, and tricarboxylic acid cycle | 29 | 50 | 0.37 | 0.0101 |
| STRING clusters | CL:4299 | Mixed, incl. pentose phosphate pathway, and phosphoenolpyruvate-dependent sugar phosphotransferase system | 57 | 131 | 0.24 | 0.0103 |
| STRING clusters | CL:4722 | Polysaccharide metabolic process, and disaccharide metabolic process | 36 | 71 | 0.31 | 0.014 |
| STRING clusters | CL:6488 | Taxis | 14 | 15 | 0.57 | 0.014 |
| STRING clusters | CL:2583 | Mixed, incl. pyruvate metabolism, and oxidative phosphorylation | 67 | 165 | 0.21 | 0.0143 |
| STRING clusters | CL:2582 | Mixed, incl. pyruvate metabolism, and oxidative phosphorylation | 70 | 175 | 0.2 | 0.0148 |
| STRING clusters | CL:101 | rRNA-binding | 14 | 16 | 0.54 | 0.0196 |
| STRING clusters | CL:168 | Ribosomal protein | 11 | 11 | 0.6 | 0.0334 |
| STRING clusters | CL:906 | Mixed, incl. external encapsulating structure organization, and cell cycle | 73 | 192 | 0.18 | 0.0334 |
| STRING clusters | CL:2586 | Pyruvate metabolism, and Valine, leucine and isoleucine biosynthesis | 38 | 83 | 0.26 | 0.0381 |
| STRING clusters | CL:1068 | Mixed, incl. membrane organization, and lipoprotein localization | 29 | 58 | 0.3 | 0.0468 |
| STRING clusters | CL:4721 | Cellular polysaccharide metabolic process, and oligosaccharide metabolic process | 47 | 112 | 0.22 | 0.0468 |
| STRING clusters | CL:1069 | Mixed, incl. membrane organization, and lipoprotein localization | 25 | 47 | 0.33 | 0.0472 |
| STRING clusters | CL:132 | Ribosomal protein | 11 | 12 | 0.56 | 0.0472 |
| STRING clusters | CL:1589 | Nicotinate and nicotinamide metabolism, and Pyrimidine metabolism | 32 | 67 | 0.28 | 0.0472 |
| STRING clusters | CL:2030 | Redox-active center, and ferredoxin-NADP+ reductase activity | 11 | 12 | 0.56 | 0.0472 |
| STRING clusters | CL:252 | Mixed, incl. rna polymerase, and transcription termination | 11 | 12 | 0.56 | 0.0472 |
| STRING clusters | CL:4309 | Glycolysis, and transketolase activity | 11 | 12 | 0.56 | 0.0472 |
| STRING clusters | CL:6493 | Chemotaxis | 10 | 10 | 0.6 | 0.0472 |
| STRING clusters | CL:1296 | Ribonucleoside monophosphate biosynthetic process, and tetrahydrofolate interconversion | 18 | 29 | 0.39 | 0.0491 |
| KEGG | stm01100 | Metabolic pathways | 503 | 1448 | 0.14 | 5.28E-13 |
| KEGG | stm01110 | Biosynthesis of secondary metabolites | 218 | 520 | 0.22 | 1.15E-09 |
| KEGG | stm03010 | Ribosome | 51 | 58 | 0.55 | 8.09E-09 |
| KEGG | stm01200 | Carbon metabolism | 82 | 146 | 0.35 | 2.47E-07 |
| KEGG | stm00010 | Glycolysis / Gluconeogenesis | 38 | 57 | 0.43 | 0.00013 |
| KEGG | stm01230 | Biosynthesis of amino acids | 77 | 169 | 0.26 | 0.00026 |
| KEGG | stm02030 | Bacterial chemotaxis | 25 | 32 | 0.49 | 0.00064 |

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|---|---|---|---|---|---|---|
| KEGG | stm00970 | Aminoacyl-tRNA biosynthesis | 25 | 34 | 0.47 | 0.0011 |
| KEGG | stm00240 | Pyrimidine metabolism | 33 | 56 | 0.37 | 0.0018 |
| KEGG | stm00250 | Alanine, aspartate and glutamate metabolism | 26 | 43 | 0.38 | 0.006 |
| KEGG | stm00620 | Pyruvate metabolism | 35 | 67 | 0.32 | 0.006 |
| KEGG | stm00020 | Citrate cycle (TCA cycle) | 22 | 34 | 0.41 | 0.0073 |
| KEGG | stm00260 | Glycine, serine and threonine metabolism | 26 | 46 | 0.35 | 0.0103 |
| KEGG | stm00230 | Purine metabolism | 44 | 99 | 0.25 | 0.012 |
| KEGG | stm00480 | Glutathione metabolism | 23 | 39 | 0.37 | 0.012 |
| KEGG | stm00520 | Amino sugar and nucleotide sugar metabolism | 40 | 88 | 0.26 | 0.0126 |
| KEGG | stm01120 | Microbial metabolism in diverse environments | 136 | 409 | 0.12 | 0.0136 |
| KEGG | stm00030 | Pentose phosphate pathway | 26 | 50 | 0.32 | 0.0181 |
| UniProt Keywords | KW-0963 | Cytoplasm | 258 | 505 | 0.31 | 6.29E-20 |
| UniProt Keywords | KW-0479 | Metal-binding | 209 | 492 | 0.23 | 2.98E-09 |
| UniProt Keywords | KW-0687 | Ribonucleoprotein | 51 | 59 | 0.54 | 2.80E-08 |
| UniProt Keywords | KW-0689 | Ribosomal protein | 50 | 57 | 0.54 | 2.80E-08 |
| UniProt Keywords | KW-0694 | RNA-binding | 63 | 92 | 0.44 | 1.15E-07 |
| UniProt Keywords | KW-0699 | rRNA-binding | 38 | 40 | 0.58 | 4.97E-07 |
| UniProt Keywords | KW-0560 | Oxidoreductase | 123 | 273 | 0.26 | 1.74E-06 |
| UniProt Keywords | KW-0648 | Protein biosynthesis | 38 | 45 | 0.53 | 3.10E-06 |
| UniProt Keywords | KW-0436 | Ligase | 53 | 84 | 0.4 | 8.69E-06 |
| UniProt Keywords | KW-0547 | Nucleotide-binding | 173 | 448 | 0.19 | 1.08E-05 |
| UniProt Keywords | KW-0460 | Magnesium | 90 | 191 | 0.27 | 1.92E-05 |
| UniProt Keywords | KW-0030 | Aminoacyl-tRNA synthetase | 22 | 24 | 0.56 | 0.00048 |
| UniProt Keywords | KW-0520 | NAD | 50 | 97 | 0.31 | 0.0009 |
| UniProt Keywords | KW-0574 | Periplasm | 40 | 75 | 0.33 | 0.0028 |
| UniProt Keywords | KW-0521 | NADP | 33 | 60 | 0.34 | 0.0066 |
| UniProt Keywords | KW-0808 | Transferase | 193 | 592 | 0.11 | 0.0078 |
| UniProt Keywords | KW-0862 | Zinc | 58 | 137 | 0.23 | 0.0111 |
| UniProt Keywords | KW-0067 | ATP-binding | 132 | 383 | 0.14 | 0.0112 |
| UniProt Keywords | KW-0324 | Glycolysis | 13 | 14 | 0.57 | 0.0146 |

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|---|---|---|---|---|---|---|
| UniProt Keywords | KW-0028 | Amino-acid biosynthesis | 40 | 89 | 0.25 | 0.0272 |
| UniProt Keywords | KW-0408 | Iron | 58 | 146 | 0.2 | 0.0296 |
| UniProt Keywords | KW-0143 | Chaperone | 28 | 55 | 0.31 | 0.0299 |
| UniProt Keywords | KW-0732 | Signal | 146 | 448 | 0.11 | 0.03 |
| UniProt Keywords | KW-0998 | Cell outer membrane | 28 | 56 | 0.3 | 0.0335 |
| UniProt Keywords | KW-0021 | Allosteric enzyme | 14 | 20 | 0.45 | 0.045 |
| UniProt Keywords | KW-0132 | Cell division | 27 | 55 | 0.29 | 0.045 |
| UniProt Keywords | KW-0816 | Tricarboxylic acid cycle | 13 | 18 | 0.46 | 0.0459 |
| UniProt Keywords | KW-0663 | Pyridoxal phosphate | 26 | 53 | 0.29 | 0.047 |
| UniProt Keywords | KW-0342 | GTP-binding | 18 | 31 | 0.37 | 0.0474 |

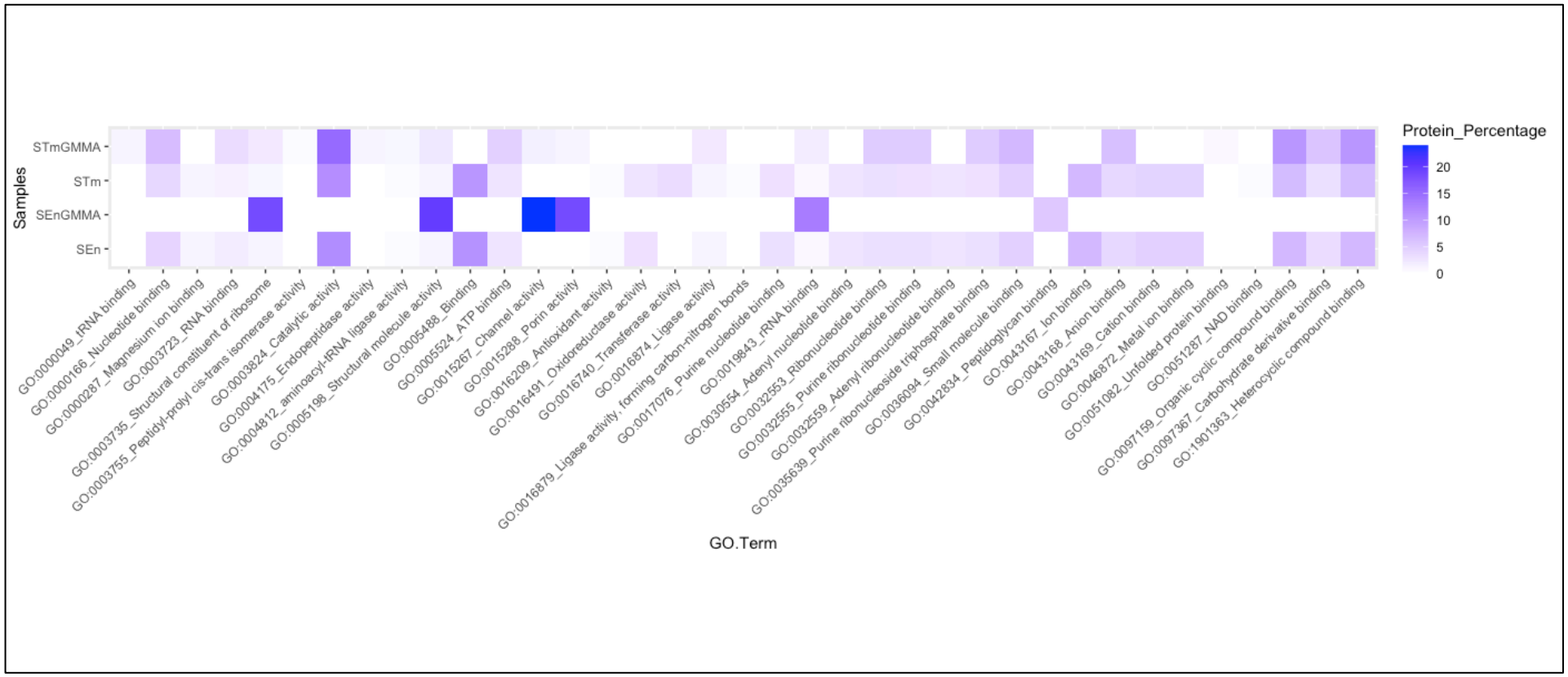



*Figure 96 Heatmap of percentage protein contribution to GO Terms (Molecular Function) across all sample types*

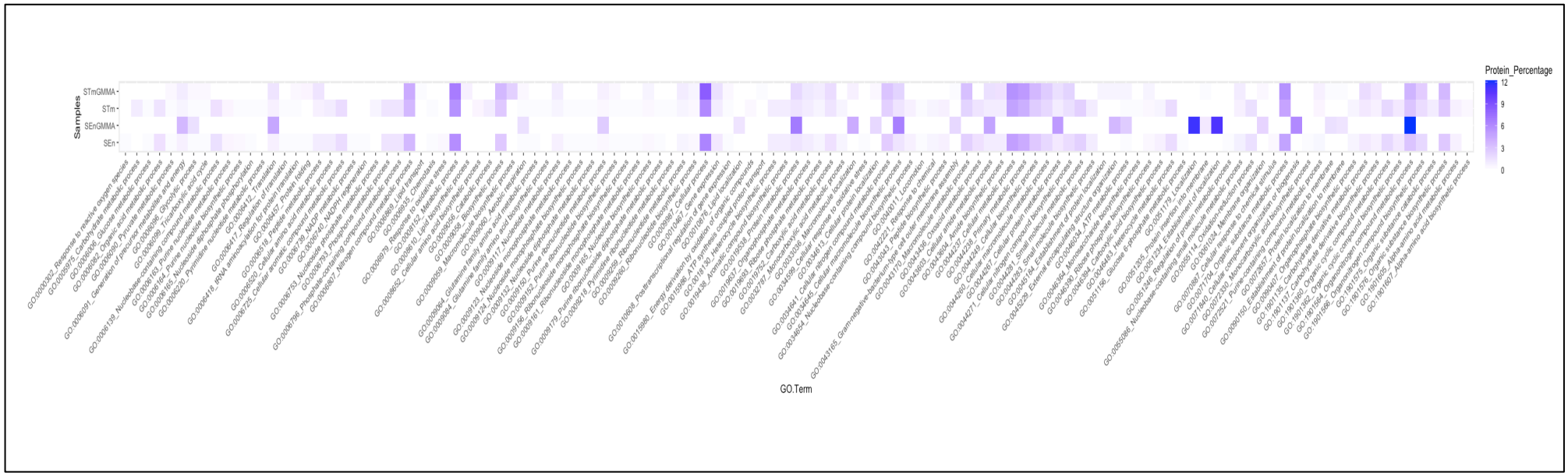



*Figure 97 Heatmap of percentage protein contribution to GO Terms (Biological Process) across all sample types*

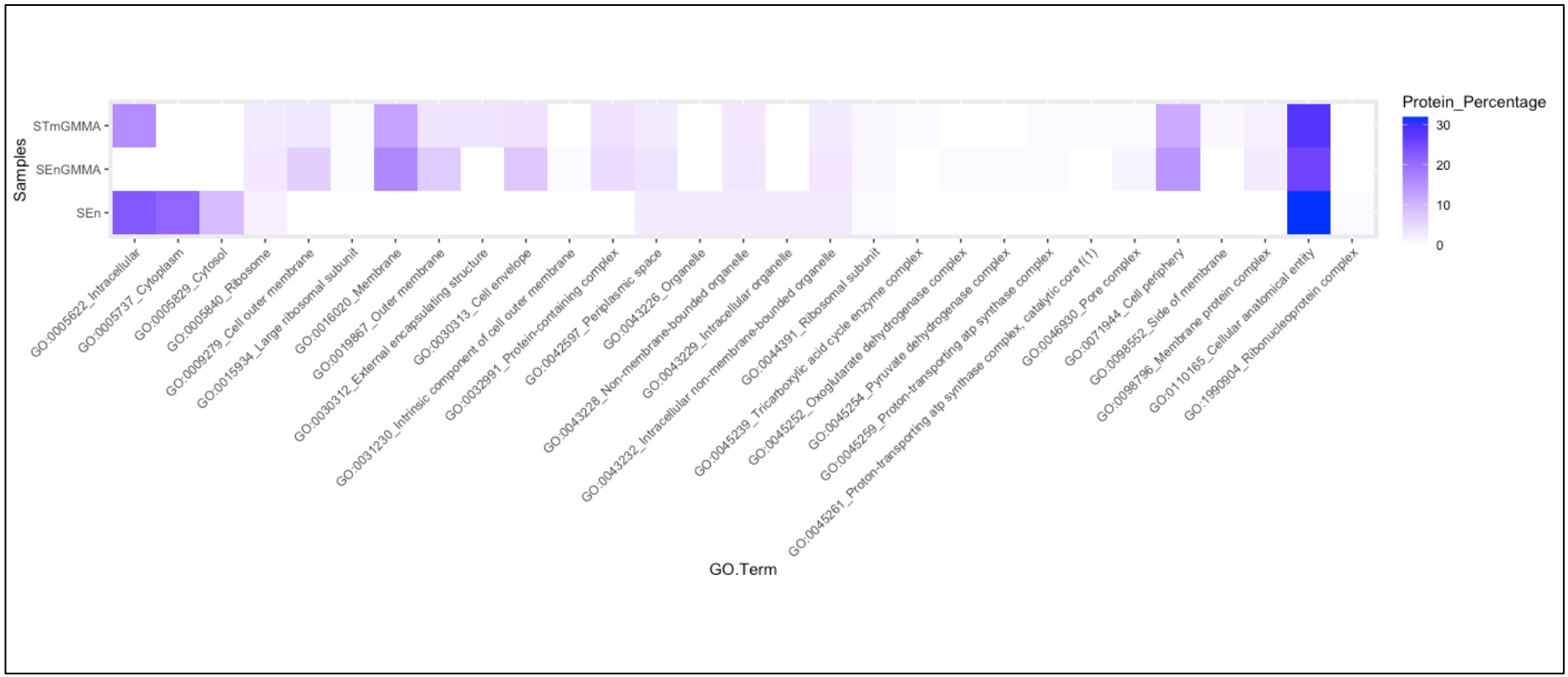



*Figure 98 Heatmap of percentage protein contribution to GO Terms (Cellular Component) across all sample types*

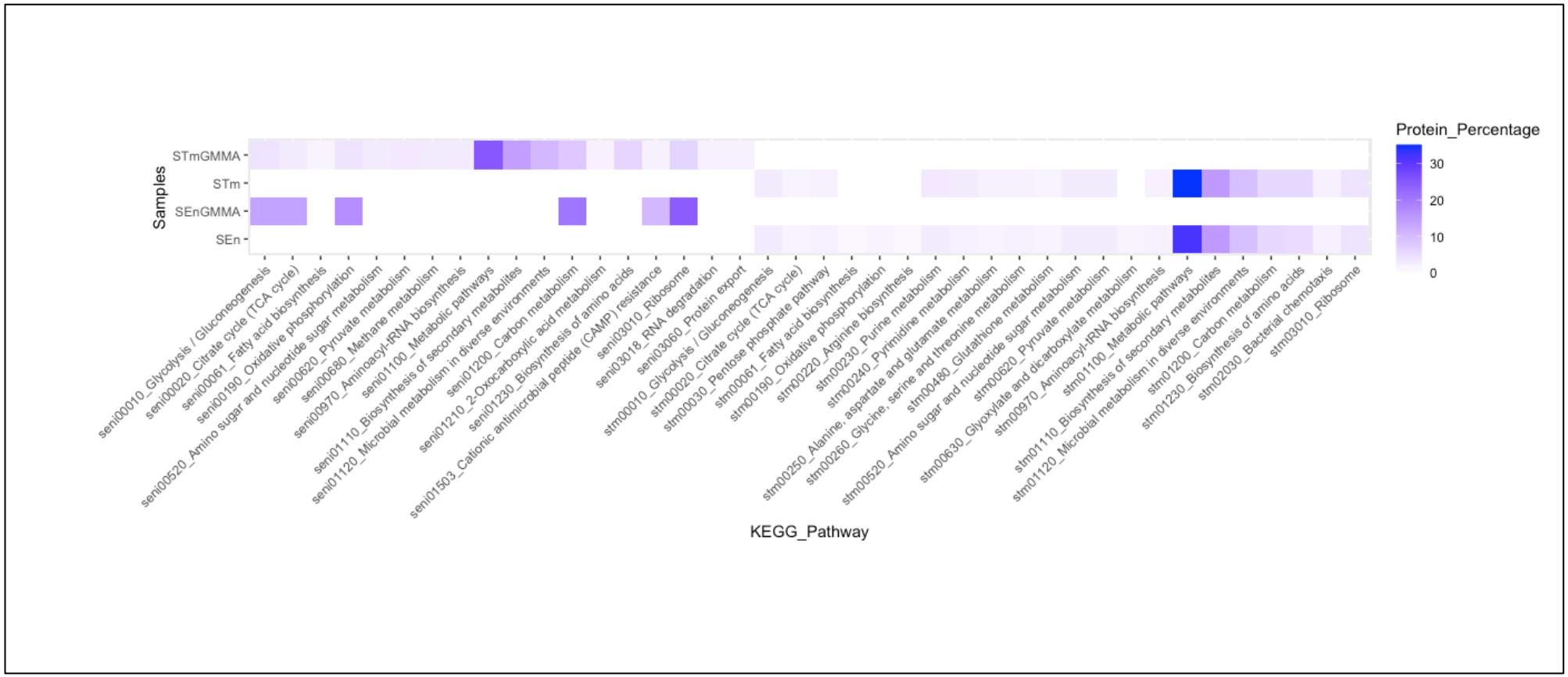



*Figure 99 Heatmap of percentage protein contribution to KEGG Pathways across all sample types*

# Appendix C

*Table 41 Shared 118 proteins between SEn, STm, SEnGMMA and STmGMMA (output from UniProt [Uniprot Consortium 2023][237]*

| Accession Number | Protein names | Gene Names | Organism |
|---|---|---|---|
| A0A6C7IHC3 | Elongation factor Tu (EF-Tu) | tuf | *Salmonella* typhimurium (strain D23580) |
| A0A6C7I7P3 | Acetyltransferase component of pyruvate dehydrogenase complex (EC 2.3.1.12) | aceF | *Salmonella* typhimurium (strain D23580) |
| A0A6C7I6P3 | Lipoprotein | metQ | *Salmonella* typhimurium (strain D23580) |
| A0A6C7I7Y8 | Possible outer membrane adhesin | pagN | *Salmonella* typhimurium (strain D23580) |
| B5R3I3 | Elongation factor Ts (EF-Ts) | tsf | *Salmonella* enteritidis PT4 (strain P125109) |
| A0A6C7I7V6 | Sec translocon accessory complex subunit YajC | yajC | *Salmonella* typhimurium (strain D23580) |
| A0A6C7I918 | Cytochrome bo(3) ubiquinol oxidase subunit 1 (EC 7.1.1.3) (Cytochrome o ubiquinol oxidase subunit 1) (Oxidase bo(3) subunit 1) (Ubiquinol oxidase polypeptide I) (Ubiquinol oxidase subunit 1) | cyoB | *Salmonella* typhimurium (strain D23580) |
| A0A6C7I928 | Peptidyl-prolyl cis-trans isomerase D (EC 5.2.1.8) | cypD | *Salmonella* typhimurium (strain D23580) |
| B5QVP8 | LPS-assembly lipoprotein LptE | lptE | *Salmonella* enteritidis PT4 (strain P125109) |
| A0A6C7I7H6 | LPS-assembly protein LptD | lptD | *Salmonella* typhimurium (strain D23580) |
| B5QX24 | Tol-Pal system protein TolB | tolB | *Salmonella* enteritidis PT4 (strain P125109) |
| A0A6C7I751 | Periplasmic serine endoprotease DegP-like (EC 3.4.21.107) | degP | *Salmonella* typhimurium (strain D23580) |
| A0A6C7I7F5 | Outer membrane protein assembly factor BamA | bamA | *Salmonella* typhimurium (strain D23580) |
| A0A6C7IA37 | Putative exported protein | ybiS | *Salmonella* typhimurium (strain D23580) |
| A0A6C7I7H4 | Outer membrane lipoprotein RcsF | rcsF | *Salmonella* typhimurium (strain D23580) |
| A0A6C7I860 | Citrate synthase | gltA | *Salmonella* typhimurium (strain D23580) |
| A0A6C7I9R0 | Succinate dehydrogenase iron-sulfur subunit (EC 1.3.5.1) | sdhB | *Salmonella* typhimurium (strain D23580) |
| A0A6C7I875 | Cytochrome D ubiquinol oxidase subunit I | cydA | *Salmonella* typhimurium (strain D23580) |
| A0A6C7I9Y4 | Possible pectinesterase (EC 3.1.1.11) | ybhC | *Salmonella* typhimurium (strain D23580) |
| A0A6C7I9F6 | Outer membrane protein F | ompF | *Salmonella* typhimurium (strain D23580) |
| A0A6C7IAA4 | Outer membrane protein A (Outer membrane porin A) | ompA | *Salmonella* typhimurium (strain D23580) |
| A0A0H3NAA4 | PTS system glucose-specific EIICB component (EC 2.7.1.199) (EIICB-Glc) | ptsG4 | *Salmonella* typhimurium (strain SL1344) |
| A0A6C7IA07 | Osmotically inducible lipoprotein E | osmE | *Salmonella* typhimurium (strain D23580) |
| B5QVW5 | 50S ribosomal protein L20 | rplT | *Salmonella* enteritidis PT4 (strain P125109) |

| | | | |
|---|---|---|---|
| A0A6C7I9A5 | Dihydrolipoyllysine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex (EC 2.3.1.61) (2-oxoglutarate dehydrogenase complex component E2) | sucB | *Salmonella* typhimurium (strain D23580) |
| A0A6C7IA06 | Pyruvate kinase (EC 2.7.1.40) | pykF | *Salmonella* typhimurium (strain D23580) |
| A0A6C7IAF0 | Putative exported protein | spy | *Salmonella* typhimurium (strain D23580) |
| A0A6C7IBS9 | Outer membrane lipoprotein SlyB | slyB | *Salmonella* typhimurium (strain D23580) |
| A0A6C7IBV7 | Putative exported protein | ydgH | *Salmonella* typhimurium (strain D23580) |
| A0A6C7ID76 | NAD(P) transhydrogenase subunit beta (EC 7.1.1.1) (Nicotinamide nucleotide transhydrogenase subunit beta) | pntB | *Salmonella* typhimurium (strain D23580) |
| A0A6C7IA00 | Formate acetyltransferase (EC 2.3.1.54) (Pyruvate formate-lyase) | pflB | *Salmonella* typhimurium (strain D23580) |
| A0A6C7IBI7 | Outer membrane porin protein (OmpD) | ompD | *Salmonella* typhimurium (strain D23580) |
| A0A6C7IAS7 | Putative lipoprotein | STM1607 | *Salmonella* typhimurium (strain D23580) |
| A0A6C7I9R2 | Putative outer membrane protein | mipA | *Salmonella* typhimurium (strain D23580) |
| A0A6C7IBB0 | Glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.-) | gapA | *Salmonella* typhimurium (strain D23580) |
| A0A6C7HRM6 | Major outer membrane lipoprotein Lpp (Braun lipoprotein) (Murein lipoprotein) (BLP) | lpp | *Salmonella* enteritidis PT4 (strain P125109) |
| A0A6C7I788 | Dihydrolipoyl dehydrogenase (EC 1.8.1.4) | lpdA | *Salmonella* typhimurium (strain D23580) |
| A0A6C7IA32 | Exported protein | ydgA | *Salmonella* typhimurium (strain D23580) |
| B5R3I2 | 30S ribosomal protein S2 | rpsB | *Salmonella* enteritidis PT4 (strain P125109) |
| A0A6C7I8V0 | Protein translocase subunit SecD | secD | *Salmonella* typhimurium (strain D23580) |
| A0A6C7I8Z0 | Nucleoside-specific channel-forming protein Tsx | tsx | *Salmonella* typhimurium (strain D23580) |
| B5R3J7 | Outer-membrane lipoprotein LolB | lolB | *Salmonella* enteritidis PT4 (strain P125109) |
| A0A6C7I7T5 | Ubiquinol oxidase subunit 2 | cyoA | *Salmonella* typhimurium (strain D23580) |
| A0A6C7ICD9 | Putative lipoprotein | slp | *Salmonella* typhimurium (strain D23580) |
| A0A0H3NDS3 | Uncharacterized protein | yfaZ | *Salmonella* typhimurium (strain SL1344) |
| A0A6C7IE98 | VacJ lipoprotein | vacJ | *Salmonella* typhimurium (strain D23580) |
| A0A0H3NE53 | Endolytic peptidoglycan transglycosylase RlpA (EC 4.2.2.-) | rlpA | *Salmonella* typhimurium (strain SL1344) |
| A0A6C7I8T8 | Succinate dehydrogenase flavoprotein subunit (EC 1.3.5.1) | sdhA | *Salmonella* typhimurium (strain D23580) |
| A0A6C7I903 | Peptidoglycan-associated protein | pal | *Salmonella* typhimurium (strain D23580) |
| B5QZJ9 | UPF0265 protein YeeX | yeeX | *Salmonella* enteritidis PT4 (strain P125109) |
| A0A6C7I9J4 | Outer membrane protein X | ompX | *Salmonella* typhimurium (strain D23580) |
| A0A6C7HSA3 | Stress response protein ElaB | elaB | *Salmonella* enteritidis PT4 (strain P125109) |
| A0A6C7ID51 | NADH-quinone oxidoreductase subunit I (EC 7.1.1.-) (NADH | nuoI | *Salmonella* typhimurium (strain D23580) |

| | | | |
|---|---|---|---|
| | dehydrogenase I subunit I) (NDH-1 subunit I) | | |
| B5R307 | NADH-quinone oxidoreductase subunit C/D (EC 7.1.1.-) (NADH dehydrogenase I subunit C/D) (NDH-1 subunit C/D) | nuoC | *Salmonella* enteritidis PT4 (strain P125109) |
| A0A6C7IFQ7 | Putative exported protein | yggE | *Salmonella* typhimurium (strain D23580) |
| A0A6C7IFZ5 | Peptidase_M48 domain-containing protein | yggG | *Salmonella* typhimurium (strain D23580) |
| A0A6C7IDL0 | Outer membrane protein assembly factor BamC | bamC | *Salmonella* typhimurium (strain D23580) |
| B5R0L8 | 50S ribosomal protein L13 | rplM | *Salmonella* enteritidis PT4 (strain P125109) |
| B5R1F3 | 30S ribosomal protein S4 | rpsD | *Salmonella* enteritidis PT4 (strain P125109) |
| B5R1F1 | 50S ribosomal protein L17 | rplQ | *Salmonella* enteritidis PT4 (strain P125109) |
| B5R285 | 30S ribosomal protein S3 | rpsC | *Salmonella* enteritidis PT4 (strain P125109) |
| A0A6C7IDY9 | Lipoprotein NlpD | nlpD | *Salmonella* typhimurium (strain D23580) |
| A0A6C7I7D7 | Chaperone SurA (Peptidyl-prolyl cis-trans isomerase SurA) (PPIase SurA) (EC 5.2.1.8) (Rotamase SurA) | surA | *Salmonella* typhimurium (strain D23580) |
| A0A6C7ICE6 | Putative outer membrane protein | ompW | *Salmonella* typhimurium (strain D23580) |
| A0A6C7IG83 | Putative outer membrane protein | yiaD | *Salmonella* typhimurium (strain D23580) |
| B5QUQ4 | Membrane protein insertase YidC (Foldase YidC) (Membrane integrase YidC) (Membrane protein YidC) | yidC | *Salmonella* enteritidis PT4 (strain P125109) |
| A0A6C7IH35 | Uncharacterized protein | yhcB | *Salmonella* typhimurium (strain D23580) |
| A0A6C7IH51 | 30S ribosomal protein S13 | rpsM | *Salmonella* typhimurium (strain D23580) |
| B5R279 | 50S ribosomal protein L5 | rplE | *Salmonella* enteritidis PT4 (strain P125109) |
| A0A6C7I801 | Putative lipoprotein | yajG | *Salmonella* typhimurium (strain D23580) |
| A0A6C7I837 | Acriflavin resistance protein A | acrA | *Salmonella* typhimurium (strain D23580) |
| B5QYD5 | 50S ribosomal protein L1 | rplA | *Salmonella* enteritidis PT4 (strain P125109) |
| A0A6C7IIZ1 | Putative lipoprotein | STM3580 | *Salmonella* typhimurium (strain D23580) |
| B5R005 | Chaperonin GroEL (EC 5.6.1.7) (60 kDa chaperonin) (Chaperonin-60) (Cpn60) | groEL | *Salmonella* enteritidis PT4 (strain P125109) |
| A0A6C7II93 | Protein HflK | hflK | *Salmonella* typhimurium (strain D23580) |
| B5QUS4 | ATP synthase subunit beta (EC 7.1.2.2) (ATP synthase F1 sector subunit beta) (F-ATPase subunit beta) | atpD | *Salmonella* enteritidis PT4 (strain P125109) |
| A0A6C7IA53 | Probable lipoprotein | ybjP | *Salmonella* typhimurium (strain D23580) |
| A0A6C7IIJ5 | CDP-diacylglycerol pyrophosphatase (EC 3.6.1.26) (CDP-diacylglycerol phosphatidylhydrolase) (CDP-diglyceride hydrolase) | cdh | *Salmonella* typhimurium (strain D23580) |
| A0A6C7IEL4 | Thiosulphate-binding protein | cysP | *Salmonella* typhimurium (strain D23580) |
| B5QYD7 | 50S ribosomal protein L7/L12 | rplL | *Salmonella* enteritidis PT4 (strain P125109) |
| A0A6C7IA94 | Outer membrane invasion protein (PagC) | pagC | *Salmonella* typhimurium (strain D23580) |

| | | | |
|---|---|---|---|
| A0A6C7IJH4 | Putative periplasmic protein | osmY | *Salmonella* typhimurium (strain D23580) |
| A0A6C7IJJ4 | Peptidoglycan lytic exotransglycosylase (EC 4.2.2.n1) | slt | *Salmonella* typhimurium (strain D23580) |
| B5QW40 | Enolase (EC 4.2.1.11) (2-phospho-D-glycerate hydro-lyase) (2-phosphoglycerate dehydratase) | eno | *Salmonella* enteritidis PT4 (strain P125109) |
| A0A6C7IB67 | Aldehyde-alcohol dehydrogenase | adhE | *Salmonella* typhimurium (strain D23580) |
| A0A6C7IFC8 | Possible lipoprotein | yraP | *Salmonella* typhimurium (strain D23580) |
| B5R1E7 | Large-conductance mechanosensitive channel | mscL | *Salmonella* enteritidis PT4 (strain P125109) |
| B5R286 | 50S ribosomal protein L22 | rplV | *Salmonella* enteritidis PT4 (strain P125109) |
| B5R297 | Elongation factor G (EF-G) | fusA | *Salmonella* enteritidis PT4 (strain P125109) |
| A0A0H3NMK3 | Hypothetical lipoprotein (Lipoprotein) | yedD | *Salmonella* typhimurium (strain SL1344) |
| B5QUS6 | ATP synthase subunit alpha (EC 7.1.2.2) (ATP synthase F1 sector subunit alpha) (F-ATPase subunit alpha) | atpA | *Salmonella* enteritidis PT4 (strain P125109) |
| A0A6C7IID9 | DNA-directed RNA polymerase subunit beta' (RNAP subunit beta') (EC 2.7.7.6) (RNA polymerase subunit beta') (Transcriptase subunit beta') | rpoC | *Salmonella* typhimurium (strain D23580) |
| A0A6C7IG37 | Outer membrane protein assembly factor BamB | bamB | *Salmonella* typhimurium (strain D23580) |
| A0A6C7IEC9 | Outer membrane protein assembly factor BamD | bamD | *Salmonella* typhimurium (strain D23580) |
| A0A6C7IFP8 | Possible lipoprotein | ygdI | *Salmonella* typhimurium (strain D23580) |
| A0A0H3NS81 | Penicillin-binding protein activator LpoA (PBP activator LpoA) | yraM | *Salmonella* typhimurium (strain SL1344) |
| A0A6C7IF42 | ATP-dependent zinc metalloprotease FtsH (EC 3.4.24.-) | ftsH | *Salmonella* typhimurium (strain D23580) |
| B5QVD6 | ATP synthase subunit b (ATP synthase F(0) sector subunit b) (ATPase subunit I) (F-type ATPase subunit b) (F-ATPase subunit b) | atpF | *Salmonella* enteritidis PT4 (strain P125109) |
| A0A6C7IGZ6 | Thiol:disulfide interchange protein | dsbA | *Salmonella* typhimurium (strain D23580) |
| B5QYD6 | 50S ribosomal protein L10 | rplJ | *Salmonella* enteritidis PT4 (strain P125109) |
| A0A6C7IL58 | Protein HflC | hflC | *Salmonella* typhimurium (strain D23580) |
| B5R0R8 | 30S ribosomal protein S6 | rpsF | *Salmonella* enteritidis PT4 (strain P125109) |
| A0A6C7HKV3 | Pyruvate dehydrogenase E1 component (EC 1.2.4.1) | aceE | *Salmonella* enteritidis PT4 (strain P125109) |
| A0A6C7HLR3 | Conserved hypothetical lipoprotein | ybaY | *Salmonella* enteritidis PT4 (strain P125109) |
| A0A6C7HNF4 | Oxoglutarate dehydrogenase (succinyl-transferring) (EC 1.2.4.2) | sucA | *Salmonella* enteritidis PT4 (strain P125109) |
| A0A6C7HNM0 | Chaperone protein HtpG (Heat shock protein HtpG) (High temperature protein G) | htpG | *Salmonella* enteritidis PT4 (strain P125109) |
| A0A6C7HRF4 | Flagellin | fljB | *Salmonella* enteritidis PT4 (strain P125109) |

| | | | |
|---|---|---|---|
| A0A6C7HRY7 | Beta-glucosidase BglX (Periplasmic beta-glucosidase) (EC 3.2.1.21) | bglX | *Salmonella* enteritidis PT4 (strain P125109) |
| A0A6C7HSD4 | Cysteine synthase (EC 2.5.1.47) | cysK | *Salmonella* enteritidis PT4 (strain P125109) |
| A0A6C7HSK8 | LPS O-antigen chain length determinant protein WzzB (Polysaccharide chain length regulator) | wzzB | *Salmonella* enteritidis PT4 (strain P125109) |
| A0A6C7HSY0 | Outer membrane protein C (Porin OmpC) | ompC | *Salmonella* enteritidis PT4 (strain P125109) |
| A0A6C7HU68 | Periplasmic serine endoprotease DegP-like (EC 3.4.21.107) | degQ | *Salmonella* enteritidis PT4 (strain P125109) |
| A0A6C7HW41 | Outer membrane channel protein TolC (Outer membrane protein TolC) | tolC | *Salmonella* enteritidis PT4 (strain P125109) |
| A0A6C7HWR3 | Peptidyl-prolyl cis-trans isomerase (EC 5.2.1.8) | fkpA | *Salmonella* enteritidis PT4 (strain P125109) |
| A0A6C7HWT6 | Cell division protein DamX | damX | *Salmonella* enteritidis PT4 (strain P125109) |
| B5QVG7 | Ketol-acid reductoisomerase (NADP(+)) (KARI) (EC 1.1.1.86) (Acetohydroxy-acid isomeroreductase) (AHIR) (Alpha-keto-beta-hydroxylacyl reductoisomerase) (Ketol-acid reductoisomerase type 2) (Ketol-acid reductoisomerase type II) | ilvC | *Salmonella* enteritidis PT4 (strain P125109) |
| B5QYD8 | DNA-directed RNA polymerase subunit beta (RNAP subunit beta) (EC 2.7.7.6) (RNA polymerase subunit beta) (Transcriptase subunit beta) | rpoB | *Salmonella* enteritidis PT4 (strain P125109) |
| B5R5E3 | ADP-L-glycero-D-manno-heptose-6-epimerase (EC 5.1.3.20) (ADP-L-glycero-beta-D-manno-heptose-6-epimerase) (ADP-glyceromanno-heptose 6-epimerase) (ADP-hep 6-epimerase) (AGME) | hldD | *Salmonella* enteritidis PT4 (strain P125109) |

# Appendix D

*Table 42 Demographics of SETA study samples, provided by Sean Elias (SETA study)[151]*

| *Laboratory ID* | *Serology / PCR Confirmed* | *Location* | *Age* | *M/F* | *HIV* | *Malaria* |
|---|---|---|---|---|---|---|
| 40155 | STm | Burkina Faso | 10m | F | N/A | N/A |
| 40535 | STm | Burkina Faso | 5 yrs | F | N/A | N/A |
| 21102 | STm | Burkina Faso | 2 yrs | F | N/A | N/A |
| 11451 | S.Typhi* | Ghana | 6 yrs | F | Neg | Pos |
| 40300 | SEn | Burkina Faso | 2 yrs | F | N/A | N/A |
| 21119 | SEn | Burkino Faso | 3 yrs | M | N/A | N/A |
| 20865 | NTS (Not Typed) | Burkino Faso | 5 yrs | F | N/A | N/A |
| 20293 | STm | Burkin Faso | 2 yrs | M | Neg | N/A |
| 20318 | STm | Ghana | 4m | M | Neg | Neg |
| 10903 | STm | Ghana | 3 yrs | F | Neg | Pos |
| 20341 | NTS (S.Poona) | Ghana | 13m | M | Neg | Neg |
| 11596 | S.Typhi* | Ghana | 12 yrs | M | Neg | Neg |

*Initially identified as NTS on site but changed to S.Typhi following qPCR identification
SEn = *Salmonella* Enteriditis, STm, *Salmonella* Typhimurium, NTS = Non-Typhoid *Salmonella*