

UNDERSTANDING IMMUNITY DIFFERENCES INDUCED BY WHOLE CELL AND ACELLULAR PERTUSSIS VACCINES IN A PAEDIATRIC POPULATION

Ana Marta Martins Valente Pinto

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Oxford Vaccine Group, Department of Paediatrics, University of Oxford, United Kingdom

Supervisors: Dominic Kelly, Elizabeth Clutterbuck, Alexander Mentzer

“Nothing in life is to be feared, it is only to be understood.
Now is the time to understand more, so that we may fear less”
Marie Curie

Aos meus filhos, Constança e Frederico, a minha luz

To my children, Constança and Frederico, my light

Ao Hugo, a minha força

To Hugo, my strength

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DECLARATIONS AND ATTRIBUTIONS

The clinical studies, laboratory work and analyses presented in this thesis are original and the work of the author together with the contributions and support from other individuals and organisations as acknowledged below.

PERtussIS COrrelate of Protection Europe (Periscope consortium):

The Periscope (PERtussIS COrrelate of Protection Europe - https://periscope-project.eu/consortium/) is an international consortium that gathers experts from several Institutions with the aim to identify biomarkers of protection against *Bordetella pertussis (B. pertussis)* that can be applied to the development of next generation pertussis vaccines. This consortium was funded by Innovative Medicines Initiative 2 joint Undertaking and Bill and Melinda Gates Foundation (grant No 115910). A substantial part of the work presented in this DPhil is from the BERT (**B**ooster against p**ert**ussis) study, focusing on the UK data, project which was undertaken as part of the Periscope consortium.

The BERT study Principal Investigator for the UK site was Dr Kelly. The author was lead doctor for the UK site for the majority of the set-up and enrolment period; Dr Sinclair and Dr Bendor-Samuel assumed that role in the latter part of the study, to enable the author to undertake essential laboratory assays. The author was involved in the protocol development, study set-up and initiation, sample processing (in collaboration with the laboratory lead team- Dr Clutterbuck and Dr Bibi- and OVG research assistants), laboratory assays and study closure and was responsible for analysis of the UK data and input in the analysis of the other sites.

The lead nurse for Periscope was Rachel White. The OVG medical and nursing team was responsible for study visits and sample collection.

As part of the BERT study the Multiplex immunoassay (MIA) was performed at Rijksinstituut voor Volksgezondheid en Milieu in The Netherlands (RIVM) under the leadership of Dr Berbers, by Pieter van Gadeldonk; the serum bactericidal assay (SBA), opsonophagocytosis assay (OPA) and complement deposition assay (CDA) were performed at Public Health England (PHE), Porton Down, UK under the leadership of Professor Andrew Gorringe, by Breeze Cavell; the bacterial adherence assay (BAI) is being performed at Radboud University, The Netherlands, under the leadership of Dr Diavatopoulos, by Evi van Schuppen.

The ELISpot assays of the UK BERT were performed at the OVG laboratory. The author was responsible for the assay optimisation for all three sites and for the execution of the assays assisted by Amy Beveridge and Katherine Sanders.

The author collaborated in protocol development of whole blood rapid T cell assay and was responsible for the performance of the assays referred to in this thesis. The supernatants from these assays were sent to Sanofi-Pasteur Research EU – Human Immunology Unit in France for cytokine measurement using a Multiplex platform, under the leadership of Dr Blanc.

OVG biobank studies and African/Asian studies serologic evaluation:

In order to complement the work started with the BERT study, a group of samples with whole genome sequencing available from previous studies was used. Samples from two studies (originally designated M14P2E1 and H1N1) of the OVG biobank were used. The MIA assay was performed at RIVM (Pieter van Gadeldonk), using part of a small grant (1297 Apr-2018) that was awarded to the author by Oxford Health Services Research Committee (OHSRC).

The African studies (Soweto Vaccine response study and VAC050 study) have been previously presented by Dr Mentzer. For the purpose of this thesis, samples were used to evaluate antibody functionality and that is the original work presented here.

The BAI assay for the biobank studies was performed by the author jointly with Evi van Schuppen, in the Radboud laboratory.

For the African studies, CDA was performed by Breeze Cavell at PHE. Although the author learnt the technique and visited the laboratory for a period during the sample processing, the assay was performed in a containment level 3 (CL3) laboratory and the author was not authorised by PHE to work at CL3 lab for that short period of time.

For the PIMMS study, the author analysed the available datasets not previously analysed, but was not involved in other aspects of the study.

Evaluation of HLA variant contribution to immune protection:

Datasets from the biobank studies and the PIMMS already had whole genome sequencing data available, so, in this case, the author was not involved in the DNA extraction process or genotyping.

For the BERT study the author was responsible for the DNA extraction and quantification, including the optimisation of the technique to extract DNA from PBMC. The whole genome sequencing was performed at the Oxford Genomics Centre, using part of the OHSRC grant (1297 Apr-2018).

The imputation analysis for the biobank and BERT studies was performed by the author under the guidance of Dr Mentzer and the imputation analysis of the PIMMS study was performed by Dr O'Connor, but the association analysis and all other statistical analysis was performed by the author.

Reactogenicity studies – iButton®

To perform the reactogenicity research the author worked as lead doctor of Sched 3 and EUCLIDS studies.

For the EUCLIDS study (funded by European Union's seventh Framework program under EC-GA no. 279185), the lead trial statistician was Ushma Galal, who was responsible for the final statistical trial report. The author contributed actively to the statistical analysis and the graphs and tables presented in this thesis were adapted and redesigned by the author based on the original figures from the statistical trial report, with permission from the trial statistician. A brief summary of the reactogenicity data as part of the final clinical trial results has been published [1,2], but the graphs and tables presented in this document were not included in those publications.

The Sched 3 study (funded by National Institute for Health Research (NIHR) Policy Research Programme – Vaccine Evaluation Consortium Phase II, 039/0031- and the Bill & Melinda Gates Foundation – OPP1126431) was performed in collaboration with PHE. Although Dr Andrews was the lead trial statistician, the author performed the full statistical analyis, graphs and tables regarding the iButton® data. The data were published [3] and the graphs and tables previously published were redesigned for this thesis, with the acknowledgement of all authors.

PUBLICATIONS

Pinto MV, Merkel TJ. **Pertussis disease and transmission and host responses: insights from the baboon model of pertussis**. Journal of Infection 2017 Jun;74 Suppl 1:S114-S119. doi:10.1016/S0163-4453(17)30201-3

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O'Connor D, **Valente Pinto M**, Sheerin D, Galal U, Dold C, Robinson H et al. **Gene expression profiling reveals insights into infant immunological and febrile responses to capsular group B meningococcal vaccine.** Molecular System Biology 2020. 16: e9888, doi: https://doi.org/10.15252/msb.20209888

Versteegen P, **Valente Pinto M**, Barkoff AM, van Gageldonk PGM, van de Kassteele J, van Houten MA, et al. **Responses to an acellular pertussis booster vaccination in children, adolescents and young and older adults: a collaborative study in Finland, Netherlands and the United Kingdom**. EBioMedicine 2021, 65:103247, doi:https://doi.org/10.1016/j.ebiom.2021.103247

IMPACT OF THE COVID-19 PANDEMIC

The covid-19 pandemic has had a relevant impact on the work that the author had planned to be part of the DPhil. The first impact was a delay in the availability of the results of the antibody functional assays of the BERT study programmed between April and May 2020. The results that were not available were the following: a small subset of the baseline and 28 days post-booster of CDA, OPA and SBA and all results of day 365. No results of the BAI assay were available for any of the defined timepoints. The lack of these data could impact the evaluation of the long-term antibody functionality in these groups.

It was also programmed to expand the functional antibody assays to biobank samples and African studies and this task was also not completed due to laboratory unavailability and to travel restrictions: it was not possible to expand the CDA to the infants from the Soweto Vaccine response study and the number of samples from the biobank studies where the BAI was performed. The relevance of these data was to allow a better comparison between the functionality of antibody in infant populations with different primary immunisations.

A significant amount of time during the DPhil was committed to developing the protocol and planning the AWARE study (pertussis **A**cellular **W**hole cell **A**dvance **Re**search) to investigate differences in immunogenicity and reactogenicity after immunisation with either an acellular pertussis vaccine (aP) or whole cell pertussis vaccine (wP) in infants using many of the assays discussed in this thesis. The AWARE study was delayed because of the complexities of providing a wP (Comvac 5® - Bharat Biotech) for the trial in the UK; and then, when ready to commence, delayed for a further 12 months because of COVID-19 pandemic. I had intended to obtain

quantitative and functional antibody data from AWARE and also to apply continuous temperature monitoring, using the iButton® method described in this thesis, to better understand reactogenicity to wP and compare with aP. This study received ethical and Medicines and Healthcare products regulatory Agency (MHRA) approval in January 2020 (South Central - Hampshire B Research Ethics Committee (REC) approval reference 19/SC/0368) to start in February 2020, but due to the pandemic, started late February 2021. A short summary of the study and current recruitment is provided in Supplementary data AWARE study.

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LIST OF ABBREVIATIONS

Table A1: List of abbreviations

Abbreviation	Definition
4CMenB	Four-component capsular group B meningococcal vaccine
ACT	Adenylate cyclase toxin
ADP	Adenosine diphosphate
AE	Adverse event
AEFI	Adverse events following immunisation
ALP	Alkaline phosphatase
aP	Acellular pertussis vaccine
ASC	Antibody secreting cells
B. pertussis	*Bordetella pertussis*
BAB	Bactericidal assay buffer
BAI	Bacterial Adherence Assay
BCIP	5-bromo-4-chloro-3-indolyl phosphate
BERT	Booster against pertussis study
BG	Bordet-Gengou
bvg	Bordetella virulence gene
bvgASR	Bordetella virulence regulatory system
cAMP	Cyclic adenosine monophosphate
CD	Cluster of Differentiation
CDA	Complement Deposition Assay
CDC	Centre for Disease Control and Prevention
CHM	Constitutive humoral memory
CHO	Chinese Hamster Ovary
CL3	Containment level 3
CM	Complete medium
CoP	Correlate of protection
CPG	Staphylococcus aureus Cowan strain

DNA	Deoxyribonucleic acid
DNT	Dermonecrotic toxin
DOB	Date of Birth
dTap	Reduced diphtheria toxoid, tetanus toxoid and reduced aP vaccine
DTaP	Diphtheria tetanus and acellular pertussis vaccine
DTP	Diphtheria, tetanus and whole cell pertussis vaccine
EC50	Half maximal effective concentration
ECDC	European Centre for Disease Prevention and Control
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ELISpot	Enzyme-linked immune absorbent spot
EPI	Expanded Programme on Immunization
EU/EEA	European Union/European Economic Area
FDA	Food and Drug administration
FHA	Filamentous haemagglutinin
fHbp1	Factor binding protein variant 1
FIM	Fimbriae
GBMC	Glucose buffer with magnesium and calcium
GM	Geometric mean
GMC	Geometric mean concentrations
GMT	Geometric mean titres
GSK	GlaxoSmithKline
GTP	Guanosine triphosphate
GWAS	Genome wide association study
HAI	Hemagglutination inhibition
Hib	*Haemophilus influenza* B
HLA	Human leucocyte antigen
IL	Interleukin

IPV	Inactivated poliovirus vaccine
IU	International Units
JCVI	Joint Committee on Vaccination and Immunisation
MAC	Membrane attack complex
MAF	Minor allele frequency
MALDI-ToF	Matrix-Assisted Laser Desorption/Ionization-Time of Flight
MBCs	Memory B cells
ME-TRAP	Multiple epitope-thrombospondin-related adhesion-protein
MenCC	Meningococcal C conjugate vaccine
MFI	Mean Fluoresce Intensity
MFI-C'	Mean fluorescence intensity minus complement background
MHC	Major histocompatibility complex
MHRA	Medicines and Healthcare products regulatory Agency
MIA	Multiplex immunoassay
MVA	Modified vaccinia Ankara
NadA	Neisserial adhesin A
NCES	National Childhood Encephalopathy Study
NHBA	Neisseria heparin binding antigen
NIHR	National Institute for Health Research
NIP	National Immunisation Program
OHSRC	Oxford Health Services Research Committee
OMV	Outer membrane vesicles
OPA	Opsonophagocytosis assay
OVC	Oxford Vaccine Centre
OVG	Oxford Vaccine Group
OxTREC	Oxford Tropical Research Ethics Committee
PAHS	Patan Academy of Health Sciences in Nepal

PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PCV-10	10-Valent Pneumococcal conjugate vaccine
PCV13	13 valent Pneumococcal conjugated vaccine
PERISCOPE	PERtussIS COrrelate of Protection Europe
PHE	Public Health England
PIrSR	Persistence in the lower respiratory tract sensor
Prn	Pertactin
PT	Pertussis toxin
REC	Research Ethics Committee
RIVM	Rijksinstituut voor Volksgezondheid en Milieu (National Institute for Public Health and the Environment)
RTX	Repeats-in-toxin
SAE	Severe adverse event
SBA	Serum Bactericidal Assay
SMEI	Severe myoclonic epilepsy of infancy
T1DGC	Type 1 Diabetes Genetic Consortium
Tcf	Tracheal colonising factor
TCR	T cell receptor
TCT	Tracheal cytotoxin
Th	T helper
TLR	Toll like Receptor
TM	Thawing media
T_{RM}	Tissue resident memory T cells
TTX	Tetanus toxoid
UK	United Kingdom
USA	United States of America
VE	Vaccine efficacy
WHO	World Health Organization
wP	Whole cell pertussis vaccine

ABSTRACT

Bordetella pertussis causes whooping cough, a life-threatening disease in infants. Both whole cell (wP) and acellular (aP) vaccines are used for infant immunisation with aP providing less robust long-term population protection. To further understand differences between wP and aP, this thesis aims: to characterise the immune response to an aP-booster in a UK cohort by age and priming backgrounds (wP/aP) using a broad variety of assays of humoral immunity; to investigate effect of HLA-DRB1-233Thr previously associated with pertussis toxin (PT) IgG antibody levels in wP-primed infants and protection from disease; to evaluate the use of continuous temperature monitoring as an objective method of evaluating reactogenicity.

An aP booster gave the best responses in adolescents (aP-primed) and adults (wP-primed) in terms of antibody (highest for pertactin (Prn) and filamentous haemagglutinin (FHA) IgG), memory B-cells and novel functional assays (SBA, OPA, CDA). Prn-IgG was the antigen most closely correlated with novel functional antibody assays (SBA, OPA, CDA). For all antigens, IgA baseline and booster response increased with age, unlike IgG, suggesting a role of continued antigen exposure in generating this response.

The association of HLA-DRB1-233Thr variant with PT-IgG antibody levels was replicated in wP-primed Asian infants and European adolescents. There was no indication of an additional association with any functional antibody assays assessed. However, an association with PT-IgG avidity and PT-IgA, suggested a role of this variant in enhancing immunological memory in addition to initial antibody production.

Continuous temperature monitoring identified a higher number of fever episodes, characterising more accurately fever, a marker of innate immune response, post-vaccination.

This thesis provides data supporting the introduction of Prn-containing aP in adolescents and adults. Investigation of the mechanisms associated to HLA-DRB1-233Thr and protection in wP-primed individuals may give further insight into differences between aP and wP vaccine responses associated with protection.

CHAPTER 1
INTRODUCTION

1.1 GENERAL OVERVIEW

Whooping cough is a highly contagious respiratory disease caused by the Gram-negative pathogen *B. pertussis* [4]. The etymology of the species assignment, from the Modern Latin, summarises the most recognisable characteristics of the disease: *per:* extensively/thoroughly/intensive; *tussis:* cough [5]. Although there are reports describing a similar disease from the Middle Ages [6,7], the first published scientific report is credited to Guillaume de Baillou, referring to an epidemic that occurred at the end of the 16th century, in France [8]. First described as the 100 days cough [9], it was associated with high mortality [10-12]. In 1678, in the UK, a new terminology was used, “chin-cough”, but in 1701 whooping cough was the agreed upon term and remains so to the present [10].

This introductory chapter aims to review the current available literature on the causative agent, disease pathogenesis, vaccines available and the innate and specific immunological responses to disease and immunisation schedules.

1.2 AN OVERVIEW OF THE WHOOPING COUGH CAUSATIVE AGENT

1.2.1 History of the discovery of *Bordetella pertussis*

B. pertussis was first isolated by Joules Bordet and Octave Gengou in 1906 [13]. Previously, several scientists, Carl Burger being the first in 1883, had

encountered these bacteria when analysing respiratory secretions, but no direct correlation with the disease was made [14].

This fastidious bacterium [4,15-17] is currently taxonomically classified as part of the order Burkholderiales, family *Alcaligenaceae*, genus *Bordetella* [18]. Previously, *B. pertussis* was included in the genus *Haemophilus*, justified by the bacteria's need for blood to be able to grow in culture [19]. This classification was challenged first in 1923 by Fildes and redefined in 1952 by Lopez, with *Bordetella* joining *Haemophilus pertussis* and another two species which share similar characteristics, *Haemophilus parapertussis* and *Haemophilus bronchiseptica* in a new genus: *Bordetella* as per Bordet's name [20].

B. pertussis, in contrast with the majority of the other *Bordetella* species, is a human-only pathogen [18,21]. It is considered to be the most common cause of respiratory disease under the *Bordetella* genus, although other species like *Bordetella parapertussis*, *Bordetella bronchiseptica* and *Bordetella holmesii* can also cause respiratory disease in humans. [22].

B. pertussis is classified as an aerobic Gram-negative coccobacillus, with an ability to be pleomorphic under specific growing conditions [4,13,17]. The first medium developed was the Bordet-Gengou (BG) medium, a solid agar containing human or rabbit blood in a glycerinate potato extract. The BG medium was modified by Eldering and Kendrick, by decreasing the percentage of blood from 50 to 17% and by removing the peptone and in the early 1950's this was recommended by the World Health Organization (WHO) to be used as the standard diagnosis cultured method for *B. Pertussis* [17]. Currently the WHO recommends the use of a fresh BG medium for the isolation of *B. pertussis*, particularly when being used for diagnosis of disease.

1.2.2 Virulence factors of *Bordetella pertussis*

There is now an increasingly detailed understanding of the characteristics, genome and behaviour of this bacterium. *B. pertussis* possesses a group of virulence factors that enable the bacteria to survive and cause disease and their expression is regulated by environmental factors. In early laboratory assays it was noted that *B. pertussis* could undergo a “change of phase”, meaning that the bacteria could switch from a virulent phase to an avirulent phase and vice versa [23,24]. In 1984, Weiss and Falkow identified a potential genetic mechanism underlying phase variation and named the genetic region responsible for this, the “vir” [23].

By the end of the 1980’s the “vir” designation was changed to “bvg” as per *Bordetella* virulence gene [25]. Nowadays, this regulator is known as the Bordetella virulence regulatory system (BvgASR) locus and is considered to be the “master regulator” of this system [26-28]. The BvgASR is composed by the proteins BvgA (23-kDa) and BvgS (135-kDa) that form a two-component system, and BvgR (32-kDa) with a repressor role; those three interact to permit or repress genes transcription [26,29]. It was identified that more than 500 genes are regulated by the BvgA protein [29].

A large number of virulence factors have been described for *B. pertussis* (Table 1.1).

Table 1.1 Description of *Bordetella pertussis* virulence factors

SECRETED TOXINS						
Virulence factor	Classification /family	Structure and molecular weight	Main Function	Pathogenesis mechanism	Location in the bacteria	Other relevant aspects
Pertussis toxin (PT)	A-B toxin of ADP ribosylating family (functional toxin exclusive of this specie)	AB_5 structure Sub-unit A (enzymatic activity) + B oligomer (S2-S5) binding properties 117 kDA	Adhesion, secreted toxin locally and systemically (Type IV secretion systems)	Inhibits G-proteins which results in an accumulation of cyclic adenosine monophosphate (cAMP) inhibiting immune responses	Periplasm	Associated with leucocytosis, hypoglycaemia, insulinemia and histamine release Included in aP vaccines
Adenylate cyclase (ACT)	Repeats-in-toxin (RTX) family	Two functional domains (N and C) activated by eukaryotic calmodulin 177 kDA	Evasion, toxin acts locally and systemically	Affects superoxide generation with increase in intracellular cAMP phagocytic and non-phagocytic targeting epithelial cells. Interference with chemotaxis, phagocytosis and bacterial killing	Extra-cytoplasmatic	
Tracheal cytotoxin (TCT)	Muramyl peptide toxin family	Peptidoglycan disaccharide tetrapeptide monomer 9.21 kDA	Evasion, toxin acts locally	Destruction of ciliated cells and ciliostasis, production of pro-inflammatory cytokines	Extracellular	
Dermonecrotic toxin (DNT)	Heat labil toxin	A-B toxin structure 160 kDA	Toxin that acts locally and systemically	Activation of the host binding protein Rho Potential targeting neuronal cells	Cytoplasm	Local necrosis and vasoconstriction Possible involvement in severe encephalopathy

OTHER TOXINS

Virulence factor	Classification /family	Structure and molecular weight	Main Function	Pathogenesis mechanism	Location in the bacteria	Other relevant aspects
Lipo-oligo saccharide (LOS)	Endotoxin	Glycol-phospholipid with a penta-acylated lipid A domain and by core oligosaccharide structure	Endotoxin causing local damage (production of nitric oxide)	Synergistic action with TCT – damage of respiratory cells Production of pro-inflammatory cytokines	Surface	

ADHESINS

Virulence factor	Classification /family	Structure and molecular weight	Main Function	Pathogenesis mechanism	Location in the bacteria	Other relevant aspects
Filamentous haemagglutinin (FHA)	Filamentous protein	Hairpin-shaped, with a heparin binding domain 232 kDA	Adhesion of bacteria to host cells and cell invasion	Attach to ciliated respiratory epithelium and to macrophages receptors, leading to phagocytosis	Cell wall	Included in aP vaccines
Pertactin (Prn)	Auto-transporter	Auto-transporter and adhesin with an Arg-Gly-Asp motif 69 kDA	Adhesion of bacteria to host cells and cell invasion	Attach to ciliated respiratory epithelial cells and to macrophages receptors (CR3) Resist clearance via neutrophils mediation	Surface of bacteria	Included in aP vaccines
Fimbriae (FIM)	Filamentous protein	Type I pili FIM 2 (22kDA) FIM 3 (21 kDA)	Adhesion of bacteria to host cells (FIM 2 and 3 to sulphated sugars and FIM D to heparin and VLA-5) and cell invasion	Attach to respiratory epithelium	Surface projections	Included in aP vaccines

References: PT: [30-37]; **ACT** [4,26,30]; **TCT** [30,38]; **DNT:** [39-41]; **LOS:** [30,42-44] [36]; **FHA:** [30,36]; **Prn** [30,45,46]; **FIM** [30,42].

1.2.3 *Bordetella pertussis* circulating genotypes

The ancestor of *B. pertussis* is a *Bordetella bronchiseptica*-like bacteria from around 0.8 to 4 million years ago [47].

The genome of *B. pertussis* is 4,100,000 bp [47]. In recent years, it has been suggested that the evolution of the *B. pertussis* genome is driven in part by the introduction of pertussis immunisation in National immunisation schedules (NIP) [48-50]. Currently, the most common circulating strain of *B. pertussis* is from *ptxP3-ptxA1* lineage [48]. This lineage replaced the *ptxP1-ptxA1 as* the most common circulating genotype around the 1990's, after 30 years of *ptxP1-ptxA1* dominance and use for vaccine development [48,49].

Isolation of Prn-deficient or FHA-deficient strains has been documented. Although these were first identified shortly after the introduction of wP, they have been noted more frequently since the introduction of aP [47,51]. A study including several European countries and conducted by the European network EUpert-labnet has shown a continuous increase in the number of Prn-deficient isolates in this continent. The overall proportion of Prn-deficient strains reported between 2012 and 2015 was 21.7%. In this study period all the countries included (in a total of nine) had already implemented aP as part of NIP [51]. Between 1998 and 2001 (EUpertstrain I project), only 3.2% of cases were found. This increase in proportion coincides with a period of pertussis outbreaks in several European countries. In Australia, between 2008 and 2012, 30% of the isolates were Prn-deficient [52], increasing to 89.7% in more recent years (period from 2013 to 2017) [53]. The number of Prn-deficient strains seemed to

be lower in countries that still use wP. In Argentina, a country that still uses an wP in the universal immunisation schedule, in a study performed between 2000 and 2017, only 2 out of 350 isolates (0.6%) were Prn-deficient [54]. One of the most common mechanisms involved in suppressing expression of Prn is the presence of an insertion sequence, IS481 (IS481F and IS48IR), but others like IS1002R and mutations involving deletions and stop codons were also described [51-53,55].

Even rarer than FHA, PT-deficient strains (I979 and FR3749) were identified in Prn-deficient strains [56,57].

The increasing prevalence of Prn-deficient strains also raised concerns about potential increased severity of the disease and reduced vaccine efficacy, although a study in Australia did not show any differences in disease severity between strains considering Prn status, but a higher number of severe cases was found in individuals infected with strains with PtxP3 allele [58].

1.3 WHOOPING COUGH AND PERTUSSIS RELATED DISEASES

1.3.1 Pertussis disease pathogenesis, clinical presentation and diagnosis

B. pertussis is transmitted via aerosolised respiratory droplets, as demonstrated thoroughly in the baboon model [59]. After exposure to the agent and inoculation into human respiratory tract, several events need to happen in order for disease to manifest [15,60-64] (Figure 1.1).

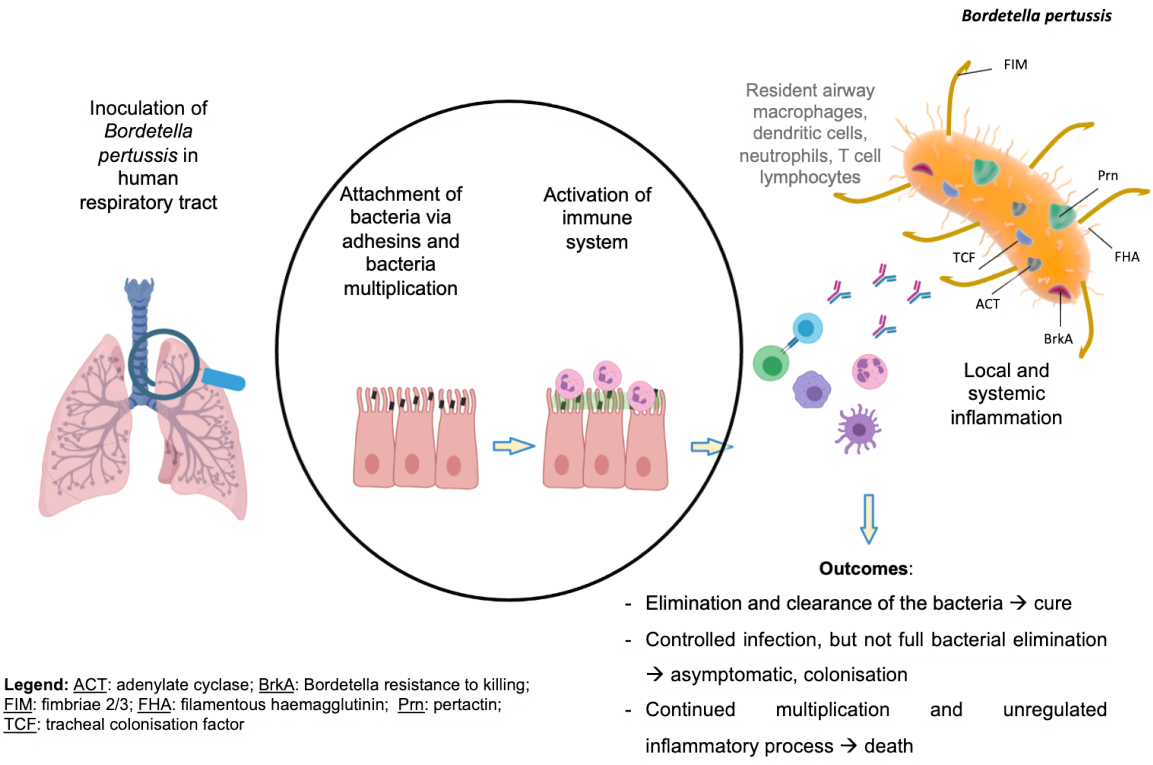


Figure 1.1 Pertussis disease pathogenesis: schematic figure

The most classical presentation of *B. pertussis* infection is whooping cough, but spectrum of disease presentation can range from a cold-like illness with rhinorrhoea, nasal obstruction and other coryzal type symptoms, to hypoxemia, apnoea or chronic/persistent cough [4,63,65,66].

In general, the disease severity depends on the immunisation status, previous infections and age, but factors such as passive immunisation (particularly infants with transfer of maternal antibodies), bacterial load and underlying disorders may also be important [4,63].

According to the Centre for Disease Control and Prevention (CDC) the disease incubation period is around 5 to 10 days but can go up to 55 days [67,68].

The classic presentation, whooping cough, is composed by three sequential recognisable phases: catarrhal phase, paroxysmal phase and convalescence phase [4,63,69-71] (Table 1.2).

Whooping cough stages	Description of symptoms and duration
Catarrhal phase	Symptoms are similar to a mild upper respiratory acute illness (rhinorrhoea and mild cough that worseness progressively) Duration: around 2 weeks
Paroxysmal phase	Severe and prolonged paroxysms of cough and a final profound inspiration effort, that may be accompanied by vomiting, cyanosis and difficulty breathing. Fever is not a common symptom and if present is normally mild Presence of respiratory mucus, that accumulates due to the loss of ciliary function of the epithelial respiratory cells Duration: around 2 weeks
Convalescence phase	Non-productive chronic cough with progressive improvement Duration: up to 8 weeks

Table 1.2 Description of whooping cough disease stages

References: [4,15,16,62,63]

The systemic manifestations are the result of the action of virulence factors causing a constellation of events [33,69], because *B. pertussis* typically does not disseminate to the blood stream [72,73].

Leucocytosis with lymphocytosis is characteristic, with first reports in 1897 [74,75]. PT is considered to be one of the main contributors to lymphocytosis [76]. It is hypothesised that the main mechanism is the down-regulation of the L-selectin CD62L [77], a lymphocyte home marker that mediates the attachment and rolling of

this cells to the lymph nodes venule's endothelium [78], causing inhibition of the migration of lymphocytes with accumulation in the peripheral blood [74]. PT [76,79], jointly with ACT [80] can also target insulin secreting β cells, causing hyperinsulinemia and hypoglycaemia [81]. The proposed mechanism for this metabolic dysregulation is the increase of cAMP in the β cells, which by activating protein kinase A leads to an increased release of insulin [80].

In the most severe forms of disease the pathological findings in the lower respiratory tract varied from necrotising bronchitis, neutrophilic bronchopneumonia, lymphangiectasia with alveolar damage, including fibrinous oedema, macrophages infiltrate and haemorrhage [62,82,83]. These events combined with the obstruction of vascular system of the respiratory tract due to lymphocytes aggregations, lead to pulmonary hypertension with consequent cardiovascular effects [82,84,85].

The WHO case definitions for pertussis disease include both clinical and laboratory elements. The clinical case definition is "a case diagnosed as pertussis by a physician or a person with a cough lasting at least two weeks with at least one of the following symptoms: paroxysms of coughing; inspiratory whooping and post-tussive vomiting without other apparent cause and apnoea (< 1 year of age)". For laboratory confirmation, a clinically confirmed case also needs "isolation of *B. pertussis* or detection of genomic sequences by means of polymerase chain reaction (PCR) or positive serology in individuals > 11 years of age, 1 years or longer after the last vaccine" [86].

The WHO considers the bacterial culture the "diagnosis gold standard" and for the highest probability of successfully isolating *B. pertussis* this should be used during the catarrhal phase or in the early weeks of the paroxysmal phase [86,87].

PCR should be used during the same period recommended for bacterial culture and it has the advantage of more rapid results compared with culture and being less susceptible to pre-treatment with antibiotics [86,88].

Serology should only be used in late stages of the disease when culture and PCR are likely to be negative (> 4 weeks after onset of disease) [89].

The recommended serological assay measures the concentration of immunoglobulin G (IgG) anti-PT antibodies in one single evaluation and the assay should be done using the WHO International standard reference sera (NIBS 06/140) [86,90]. This assay is not recommended in groups in whom the IgG PT concentrations are likely to be already elevated, like infants born from pertussis immunised mothers or in the first year after pertussis immunisation [86]. Several studies tried to define a cut-off to PT IgG to determine exposure to disease and determined the sensitivity and specificity [91]. This value ranged from 50 IU/ml to 200 IU/ml, all with high specificity and with a sensitivity that was inversely proportional to the higher the cut-off [91]. It is widely accepted that values of PT IgG between 100-125 IU/ml are associated with recent exposure to pertussis antigens. The WHO, according to a mathematical model from the Dutch, in the pertussis immunological basis for immunization series report [91], reported that a cut-off of 100 IU/ml allows the detection of the majority of the recent pertussis contacts and levels between 40 and 99 a contact with *B. pertussis*, likely more than 1 year ago. If no contact with disease or lack of any immunisation, levels below 10 IU/ml were likely after 5 years.

1.3.2 Disease treatment

Supportive treatment is commonly used for all patients, including oxygen therapy in case of hypoxemia and respiratory support in more severe cases [92].

Extremely severe leucocytosis is associated with an increased risk of death (WBC>100,000/μl) [93]. One of the first described treatments that has shown to be effective in reducing leucocytosis was leukodepletion by exchange transfusion [94], contributing, according to some case series, to a decrease in fatality rates [95,96]. One promising treatment, with potential impact on leucocytosis and in disease progression, is the use of monoclonal PT antibodies with the ability to neutralise PT and the effects associated with this toxin, demonstrated in the murine and baboon model (m1B7 and m11E6) [97].

A Cochrane review exploring the effect of different treatments in the cough during the paroxysmal phase, including corticosteroids, salbutamol and immunoglobulins, did not show any statistically significant differences when compared with placebo [98]. For bacterial eradication the treatment of choice are macrolides, like azithromycin used as a short course [92,99,100]. Antibiotic resistance to macrolides has increased in some areas of the globe, since the first case was described in Arizona, USA in 1994 [101].

An alternative is trimethoprim-sulfamethoxazole for a period of seven days [99]. The earlier the treatment starts, the higher the likelihood that symptoms will be less severe, and the disease period shortened [92,102-104]. If the treatment is performed at a late stage of disease, although the bacteria can be eliminated, the disease symptoms would prevail. According to PHE, prophylactic antibiotic treatment is not normally recommended in healthy contacts and it is limited to specific vulnerable groups and under specific conditions [105].

1.3.3 Disease epidemiology

Worldwide it is still challenging to define the number of annual pertussis cases, due to the differences and sometimes non-specific nature of disease presentations, with a high number of cases lacking use of laboratory tests.

According to the most recent WHO figures from 2019, 132,754 cases of pertussis disease were officially reported in that year, although is not clear in the report if the number of cases were confirmed clinically or based in laboratory criteria [106]. One of the latest figures of estimated deaths from the WHO is from 2008, reporting 89,000 deaths worldwide [107]. According to a modelling with data from 2014, these numbers are underestimated and 160,700 deaths in children under five years of age for a total 24.1 millions of cases have been predicted [108].

In the pre-vaccination era, pertussis had higher incidence rates and deaths. The number of cases started to decrease with the improvement of medical care, but it is with the universal implementation of the wP, that a significant decrease in the number of cases and deaths was reported (Figure 1.2) [109].

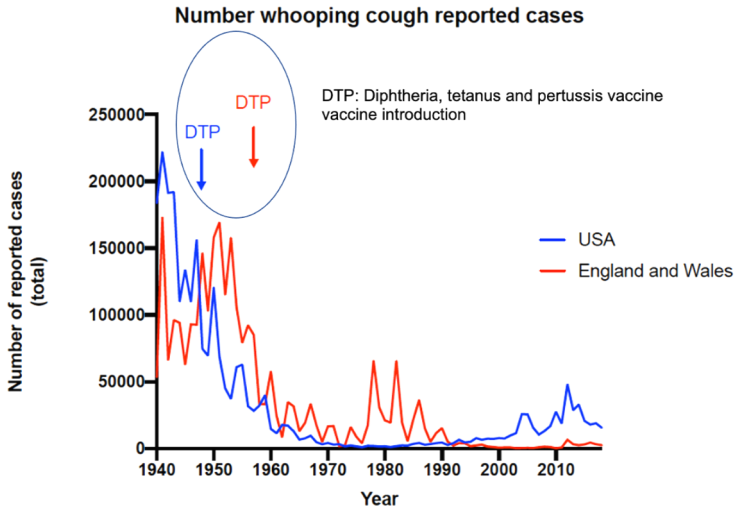


Figure 1.2 Number of whooping cough reported cases in USA and England and Wales from 1940 to 2018

Figures produced by the author with data publicly available from England and Wales available at PHE Notifiable diseases databases [110] and data from USA available at CDC Surveillance and Reporting [111].

The decrease continued until the early 1970's, when concerns about high reactogenicity and neurologic sequelae in association with the vaccine emerged, leading to declining in vaccine coverage and increase of outbreaks [109,112,113].

Subsequent studies including the National Childhood Encephalopathy study (NCES) [113,114] investigated the occurrence of adverse events after wP and helped to restore confidence in the safety and efficacy of the vaccine.

Pertussis disease behaves in a cyclic pattern, with an increase in the number of cases every 2 to 4 years, depending on the region. Factors like high vaccination coverage, low birth rates or low population size, seem to contribute in part with the cyclic pattern periodicity of pertussis, the first increasing the time between peaks and the latest two decreasing that time period [115].

Based on the European Centre for Disease Prevention and Control (ECDC) last available report in European Union/European Economic Area (EU/EEA) countries in 2017 the disease rate was 9.4/100,000 population. In 2017 the European rates per 100,000 population of disease varied from 46.1 in Denmark to 0.5 in Romania. The ages with highest rates were infants <1year (53.9/100,000) followed by children aged 10-14years (25.0/100,000) [116].

The actual burden of disease extends beyond just numbers of cases and deaths and can be associated with the time away from work or other related activities, disruption of sleep, decrease in the quality of life in symptomatic patients and household contacts [117].

WHO recognised that around 19.4 millions of children around the world did not complete the primary immunisation course for this disease and of those 13.5 million did not receive the first dose [118]. From all countries evaluated, African countries showed the lowest coverage (76%) [118].

1.3.3.1 Disease outbreaks in the last decade

Despite over 50 years of using pertussis vaccines, the last decade has seen a concerning trend. There have been pertussis outbreaks around the globe, particularly in high income countries, even with high vaccination coverage [119].

Several reasons could potentially contribute to the disease outbreaks, including changes in circulating pertussis strains, resurgence of new strains, increase awareness and disease reporting and a switch from wP to aP in high income countries [120].

In Europe, outbreaks were reported between the end of 2011 and 2012 in several countries. In the UK, before this outbreak the disease rates were low, with

incidence of 3.7/100,000 population in 2010 [121,122]. By the second half of 2011, the disease rates started to increase. According to ECDC, in 2011, the UK reported 1,256 cases and in 2012 reached 11,986 cases [119], with 14 deaths in England and Wales in 2012, all in children under three months [123,124]. Other European countries with a significant increase in the disease rates included Denmark (2011:6.7/100,000 population; 2012: 7.6/100,000 population) and Ireland (2011: 5/100,000 population; 2012: 10/100,000 population). At the time of the outbreak The Netherlands was the country with higher rates of disease with 76.8/100,000 population.

The outbreaks were not confined to Europe and in high income countries such as the USA, Australia and Canada were also reported [55,125].

In order to control the pertussis outbreaks around the world, several interventions were proposed. One that showed higher efficacy in controlling the outbreak was maternal immunisation, detailed in section 1.4.3.

The outbreaks were also reported in middle and low-income countries where vaccination coverage is lower in some regions when compared with high income countries [118,126]. Also, in some Southeast Asian countries, like Laos, Singapore and Vietnam an increase in the reported cases around 2015 was identified, less than 10 years after the introduction of aP [126]. In countries like Chile in the beginning of the 2000's an increase in the number of cases was identified in the paediatric population wP primed [115].

1.4 PREVENTION OF WHOOPING COUGH: A HISTORY ON IMMUNISATION

1.4.1 Vaccines general overview

One of measures that is considered most cost-effective in preventing disease and associated deaths is vaccination [127].

The principle of vaccination is that by the inoculation or contact with an antigen or specific antigenic structures, individuals are able to mount an immune response that mimics the natural response to the pathogen, thus conferring protection [128]. The response is initiated by the components of the innate immune system and the interaction with the adaptative immune systems allows the development of memory, conferring long-term protection [129,130].

1.4.2 History of pertussis vaccine development

After the discovery of *B. pertussis,* the development of a safe vaccine with significant impact on disease rates was a priority. One of the first experiments showing protection against disease was performed in the Faroe Islands, in the early 1920's (1923-24) and then repeated in 1929, using a wP [6,131]. The vaccine was developed in the State Serum Institute in Copenhagen by freshly culturing *B. pertussis* and by emulsifying the colonies in formaldehyde. The vaccine was administered to a large number of individuals in a three doses regime, during two epidemics, although the number of colonies in the vaccine was higher in the second trial and individuals were immunised at an earlier stage of the epidemic [132]. Although better results were obtained regarding disease prevention in the 1929 outbreak, in both experiments the

vaccine resulted in a decreased number of deaths and reduced disease severity [132]. The breakthrough came from the work of Pearl Kendrick and Grace Eldering [133]. The vaccine developed by Kendrick and Eldering resulted from the processing of *B. pertussis* colonies previously isolated from infected individuals from a region in Michigan [131]. After growing for over 72 hours in Bordet-Gengou media, the bacteria were killed with a chemical agent (merthiolate or phenol) and resuspended at a concentration of 10,000,000 microorganisms per 1 millilitre [133]. This vaccine was administered as a four-dose regimen, with one-week intervals, to 4212 individuals, aged between eight months and four years, from 1934 to 1937. The study showed that the disease incidence was lower in the vaccinated group (n=1815), with a total of 2.3 disease episodes/year when compared with the control group (n=2397; 15.1 episodes/year) [133]. This project resulted in the mass distribution of pertussis vaccine in 1940, throughout USA [131].

Despite the positive results of the vaccine in the USA, other studies failed to show protection against whooping cough. The UK was one of those examples and the inconsistency between trials with the lack of definitive proof of disease protection delayed the wider introduction of the pertussis vaccine in immunisation schedules [131,134].

The UK Medical Research Council performed the first clinical trial in the country, between 1942 and 1944 [135]. The vaccines used in the study were manufactured in the UK (*Messr. Burroughs Wellcome and Co)*, but substantial differences between the vaccines were described: number of colonies, preservative and chemical killing agent, number of doses and interval between doses [135].

To try to overcome this problem ten clinical trials in different geographical areas of England were developed by the Medical Research Council comparing a broader

number of vaccines manufactured in different countries [136]. For these 10 trials, three different vaccines were used: Parke Davies Co (1 batch), Glaxo Laboratories (2 batches) and Michigan Department of Health (2 batches) [136], composed with different concentrations of bacteria or excipients/preservatives. Across all trials children with ages between 6 and 18 months were included, 4515 randomised to the group that received pertussis vaccine (test) and 4412 in the group that was not immunised. In this study the number of cases of pertussis disease were considerably less in the test group, giving a disease attack rate per 1000 child-months maximum of 2.64 in the test group and of 7.04 in the control group [136]. Those findings lead to the development of another set of clinical trials in using wP developed in the UK designed under the same manufacturer procedures as the Michigan Department of Health vaccine, in a total of 14 batches [137]. Differences were found in the effectiveness of the vaccines used, with the last batches showing lower home-exposure attack rates, probably related with the number of times the bacteria were sub-cultured before incorporation in the vaccine, since the last batches were manufactured with freshly cultured bacteria when compared with the first batches [137].

The wP was introduced universally in UK immunisation schedule in 1957 in a 3-dose schedule at 3, 4.5-5- and 8.5-11-months schedule. This regimen was maintained until June 1990, when there was a switch to an accelerated regimen at 2, 3 and 4 months of age [109].

The wP efficacy also showed differences between different age groups. A Swedish study in children aged between 6 and 23 months, that compared fully vaccinated and unimmunised children, showed an overall wP VE of 80.1% [138]. The VE was higher in the younger children, aged 6 to 11 months (VE= 88.8% 95%CI 52.6;

97.3) [138]. Although superior to 70% of estimated VE, the group of children aged 12 to 17 months showed the lower VE (VE= 71.6% 95%CI 0.0;92.3) [138].

A retrospective review on wP VE using the WHO pertussis case definition showed a vaccine efficacy between 84 and 100% [139]. Although the majority of the studies showed that the wP was highly efficacious, this consistency in some studies was not present. As an example, two different studies showed a VE lower than 50% in a wP from the same manufacturer [140,141]

In 1974, with the introduction of Expanded Programme on Immunization (EPI) wP vaccines were also introduced universally in low-income countries [142]. Due to the high reactogenicity of these vaccines, there was a drive to produce safer types of pertussis vaccine. During the 1980's, in Japan, *Sato et al* developed an aP, composed of highly purified protein antigens capable of inducing high antibody levels in immunised individuals [143,144]. A group of trials around the world, particularly in 1990's were performed, leading to the introduction of aP in primary immunisation schedules replacing previous wP schedules or introduced as booster dose [144]. A review by *Cherry* looking at seven different combined aP, five at the regime of three primary doses and two with an extra booster dose, showed a VE between 59% (95% CI 51; 66) and 96% (95% CI 78;99) [145]. As found for the wP, different case definitions were also associated with differences in efficacy in the same vaccine [145]. The number of antigens in the vaccine was also associated with differences in efficacy, as shown by the Swedish study that compared a 2-component (PT and FHA) and 5-component (PT, FHA, Prn and FIM 2,3) aP. The 2-component aP had an efficacy of 58.9% and the 5-component aP of 85.2% [140].

In the UK, before an official switch from a wP regime to aP schedule, between 2000 and 2001, due to a shortness of wP in the country, in specific areas of the country

a multicomponent aP (diphtheria, tetanus and a 3-component aP) was administered [109]. The first official introduction of the aP was as a booster dose for children of 3 years and 4 months of age in November 2001 [146]. A full switch to an aP schedule occurred in September 2004, when the aP was implemented in the primary immunisation schedule at 2, 3 and 4 months, replacing the wP [109,146].

1.4.3 Differences between acellular and whole cell pertussis vaccines

The wP are composed by suspension of formalin of chemically or heat inactivated *B. pertussis,* adsorbed with aluminium [131] and the aP is composed by highly purified proteins from *B. pertussis* including toxins and adhesins adsorbed to aluminium [147].

Thiomersal, a substance with anti-fungal and anti-septic properties, is a common preservative used in wP [148,149]. The possible relationship with poisoning after exposure and with other medical conditions, mainly neurodevelopment disorders was questioned [150,151], but refuted by US Food and Drug administration (FDA), WHO and CDC [149,152,153].

The aP also differ, particularly in the number, type and concentration of antigens, but also in bacterial clone, adjuvants and preservatives [154]. In current commercially available aP, PT, FHA, Prn and FIM 2/3 are the antigens of choice. All aP contain PT combined with a variable number of pertussis antigens. Currently, the WHO recommends the use of an at least 3-component vaccine, since it was shown to be more efficacious in preventing mild to severe disease when compared with one or two pertussis components vaccine [147]. The antigens used in aP vary in

concentration and the vaccines with reduced content are more commonly used in booster doses and not recommended as primary immunisations [155].

High reactogenicity is associated with wP, with concerns in the 1970's of neurologic sequelae with encephalopathy and permanent brain damage [112,114]. To evaluate the increased risk of neurological illness, a National study across England, Wales and Scotland (NCES) was performed [114]. After this study the Joint Committee on Vaccination and Immunisation (JCVI) stated in 1981 that the risk associated with wP immunisation was low and outweighed by the benefits of the vaccine [109]. A follow up NCSE study considered that the low number of cases did not allow to determine if there was a causal effect between the vaccine and the event and attribution of a cause was considered to be speculative [113]. More recently, in the cases of children that received wP and developed encephalopathy without previous identified neurological disease, an underlying genetic condition, designated Dravet Syndrome, a severe form of myoclonic epilepsy of infancy (SMEI) has been described [156,157].

Although high reactogenicity is attributed to the wP, the characteristics of the adverse events following immunisation (AEFI) are similar for both aP and wP and to other routine immunisations, although higher with wP [147,158-161]. Of those, high fever (≥ 40.5 ºC), severe irritability, hypotonic hyporesponsive episodes and severe local skin reactions were among the ones of higher concern [112,159].

Nowadays, aP are used more commonly in industrialised/high-income countries and wP is still the vaccine implemented in the primary schedules of low-income countries. The WHO continues to recommend the use of wP as a primary immunisation in the countries that have this vaccine already implemented in NIP, but do not recommend the use as booster immunisation, due to possible interference with other vaccines antigens [162].

Several different schedules can be implemented with aP, since it has been shown that they can induce immunogenicity with similar antibody titres: a three dose primary schedule at 2, 4 and 6 months of age, that can be also applied in a more accelerated form at 2, 3 and 4 months of age or a two dose primary schedule at 3 and 5 months [163]. In addition to the booster dose given in the first year of life, pre-school booster dose is recommended since increases herd immunity in the paediatric population reducing disease transmission [164]. With the increase of cases in older age groups booster doses in different ages have been considered and, in some countries, implemented. Adolescent booster dose, under the rational of individual protection and reduction of disease transmission, was implemented in countries like Austria, Finland, Germany, Belgium and Italy [164].

Maternal immunisation is one additional vaccination strategy that in addition to pertussis immunisation in infants and pre-school age is considered to be most cost-effective and currently recommended by WHO [154,165]. This strategy has shown to reduce the mortality and morbidity of pertussis disease in the UK after implementation in 2012 and reported a protection level superior to 90% of infants born from immunised mothers [166,167].

Although there is an impact on the number of disease cases, immunogenicity after primary immunisations can be reduced [165,168-170]. The blunting described after primary immunisation is not present at booster immunisations [171]. The clinical meaning of this blunting is not known and neither the real impact for both aP and wP primary immunised infants.

1.5 PROTECTION AGAINST DISEASE: OVERVIEW OF IMMUNOLOGICAL MECHANISMS

The innate and adaptative immune responses are the two mechanisms that allow the elimination of bacteria and protection against disease. The innate immune response is the first line of defence against bacteria. Considering pertussis disease pathogenesis (section 1.3.1), the control of the bacteria by innate immunity at the site of inoculation seems to be relevant to control the infection (Figure 1.1). For *B. pertussis* one of the first components of this line of defence is the respiratory epithelial cells and the produced mucus [26]. Beside this physical barrier, antimicrobial peptides, the complement system (described in CHAPTER 2 and CHAPTER 3) [172] and other immune cells like neutrophils, macrophages and natural killer cells are activated to start the elimination of the bacteria or control proliferation [26,173]. The dendritic cells and macrophages, part of the innate immune response, play a very relevant role in this process, by the expression of pattern recognition receptors that sense pathogen-associated molecular patterns of *B. pertussis* and process the bacterial antigens to be presented to the cells of the adaptative immune system, building the bridge between the two systems [26].

Pertussis virulence factors can interfere with ability of the innate immune system to eliminate the bacteria including the adenylate cyclase (ACT) and the ability to evade neutrophil killing [4,30], the role of Vag8 in increasing the bacteria resistance to complement bacterial killing [172] and interference with the maturation of dendritic cells and the work of macrophages by pertussis virulence factors [174]. The adaptative responses are explored below.

It has been challenging to define when an individual is protected against disease. Although pertussis vaccines have been used for more than half a century duration of immunity is not yet established. Depending on the design of the study and mathematical models applied, significant differences in duration of immunity have been reported. A retrospective study in Australia showed that primary immunisation with wP, particularly the first dose, was associated with a lower risk of disease when compared with aP primary immunised children [175]. Although those differences between the vaccines were described, duration of immunity is still controversial. In a review from *Wendelboe et al* immunity after a natural infection could wane after 4-20 years [176], although more optimistic results were described by *Wearing et al* that according to the developed mathematical model, natural immunity could last on average 30 years [177].

The same review from *Wendelboe et al* showed that immunity against *B. pertussis* after vaccination can last between 4-12 years in children, with no significant differences between aP and wP [176]. In consideration with the effectiveness of the two vaccines, *Sheridan et al* in a retrospective study described that infants fully primed with wP would have a lower associated risk of developing disease when compared with infants primed with aP, particularly if this vaccine was used in the first administration [178]. Although the time of duration of immunity seems more extended when compared with simple evaluation of specific pertussis antibody, waning after immunisation is estimated to be around two to three years [179].

1.5.1 Definition of correlates of protection

Before addressing if we have a “correlate of protection (CoP)” for pertussis, it is relevant to define terminology. An attempt to normalise this definition for

immunisation trials has been done by several authors, but two main references in this field are from *Qin et al* [180] and Plotkin [181,182].

The WHO acknowledged the lack of consistency in the use of the terms among scientific community and in 2013 suggested that a CoP should be used if the immune marker or substitute endpoints are statistically associated with protection against disease or the clinical defined outcome, with no need to be part of the causal mechanistic pathway [183].

Until today, no definitive CoP was determined and accepted widely by the scientific community for pertussis. The existence or not of those correlates is controversial, and if some defend that no correlates exist for pertussis, Plotkin believes that they are multiple [184]. High antibody levels, presence of specific MBCs and cytokines levels after induction of T cells are among the factors that have been considered as markers of protection but, so far, the integration of these data to determine a correlate or co-correlate has not been achieved.

During the 1940-50's, associated with the wider introduction of the wP, several clinical trials were performed, some focusing on the identification of laboratory markers that could work as a CoP. The presence of agglutination titres were the first marker pointed as a potential CoP [185]. *Sako et al*, described that agglutination titres of 1:320 or above were associated with protection against disease in children with direct exposure by household contacts [186]. In the field trials reported by whooping cough immunisation committee in the 1950's the vaccines with the ability to produce higher agglutination titres were also correlated with better protection against disease when compared with vaccine with a poorer response [187].

Despite this potential correlation, it was also shown by *Sako et al* that children with low or no agglutination titres could also escape disease after immunisation,

showing that other factors should be taken into account when considering a CoP [186]. The evaluation of serological markers, similar to other infectious diseases, could be theoretically a good CoP, because of costs, low blood volume and low time-consuming technique.

Although the advantages of the use of pertussis specific antibodies, so far, no consistency in the correlation between antibody levels and protection against disease has been described. Association between the presence of PT and Prn antibodies and protection against disease after exposure to disease in household contacts, but not FHA, was described by *Cherry et al* [188]. Different cut-offs were defined for each antigen, with lower antibody levels being associated with protection for Prn IgG specific antibodies (≥ 7 EU/ml) when compared with PT (≥ 66 EU/ml) [188].

No correlation between MBCs frequencies and pertussis specific antibody levels to our knowledge are yet described, but *Marcellini et al* concluded that the presence of MBCs in an adult population of non-immunised mothers, protected against severe disease, but not against mild disease or colonisation [189].

1.5.2 Animal models: a summary of the available knowledge

Human challenge studies are considered to be the best models to better understand a disease pathophysiology. The disease transmission route and severity, with potential need to use self-contained facilities for long periods and the inability to clear bacteria if antibiotic treatment is provided in later stages of the disease makes pertussis human challenge models difficult to apply and are confined to limited number of researchers, with high budgets and specific facilities [190,191]. The Periscope consortium developed the first pertussis human challenge model according to the

Declaration of Helsinki and International Conference on Harmonization Guidelines for Good Clinical Practice [192].

Several animal models have been developed for *B. pertussis*, particularly murine, pig model and the baboon model [193,194].

The murine model is the easiest and most frequently used, and although the animal share a group of typical signs, like leucocytosis, and features of pathophysiology like adherence of bacteria to respiratory epithelial cells, the most characteristic symptom of pertussis disease, cough, is not developed by this animal [193]. The pig model has the advantage to present with a range of symptoms that mimics human disease in a more complete way, including the presence of leucocytosis and hypoglycaemia and of a range of respiratory symptoms including cough (but not paroxysmal) and respiratory distress [193].

The baboon model has the advantage of mimicking the human infection as seen with no other animal model. Baboons, when infected, are able to present with classic signs and symptoms of whooping cough, including paroxysmal cough, leucocytosis and low-grade fever. Also, the baboon has the advantage of having the four IgG subtypes (IgG1, 2, 3 and 4), as humans [195]. In this model, after exposure, it was possible to develop a model showing all of the phases of infection [194].

In a study by *Stenger et al* using a mouse model, animals primed with either DTP vaccine or a 5-component diphtheria, tetanus and aP, DTaP (PT, FHA, Prn, FIM 2/3), showed that IgG specific pertussis responses peaked at day 98 after the last immunisation at day 28. Antibody levels were higher for all study timepoints in aP primed mice when compared with wP, and specifically no IgG PT antibodies were detected in wP primed mice [196]. In the baboon model, this significant increase in

IgG antibody titres in animals natural infected or as result of previous immunisation of aP and wP was described, with higher titers also found in aP immunised animals [197].

In the *Stenger et al* murine model study, with follow up of antibody titres for more than a year since intervention, after this peak, for both interventions (aP and wP) IgG antibodies for pertussis specific antigens waned significantly, although with levels higher than pre-intervention [196]. This study also gave some insights of mechanisms and differences in the MBCs pool in the most relevant niches for B cells, like lymph nodes and bone marrow. Despite the fast decline in IgG, the long-lived bone marrow plasmablasts increased when compared with the early time-points, although the spleen specific MBCs were undetectable more than a year after immunisation for PT, Prn and FHA. In this mouse model the described recall process was sub-optimal, with no rapid peak of specific IgG levels after a booster vaccination [196].

Another relevant aspect that the animal model tried to explain is the timings and the associated mechanism for bacterial clearance, relevant to understand potential carriage, even in immunised individuals. In baboons previously immunised, independently of the type of vaccines given, it was shown that the animals could equally be infected, but the clearance of the bacteria was quicker in wP animals when compared with aP [197]. A natural infection and immunisation stimulate our immune system as a response to the presence of antigens that would trigger an inflammatory process. One of the key regulators are the T helper cells (Th) [198,199]. Three independent Th cell pathways are considered: Th1, Th2 and Th17. Th1 cells secrete several pro-inflammatory cytokines including Interferon- γ (IFN- γ), Interleukin (IL) 2 and tumor necrosis factor- α (TNF-α), inducing cell-mediated immunity and phagocyte dependent inflammation, stimulating mainly macrophages and natural killer cells [198,200]. Th2 cells are responsible for inducing a high antibody response, including

IgE and stimulate the eosinophils, by the secretion of several cytokines (IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13) [198,200]. The Th2 response is associated with a higher tolerance and inhibits several phagocytic functions, and stimulates mainly eosinophils, basophils and mast cells. Th17 responses, lead to the stimulation of neutrophils, leading to the clearance of extra-cellular pathogens. The main secreted cytokines are IL-17A and IL-17F [200].

Previously immunised mice were challenged with aerosol containing *B. pertussis.* In comparison with animals that received aP, the animals that received wP had a lower bacterial load in the lungs and lower antibody levels [201]. The immunisation with wP induced a Th1/Th17 response and the IgG antibodies were predominantly type IgG2a/c, with an opsonising function [202,203]. The mice primed with aP, mounted a Th2 response and IgG1 antibodies with a toxin-neutralisation role were predominant. In this model, CD4+ tissue resident memory T cells (T_{RM}) of mice with previous contact with *B. pertussis* were able to clear the bacteria after a re-infection [203,204]. If those cells were transferred from convalescent mice to naïve mice, it was able to protect the mice from pertussis infection. A similar response was seen in wP immunised mice, with the ability of T_{RM} cells able to expand after infection, but not for previously aP immunised mice [201,203].

Baboons immunised with aP showed skewed Th1/Th2 responses, represented by an increase of IL-5 production and wP animals had a type Th1/Th17 response, similar to natural infection, although the levels of IL-17 and IFN- γ were lower in magnitude [197,202].

1.5.3 Serological responses to pertussis immunisation

The first methods to measure antibody titers were based on the measurement of agglutinin titres in serum, that seem to correlate with protection. With the development of new methods, the use of Chinese hamster ovary (CHO)-cell neutralization titres was introduced, followed by the more generalised quantitative measurement of specific pertussis antibody titres against PT, FHA, Prn and FIM 2/3 by ELISA or ELISA like methods [143,205,206].

Not all wP available studies show the same immune response after administration. In 1956 a study evaluated 14 vaccines administered to 28799 children, showing inconsistent results in the ability to produce detectable immune response, measured by agglutination assay [187]. After WHO defined the parameters to produce wP, all vaccines commercialised needed to achieve specific parameters of immunogenicity [162]. Also, it was evaluated if DTP vaccines given alone or in combination with a *Haemophilus influenza* B (Hib) vaccine would change immunogenicity. A study from *Begg et al* showed that both options were associated with high geometric mean titres (GMT) of specific pertussis antibodies [207]. The GMT varied between 367 to 736 EU/ml for pertussis specific antibodies and from 3404 to 4732 EU/ml for FHA [207].

In high income countries this evidence led to the introduction of aP in the NIPs, although immunogenicity data is not completely consistent across all studies. As described by *Kitchin et al* in a study comparing two infant cohorts vaccinated with 3 primary doses of either wP (Act-Hib DTP®) or a 5-component aP (Pediacel®) the PT geometric mean concentration (GMC) four weeks after the last primary dose was highest of all tested antigens, with the exception of FIM, in both groups (wP group:98 EU/ml; aP group:78.7 EU/ml). The wP vaccinated group had a higher GMC for FIM

(wP group:591.4 EU/ml; aP group:278.7 EU/ml) and Prn (wP group:68.9 EU/ml; aP group:32.7 EU/ml) when compared with aP group [208]. In a study comparing six different aP (with or without FIM) with a wP in different combinations (only aP, mixed aP/wP and only wP), ≥ 90% of children had at least a four-fold increase in the PT IgG GMC from baseline to post-booster [209]. The children that received at least one dose of wP were the ones with higher GMC for PT, when compared with other with aP only schedules [209]. For other pertussis antigens (FHA, FIM, Prn) the percentage of response was in general lower, when compared with PT [209]. Some children vaccinated with a full aP schedule showed a response to antigens that were not contained in the vaccine. Considering that the only exclusive *B. pertussis* virulence factor is PT and both Prn, FHA or FIM can be present in other *Bordetella* species [63] cross-reactivity needs to be considered.

When focusing on more long-term protection after the booster doses the wP vaccinated population seems to have a higher waning of the antibodies when compared with an aP, besides the better immunogenicity for PT that was described in previous studies [210]. In a study by *Guiso et al,* the percentage of responders (defined cut-offs: anti-Prn:4 EL U/ml; anti-PT and anti-FHA:8 EL U/ml) six years after the first booster dose, were always higher for aP vaccinated, showing a higher waning of antibody levels for wP vaccinated individuals. PT antibodies achieved the lowest GMC and the lowest percentage of responders in both groups, around 21% for aP vaccinated group and 17.5% for wP [210].

Antibody titres *per se* are not able to predict duration of protection. Exploring antibody functionality as a measure of potential correlation and prediction of long-term memory should be considered. Antibody avidity as a measure of antibody functionality also seems to influence the maintenance of IgG MBCs [211].

IgA antibodies are normally stimulated by mucosal antigens, a route not used by the currently available pertussis vaccines, explaining why immunisation does not induce strong IgA responses [173,212]. The IgA responses are mainly induced by the mucosal antigens, after the contact of the bacteria with the epithelial respiratory cells [212], with higher levels in nasal secretions [213]. Mucosal IgA (secretory IgA) inhibits the adherence of *B. pertussis* to the epithelial cells [214] showing the potential role in disease protection. Although the first animal studies did not show a crucial role of secretory IgA in protection against *B. pertussis* [215], a recent study testing a new live attenuated nasal vaccine against this bacteria (BPZE1), showed the role of secretory IgA in protection against bacterial colonisation and the relevance of mucosal immunity in the induction of local memory [216].

1.5.4 Memory B cell responses to pertussis immunisation and natural infection

MBCs are considered a heterogeneous population, and the different functions attributed to each cell of this group increases the complexity of understanding their role [217,218]. MBCs can be generated by T cell dependent or independent processes [219]. Also, MBCs can express different isotypes that would also contribute to different survival rates and function.

As previously described the circulating B cells just represent a fraction of the available B cells. Since the most easily accessible physiological compartment for use in human trials, is peripherical blood, it is relevant to take into consideration and question how much the detected MBCs in the circulating blood correlates with the total amount of available B cells. This question is also valid for other type of immune cells.

Although the animal model theoretically is the best model to give some insights of potential differences in different immune compartments (since are easier to access by biopsy or even by analysing the full tissue, when compared with humans), differences between humans and animals can fail to fully translate immune responses between these species [220]. For the MBCs pool, the most relevant body structures for B cells are the lymph nodes, bone marrow, spleen, Peyers patches and tonsils, compartments where the antigens contact and stimulate the immune cells [221]. For most human studies sampling of MBCs is from peripheral blood and only occasionally lymphoid tissue or bone-marrow and it is important to recognise this limitation when interpreting the results of studies where only recirculating MBCs from peripheral blood can be measured [221].

A lack of consistent correlation between the pertussis specific IgG antibody levels and antigen specific MBCs has been reported [222,223]. This makes the assumption that MBCs could contribute independently to long-term immunity and on that basis, it is likely they should be considered when a CoP is defined for pertussis.

After a booster immunisation or a second contact with the infectious agent, it is expected that a rapid recall response will be mounted by the antigen specific MBCs pool [219,224]. In response to pertussis, however, it remains unclear whether the maintenance of long-term memory is dependent of ongoing contact with pertussis antigens. This contact could allow activation of MBCs, inducing differentiation into antibody secreting cells (ASC), which could also occur without any type of re-exposure but instead be dependent on other factors related with the primary exposure (or primary vaccinations). Factors such as timing of vaccination or primary infection may be involved [196,225]. In a study by *Nanan et al* they were able to correlate the number of booster immunisations with the accumulation of specific MBCs in peripheral

blood, showing not only the potential role of booster immunisation or natural boosting as a relevant factor to induction and maintenance of long-term memory, but also the idea that analysis of circulating MBCs peripheral blood to determine long-term memory is representative of the human immunologic memory [221].

1.5.5 Final considerations

As initially stated on section 1.5.1, no definite CoP was defined for pertussis, and it is not clarified if for each stimulus (type of vaccine, natural infection) a different CoP should be determined.

Expanding our knowledge about what other players can contribute to disease protection and integrate that with current knowledge can be crucial in finding a CoP, key to the development of new vaccines or improvement of current ones, ideally with low reactogenicity and conferring long-term protection against disease and colonisation.

The major histocompatibility complex (MHC) has a recognised role in the immune responses and particularly the molecules classified as part of the HLA class I and II participate in the antigen presenting processes. In recent studies the role of these molecules and associated polymorphisms in response to immunisation have been described, leading to the hypothesis that these genetic variations could be relevant to define a novel CoP [226-229]. For pertussis, an association between specific HLA variants, antibody levels and protection against disease has been described in specific populations and conditions [230]. In CHAPTER 5 a review of HLA and the role of these molecules in disease prevention and response to immunisation is presented.

1.6 DPHIL AIMS AND THESIS OUTLINE

This DPhil is focussed on studying the human immune response to pertussis vaccines in the context of known differences between aP and wP and evidence that aP used in routine immunisation schedules are less effective in terms of control of *B. pertussis* transmission and long-term protection against disease. Due to the relevance of safety and reactogenicity of vaccines, part of the DPhil is dedicated on understanding reactogenicity after immunisation, focusing on fever events.

This DPhil has three main aims, described below:

A. To characterise the immunological response to an aP booster in a UK cohort with different ages and priming backgrounds, in order to better understand the effect on duration of immunity and MBCs (UK-BERT study). This includes a first assessment of novel functional antibody assays against pertussis and their relationship to more conventional measures of pertussis specific immune response. I hypothesised that individuals of all ages will respond significantly to booster vaccine, with groups primarily vaccinated with aP, will have higher serological responses, but wP primed individuals will show better long-term memory responses. Also, antibodies against adherence virulence factors are likely to correlate better with antibody functionality when compared secreted virulence factors.
B. To investigate the effect of human leucocyte antigen (HLA) DRB alleles previously associated with protection from whooping-cough (HLA-DRB1-233Thr) in determining pertussis-specific immune responses in i) wP immunised infant cohorts, ii) a cohort of wP immunised adolescents and iii) children and adolescents in the UK-BERT study. I hypothesised that the HLA-DRB1-233Thr association with PT IgG antibody levels in wP primed

populations will be replicated in other populations of wP-primed individuals including those of different ethnicities and this association would persist at older ages.

C. To investigate the use of continuous temperature monitoring as a method for more detailed study of reactogenicity in childhood vaccine studies. I hypothesised that the continuous temperature method will permit a better detection and characterisation of post-immunisation fever events.

After the overview of different aspects related to pertussis disease, immunisation and the immune mechanisms after natural infection and vaccination presented in this chapter (CHAPTER 1), CHAPTER 2 describes the laboratory methods and statistical analysis applied to achieve the aims of this project. CHAPTER 2 presents detailed description of the study design, study population and all of the laboratory assays used as part of BERT-UK study. An overview of the other selected studies and methods applied, where samples were used to complete a set of laboratory assays are also included.

To answer aim A, an evaluation of the serologic responses (CHAPTER 3) and cellular immune responses (CHAPTER 4) to pertussis in different age groups and with different immunisation backgrounds is performed. CHAPTER 3 explores the antibody titres, antibody functionality and specific correlations in these populations. In CHAPTER 4 plasma cells and MBCs responses in the UK-BERT population are evaluated. In the same chapter the correlation between the different serologic evaluations and B cell responses is determined. As part of the evaluation of the cellular responses a pilot optimization of whole blood T cell assay in the BERT-UK populations is also presented. In the following chapter (CHAPTER 5), in order to identify other

factors that can also contribute to protection against disease, as described in aim B, the contribution of specific HLA alleles to the immune responses against pertussis in a variety of populations with different immunisation backgrounds, is evaluated. CHAPTER 5 focuses on a specific HLA allele (HLA-DRB1-233Thr) previously described in an infant African population as being likely associated with protection and their association with the different immunological parameters explored in CHAPTER 3 and 4.

To answer aim C, I studied the post-immunisation fever events in a high reactogenic vaccine, by comparing the performance of novel continuous temperature method with a standard intermittent method (CHAPTER 6).

To conclude this thesis, the last chapter (CHAPTER 7) discusses the main findings of each chapter and integrates all knowledge and discusses potentialities for future work in this area.

CHAPTER 2

METHODS

2.1 GENERAL INTRODUCTION

For this DPhil project, in order to determine the immunological differences after pertussis vaccination the author used a range of samples and datasets from infants, children and teenagers primed with wP or aP, adults primed with wP and adults with no pertussis primary immunisations, with distinct genetic background (**A.** Immunological studies). To evaluate post-immunisation fever events after administration of a highly reactogenic vaccine, two infant randomised clinical trials were included (**B.** Reactogenicity studies).

This chapter describes all materials and methods used in this DPhil project and includes the following sections:

A. Immunological studies (CHAPTER 3, CHAPTER 4 and CHAPTER 5)

- General description of the studies design, the sample sets and procedures of each study including interventions and sample handling;
- Description of the laboratory assays;
- Statistical analysis.

B. Reactogenicity studies (CHAPTER 6)

- General description of the study design and participants;
- Description of the iButton® procedures;
- Statistical analysis.

In each chapter, when considered relevant, a more detailed description of those methods is also presented.

A. IMMUNOLOGICAL STUDIES

2.2 GENERAL DESCRIPTION OF THE STUDIES DESIGN, PARTICIPANTS AND STUDY PROCEDURES

2.2.1 Booster against pertussis (BERT): "Immunological effects of an acellular pertussis booster vaccination in children, young adults and elderly with different immunisation backgrounds" - UK recruitment and data analysis of an International study in Finland, The Netherlands and UK

2.2.1.1 Study design and participants

The BERT study (East Midlands-Nottingham 2 Research Ethics Committee (REC) approval reference 18/EM/0022) was a prospective, interventional, multicentre study, performed in collaboration with Finland and The Netherlands, with each site acting as their own sponsor.

The participants were divided in four cohorts: children in cohort A (7 -10 years), adolescents in cohort B (11-15 years), adults in cohort C (20-34 years) and elderly in cohort D (60-70 years). The sample size was calculated based on recruitment across all 3-sites, focusing on the paediatric cohorts. It was determined that a total of 108 children per paediatric cohort (A and B), considering a maximum sample loss of 15%, was needed to provide 80% power to detect a standardised difference in log-anti-PT IgG 28 days after the aP booster dose of 0.42 between age cohort.

Per country, a recruitment of a total of 36 children in cohort A, 36 teenagers in cohort B, 25 adults in cohort C and 25 elderly in cohort D was determined, giving a total of 122 participants per country and a total of 366 participants when combining the 3 sites. It was determined that in case of loss of follow-up or withdraw, the participant could be replaced while the recruitment process was ongoing. After recruitment closed, further participants could not be enrolled.

In the UK site, the recruitment started on the 18th of April 2018 and the last visit of the last participant performed on the 14th of January 2020.

The inclusion and exclusion criteria of this study are presented in Table 2.1. Although it was mandatory for cohorts A and B availability of a record with a full immunisation status, for cohorts C and D recollection of immunisation status by the participant in the last 5 years previous to recruitment was considered to be sufficient. If no full immunisation records were available for those participants, an extrapolation of the primary immunisation background was done based on the DOB and the NIP implemented at the time.

At baseline, informed consent was obtained from all participants before any procedure was performed.

Figure 2.1 shows an overview of the study design.

Table 2.1 Inclusion and exclusion criteria defined for the BERT study

Inclusion criteria	Exclusion criteria
• Normal general health; • Received all regular vaccines for their age group according to the country of recruitment national immunisation schedule; • Cohorts A and B availability of a record with a full immunisation status; • Willing to sign written informed consent; • Willing to adhere to the protocol and be available during the study period.	• Presence of serious disease in the last 3 months before inclusion requiring immunosuppressive or immunomodulating treatment; • Chronic infection; • Known or suspected immunodeficiency; • Administration of serum products in the last 6 months before inclusion (including immunoglobulins); • Known or suspected allergy to any of the vaccine components; • Occurrence of serious adverse events after immunisation with a combined whole cell pertussis (wP) or acellular pertussis vaccine (aP); • Immunisation with a pertussis vaccine outside the NIP of the country of recruitment. • Diphtheria, tetanus, acellular pertussis vaccine, inactivated poliomyelitis vaccine (DTaP-IPV) given as part of a maternal immunisation schedule in the last 5 years prior to recruitment; • Immunisation with other diphtheria, tetanus, inactivated poliomyelitis vaccine (DT-IPV) in the last 5 years; • Mixed aP/wP primary immunisation; • Pregnancy.

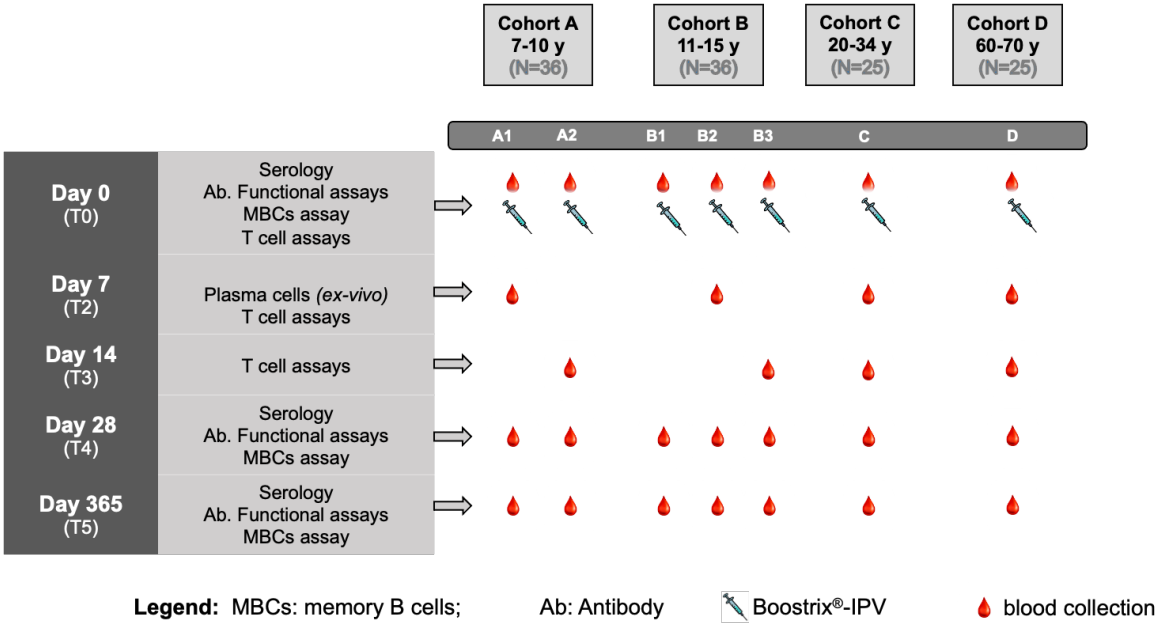


Figure 2.1 BERT study design with study timepoints and procedures, per cohort with sample size per country

2.2.1.2 Study objectives

The primary objective of the BERT study was "to assess magnitude and changes in PT specific IgG antibody levels just before/baseline (D0) and 28 days (D28) after the booster vaccination". PT was chosen as a *B. pertussis* specific virulence factor with well-established assays to evaluate antibody levels against PT.

Secondary objectives included: to determine PT specific IgG antibody levels 1 year (D365) after booster immunisation, to determine PT specific IgA antibody levels at D0, D28 and D365 after booster immunisation , to determine other pertussis specific antibody levels (IgG and IgA FHA, Prn and FIM 2/3) at D0, D28 and D365; to evaluate functional pertussis specific antibody levels, by using assays like BAI, SBA, OPA, to evaluate the magnitude of MBCs responses against pertussis specific antigens (PT, FHA, Prn, FIM 2/3) at D0 and at different timepoints after booster immunisation.

2.2.1.3 Study procedures

After collecting the general medical history to confirm that all inclusion and no exclusion criteria were present, blood samples were collected at different timings, including for all participants at D0, D28 and D365 after booster immunisation (Figure 2.1). For all adult cohorts and a sub-group of participants from the paediatric cohorts whole blood samples were also collected seven (D7) and 14 days (D14) after vaccination.

All participants at D0 (time of recruitment) received intramuscularly in the upper arm a Boostrix®-IPV vaccine, GlaxoSmithKline (GSK) (UK authorisation number PL 10592/0214, renewed in September 2008). This vaccine contains a reduced diphtheria toxoid (at least 2 International Units (IU)), tetanus toxoid (T) (at least 20 IU) and reduced aP containing 8 micrograms (µg) of pertussis toxoid, 8µg FHA and 2.5µg Prn combined with inactivated polio (IPV)), adsorbed on hydrated aluminium hydroxide (0.3 mg Al^{3+}) and aluminium phosphate (0.2 mg Al^{3+}). The IPV component contains 40 D-antigen units of type 1 (Mahoney strain), 8 D-antigen units of type 2 (MEF-1 strain) and 32 D-antigen units of type 3 (Saukett strain), all propagated in VERO cells [231].

2.2.1.4 Data collection

The study data were collected using an electronic software secured at the University of Oxford server and in compliance with the Data Protection Act 2018 and GDPR designated Open Clinica™. The demographic data collected for each participant were date of birth (DOB), ethnicity, gender and consent study details. Regarding medical history, the following was recorded 1) general health and previous

illness; 2) medication history; 3) immunisation background and 4) scheduled hospital admissions.

The inclusion criteria required that participants needed to have had their routine vaccines in the UK. Given this it is likely that the participants from the youngest cohorts had spent most of their life in UK. However, since specific information about where individuals had lived previously was not collected there is uncertainty about the pertussis epidemiology to which any individual had been exposed during their life. Furthermore, no data was specifically collected regarding nutritional status or household structures. Given that inclusion in the study required individuals to be generally considered healthy it is likely in the UK that nutrition was adequate. However, differences in exposure arising from household structure could not be accounted for.

In this study, only the adverse events (AE) that occurred in the first 28 days after immunisation were collected by the study team. Throughout all the study period, serious adverse events (SAE) were collected and reported within 24 hours after acknowledging the event.

2.2.1.5 Sample processing

All the fresh whole blood samples were collected in serum tubes for serum collection and/or sodium heparin tubes for collection of blood components like PBMC's and plasma or for direct use of whole blood (T-cell assay, described in section 2.3.5). The samples were processed within the first six hours after blood collection.

For serum collection, whole blood samples were centrifuged at 2000g and 4°C for 10 minutes and the supernatant was collected and aliquoted into a micronic rack system, stored at -20 to -30°C.

Whole blood samples collected in the sodium heparin tubes were first centrifuged at 700g for 10 minutes for plasma collection. After aliquoting the supernatant into 15 mls falcon tubes, a second centrifuging cycle at 800g for 10 minutes was performed and plasma aliquoted in 2 to 4 mls vials and stored at -80°C. For PBMC isolation, the cell pack that remained in the original sodium heparin tube, after removal of the supernatant, was reconstituted to original volume with a solution of phosphate buffered saline (PBS) (Gibco, Life Technology Corporation, Grand Island, USA) and Heparin. After reconstitution, the blood diluted in 1:1 PBS was layered slowly into a falcon tube containing lymphoprep (Alere Technologies, AS, Oslo, Norway) and centrifuged at 800g (brake off) for 30 minutes. After centrifuging, the PBMC layer was removed with a Pasteur pipette and transferred to a new falcon tube for new centrifuging cycle (300g; 10 minutes), after adding PBS wash buffer. After the first centrifuging, the wash buffer was removed, and the cell pellet resuspended again in the wash buffer for a second wash (300g; 10 minutes). After the centrifuging cycle and removal of wash buffer, the cell pellet was resuspended in a small volume of PBS (1ml per 10 mls of blood) and counted in the Muse (Luminex corporation, Austin, TX, USA). After the cells were counted, a final centrifuging step was performed, and the cell pellet resuspended in cell freezing medium (Gibco - Life Technologies Corporation, Grand Island, NY, USA) to achieve a concentration of $10x10^6$ cells/ml. PBMC's aliquoted in cryovials were stored in the first 24 hours at -80°C in Mr Frosty and transferred to liquid nitrogen for long-term storage at -135°C.

2.2.2 Biobank studies: description of samples and datasets used for pertussis immunological investigation in individuals of different age groups, different country/continents of origin and different pertussis immunisation backgrounds

The biobank studies used in this thesis were not designed to assess pertussis responses, but residual serum and plasma samples were used: to further evaluate novel functional antibody assays; to extend previous observations of the association between specific HLA variants and pertussis-specific antibody responses and as a wP-primed comparison for the aP-primed child and adolescent cohorts in the BERT study.

For these populations, we only had access to limited metadata, and no information regarding nutritional status or household structure were available for any of the studies to include in the analysis. Also, it is relevant to highlight that no available information regarding environmental exposure to pertussis/disease epidemiology was available in these populations and no extrapolations could be made in the analysis.

2.2.2.1 UK children and adolescent samples

A set of samples from the OVG biobank from studies undertaken in the UK were used for analysis. Based on the year of blood collection and the age of the participants, samples from wP-primed paediatric populations, currently difficult to obtain in prospective studies, due to the introduction in the UK of the aP as a primary immunisation in 2004 [109], were selected. Furthermore, whole genome sequencing data were available for the participants in these studies [232], and this permitted HLA-imputation and association analysis with no further costs.

The studies selected are stored as part of the Oxford Vaccine Centre (OVC) Biobank, ethically approved by South Central – Hampshire B Research Ethics Committee (165/SC/0141). The use of the samples for research relating to vaccine immune responses is included under this approval and the samples were made available after submission of a study protocol approved by that entity.

The two studies selected were the H1N1 study and the M14P2E1 study. For both, immunisation background was not recorded in the available metadata and was assumed to be the UK routine immunisation schedule based on the DOB. The samples included in this project were for participants born before any aP was used in the UK and the majority of other countries in the world, decreasing the risk of choosing samples of individuals with a mixed or aP primary schedule and mitigating potential wrong assumption of the immunisation background and interpretation of the results.

The **H1N1 study** (2009/08 H1N1; original ethics reference: 09 H0604/107), was an open label, randomised, multi-centre study to evaluate the safety and immunogenicity of Baxter H1N1 vaccine and GSK H1N1 vaccine in children 6 months to 12 years of age. The study was performed by OVG in collaboration with other research centres (St. Georges Vaccine Institute, Health Protection Agency Bristol Children's Vaccine Centre) during the winter 2009. For the purpose of this thesis only individuals born before 2000 and with serum available were selected as part of the wP-primed children's cohorts.

From the original study, information on gender, DOB and date of the blood visit was available for a group of individuals. Ethnicity records were not available.

Based on DOB, all participants had the same schedule of pertussis primary immunisation, with a wP given as part of a combination vaccine (two, three and four

months of age). It was estimated that 2/3 of the selected individuals had also received an aP booster vaccine in pre-school age, since this vaccine was implemented in the UK in November 2001, affecting all children born after May 1998 [109]. In other European countries implementation of aP booster immunisation happened in similar periods [233,234] or in the end of the 90's [235].

Only one blood sample was collected in this population.

The **M14P2E1** study was a phase IV follow-up study, open-label, designed to evaluate the persistence of antibodies in adolescents with 14 to 16 years of age, vaccinated with Chiron Meningococcal C conjugate vaccine (MenCC - Menjugate®) during 1999-2001 UK MenCC Immunisation Campaign. In parallel, this study also aimed to evaluate subjects aged 11-20 years, four years after UK MenCC Immunisation Campaign during 1999-2001. The original study received ethical approval Midlands and South Bucks (REC NC 1165/703).

This study was performed in 2005 and the individuals recruited were born in a period of time that aP was not available in the UK and not used in the NIP's of the majority of high-income countries [109]. It was assumed that all individuals were wP primed. The metadata collected included: DOB, gender, ethnicity and date of the blood visit. The serum samples were collected at a single time-point.

The selection of samples of each one of the studies to perform the MIA assay was based in convenience sampling, that fulfilled the following: DOB prior to 2000 and sample volume availability to perform the assays. For the functional antibody assays a sub-group of samples using the same method was selected. A set of criteria were defined for that selection: having enough volume to perform the requested assay, age

at time of collection in the range of the BERT study cohort A and B (7 to 15 years of age) and having genome sequencing data available.

Sample processing of the children and adolescent UK samples

In the H1N1 study whole blood was collected in serum tubes, stored in the fridge (2-8°C) for a maximum of 24 hours and after centrifuged, serum aliquots were storage at -30°C. Similar method was applied to the M14P2E1 study.

For an easier understanding of the relevant studies in terms of pertussis immunogenicity, from this point, **H1N1 study** will be referred as **Children_UK_wP** and the **M14P2E1 study** referred as **Adolescent_UK_wP.**

2.2.2.2 African infant samples

In collaboration with Dr Mentzer and the Jenner Institute, samples and data from the **Soweto Vaccine response study and VAC050 ME-TRAP Malaria Vaccine trial** were made available by the research team of the study.

Both studies evaluated immunogenicity data, which included pertussis-specific antibodies (PT IgG, FHA IgG and Prn IgG). The research team of the original studies had previously performed a genome-wide association analysis (GWAS) which had identified HLA-specific variants associated with pertussis-specific antibody levels [230]. In the original studies, ethnicity was collected and presented by *Mentzer* [236].

The **Soweto Vaccine response study** recruited infants specifically to understand the impact of human genetic variation on childhood vaccines [237]. In this study, six months infants from the Soweto Region in South African were identified

[238]. The study was originally ethically approved by the University of Witwatersrand Human Research Ethics Committee (reference M130714) and Oxford Tropical Research Ethics Committee (OxTREC) (1042-13) [230]. As part of the EPI schedule, this population received a combined aP as part of the primary immunisations at 6, 10 and 14 weeks of age. The vaccine received intramuscularly was Pentaxim® (Sanofi Pasteur, Ltd) containing diphtheria toxoid (≥ 30 IU), tetanus toxoid (≥ 40 IU) and aP containing 25 µg of PT and 25 µg FHA with an IPV adsorbed on hydrated aluminium hydroxide (0.3 mg Al^{3+}). The IPV component contained 40 D-antigen units of type 1 (Mahoney strain), 8 D-antigen units of type 2 (MEF-1 strain) and 32 D-antigen units of type 3 (Saukett strain), all propagated in VERO cells. This vaccine was combined with a polysaccharide of *Haemophilus influenza type b* (10 µg) conjugated with tetanus protein (18 to 30 µg) [239].

Whole blood samples were collected at six and 12 months of age [230].

To be able to use the plasma samples for the purposes of the DPhil an amendment to the original protocol and new local REC submission was performed (approval locally (M190208); OxTREC (1042-13 and 42-14).

The **VAC050 ME-TRAP Malaria Vaccine trial** was a phase I/IIb clinical trial, designed to test the safety, immunogenicity and efficacy of a prime-boost novel malaria vaccine using an MVA-viral vector [230] in children aged 6 to 18 months after completing primary immunisation schedule according to the EPI schedule of that country (wP at 6,10 and 14 weeks of age) [240]. Blood samples were collected at several time-points after the intervention between 6 and 18 months of age as per protocol. The study received ethical approval from Ministere de la Recherche Scientifique et de l'Innovation in Burkina Faso (2014-12-151) and of OxTREC (41-12).

Based on the studies performed by Dr Mentzer on pertussis immunological responses, protection against pertussis disease and HLA genetic variations, the same REC application was applicable to this investigation.

The samples provided had available MIA assay results for pertussis specific antibody levels. A convenience sampling was used for sample selection, based particularly in the existence of enough volume to process functional antibody assays.

Sample processing of African infant studies

Whole blood samples were collected in ethylenediaminetetraacetic acid (EDTA) tubes in the **Soweto Vaccine response study** and in heparin lithium tubes in the **VAC050 ME-TRAP Malaria Vaccine trial** study. Samples were processed according to the study specifications. The samples were centrifuged, and the plasma was stored at -80°C. In the **Soweto Vaccine response study** studies, the DNA was extracted from the erythrocyte layer and in the **VAC050 ME-TRAP Malaria Vaccine trial** studies from buffy coat.

For an easier and quick understanding of the relevance studies in terms of pertussis immunogenicity, from this point, the **Soweto Vaccine response study** will be referred as **Infants_Africa_aP** and the **VAC050 ME-TRAP Malaria Vaccine trial** as **Infants_Africa_wP.**

2.2.2.3 Nepali infant samples

The **PIMMS** study was a phase IV, open label randomised (1:1) trial, designed to compare two-dose priming of a 10-Valent Pneumococcal conjugate vaccine (PCV-10) administered at two different schedules (six and ten weeks of age *versus* six and

14 weeks of age) in a Nepalese infant population. This study was performed as a collaboration between OVG and Patan Academy of Health Sciences in Nepal (PAHS). It received ethical approval from OxTREC (25-15) and from the Nepal Health Research Council (NHRC:90-2015).

A total of 304 healthy infants, with an age range from 40 to 60 days were randomised in two groups (N=152 per group). A total of three blood samples were collected: at 14 weeks (group that received the 6+10 weeks PCV-10 schedule) or at 18 weeks (group that received the 6+14 weeks PCV-10 schedule) and at 9 and 10 months of age in both groups. All infants received their primary immunisations during the study period [241]. All infants where Nepalese and were residents in Kathmandu. No other specific information on ethnicity was available.

Pertussis immunisation was performed according to the Nepal's NIP, consisting of a three primary doses at six, 10 and 14 weeks of age of a wP vaccine. The vaccine administered intramuscularly into the left thigh consisted in a DTwP-HBV-Hib vaccine from the Serum Institute of India, composed by diphtheria toxoid (≥ 30 IU), tetanus toxoid (≥ 40 IU) and wP *B. pertussis* ≥ 4 IU, combined with 10 µg Hepatitis B surface antigen (rDNA) and a purified capsular polysaccharide of *Haemophilus influenza type b* (10 µg) conjugated with tetanus toxoid (10 µg) adsorbed in ≤ 1.25 mg Al^{2+}. The vaccine contained 0.005% thiomersal.

For this study, participants immunogenicity data of pertussis specific antibodies (PT, FHA and Prn IgG) and DNA sequencing were readily available for analysis. Since no extra laboratory assays were performed in these samples, no amendment or new ethical approval was needed.

Sample Processing of Asia Infant studies

In the PIMMS study the blood was collected into serum tubes and stored at temperature of 2-8°C for a period of 12 hours, before transferring to the local laboratory at Patan Hospital, in Nepal. After the whole blood sample was centrifuged, serum for immunogenicity studies and blood clot for Deoxyribonucleic acid (DNA) extraction were kept in the freezer at -20°C.

For the purpose of this project this study will be referred, from this point, as **Infants_Asian_wP.**

Table 2.2 gives an overview of the data available for all sample datasets that allowed different comparisons for different age groups and immunisation backgrounds.

Table 2.2 Description of the different studies and sample sets by age and immunisation background and the assays performed in each study for the DPhil project

	Pertussis immunisation background			
	Whole cell pertussis vaccine/ Natural infection		Acellular pertussis vaccine	
	Study	Assay	Study	Assay
Infants	Infants_African_wP (VAC050)	A, B, HLA	Infants_African_aP (Soweto Vaccine response study)	A, B, HLA
	Infants_Asian_wP (PIMMS)	A, HLA		
Children	Children_UK_wP (H1N1)	A, B, HLA	BERT – cohort A	A, S, C, O, MBC, HLA
Adolescents	Adolescent_UK_wP (M14P2E1)	A, Av, B, HLA	BERT – cohort B	A, S, C, O, MBC, HLA
Adults	BERT – cohort C	A, S, C, O, MBC, HLA		
Elderly	BERT – cohort D	A, S, C, O, MBC, HLA		

Legend: A: antibody levels; Av: Avidity assay; B: Bacterial adherence assay (BAI); S: Serum Bactericidal Assay (SBA); C: Complement Deposition Assay (CDA); O: Opsonophagocytosis (OPA); HLA: Human leukocyte antigen

2.3 LABORATORY ASSAYS AND OPTIMISATION

2.3.1 Multiplex immunoassay for determination of antibody levels

Background

The MIA was developed based on the Luminex™ multiple analyte profiling technology (Luminex™, Austin, TX, USA). This highly sensitive and reproducible assay uses a flow-cytometry-like system that allows test of several analytes of interest

in the same sample, by the use of pre-coated fluorescent beads, using lower volumes and by being less time consuming when compared with traditional enzyme-linked immunosorbent assay (ELISA) [242,243].

Methods

For immunogenicity studies fluorescence carboxylate beads/microspheres are covalently coupled after a two-step carbodiimide reaction with a specific antigen of interest, a step that allows the recognition and determination of what antigen is being detected (a). To the coated microspheres, a serum or plasma sample is added allowing the formation of an antigen-antibody complex. In order to detect the magnitude of the specific antigen-antibody biding and ultimately quantify the antibody concentration, a goat anti-human conjugate is labelled with R-phycoerythrin. The reaction is read by a dual-laser detection instrument, that based on fluorescence reads of each laser, detects the type of bead (a) and the magnitude of the signal transmitted by the phycoerythrin (b) [244].

The MIA assay was performed at RIVM, according to the technique described by van *Gageldonk et al* [243,245]. In summary, purified pertussis specific antigens (PT: GSK, Brentford, Middlesex, UK; FHA: Sanofi-Pasteur, Lyon, France; Prn: GSK, Brentford, Middlesex, UK; Fim 2/3: Sanofi-Pasteur; Lyon, France), Tetanus toxoid from *Clostridium tetani T3194* (TT) (Sigma-Aldrich, St Louis, MO, USA) and Diphtheria toxoid (Dt) (Netherlands Vaccine Institute, Bilthoven, Netherlands) were coupled with carboxylated microspheres (Bio-Rad Laboratories, Hercules, CA, USA) in pre-determined regions of the bead. A solution of beads ($12.5x10^6$ beads/ml) washed in PBS (Gibco, Life Technology Corporation, Grand Island, NE, USA) were activated by the addition of 5 mg 1-ethyl-3-(-3-dimethylaminopropyl)-carbodiimide hydrochloride

(Thermo Fisher Scientific, Waltham, MA, USA) and 5 mg of N-hydroxy-sulfosuccunimide (Thermo Fisher Scientific, Waltham, MA, USA) in 1 ml of PBS. After activation and three washes with PBS, the microspheres were resuspended to achieve a bead/antigen ratio of 10μg/12.5x10^6 for the pertussis specific antigens, 25μg/12.5x10^6 for TT and 100μg/12.5x10^6 Dt. After incubation for two hours using a rotating incubator and further washing steps, the coupled beads were stored in the dark at 4°C in in PBS containing 0.03% (wt/vol) sodium azide and 1% (wt/vol) bovine serum albumin at 4°C until use (Sigma Aldrich, St. Louis, MO, USA).

In-house reference standards were used: in--house reference standard for pertussis calibrated against WHO 1st IS Pert 06/140 and in-house reference standard for Dt and TT calibrated against WHO NIBSC DI-3 and TE-3.

In a 96 Multiscreen HTA filter plate (Millipore Corporation, Billerica, MA, USA), diluted sera from study and reference samples (1/200 and 1/4000 in PBS with 0.1% Tween-20 and 3% BSA) were mixed (1:1) with the pre-conjugated beads and incubated in a plate shaker for 45 minutes under dark conditions. Beads were collected using a vacuum system and after three washing steps the beads were incubated with R-phycoerythrin-labelled goat anti-human IgG antibody (Jackson Immunoresearch Laboratories, West-Grove, PA, USA). or Goat F(ab)2 anti-human IgA-PE (Southern Biotech). After a last washing step, beads were resuspended in PBS and shaken for data aquisition in Bio-Plex 200 or Flexmap 3Dand Bio-Plex Manager software (Bio-Rad Laboratories, Hercules, CA, USA), that determined the Mean Fluoresce Intensity (MFI) for each sample and specific bead.

The final specific antibody concentrations expressed in IU/ml were determined using a 5-parameter logistic regression model of the mean of two sample dilutions conditional to fitting the linear part of standard curve.

This assay, using the same or equivalent reagents, was performed at RIVM to determine the antibody titers for the BERT study and for all biobank samples (Infants_African_wP, Infants_Asian_wP, Children_UK_wP, Adolescent_UK_wP and Infants_African_aP).

2.3.2 PT IgG avidity assay

Background

Measurement of antibody avidity allows us to determine the functionality of antibodies [246]. The basic principle is to determine the ability to disrupt the binding of antibody-antigen complex by adding a chaotropic agent[1], at different dilutions, by evaluating the strength of this bond [246-248]. Different chaotropic agents, including ammonium or sodium thiocyanate, urea and diethylamine can be chosen to achieve this objective and have been used in previous assays [246].

Methods

The avidity assay was performed by Pieter van Gageldonk at RIVM, using a technique previously described by the same group [222,249].

The avidity of PT IgG was measured in plasma biobank samples, using the same principles of MIA assay, with some modifications. The chaotropic agent used was ammonium thiocyanate (NH_4SCN).

Samples were diluted 1/200 (3%BSA and 0.1% Tween-20 in PBS). 25µl of a bead suspension was added to 25µl of the plasma sample and incubated for 45 minutes. After the incubation period the diluted sample was washed three times with

[1] An agent that disrupts the hydrogen bonding between molecules in a water solution

PBS. The same sample was incubated either with PBS or with 1.5M of NH_4SCN for 10 minutes. The mixture was washed again three times with PBS. The samples were incubated with R-Phycoerythrin conjugated goat anti-human secondary antibody for 30 minutes. A final washing step (three washes with PBS) was performed before reading the plate on Flexmap 3D (Luminex).

After reading the plates, an avidity index (AI) was calculated by the levels of IgG or IgA after adding NH_4SCN in comparison with the original levels in the presence of PBS (determined as 100%) and expressed in percentage (AI in %; [1.5M NH4SCN/PBS]*100).

2.3.3 Other functional antibody assays

2.3.3.1 Serum bactericidal antibody assay (SBA)

Background

The complement system is a well organised network with more than 30 proteins, that work in the first line of defence, establishing a link between innate and adaptive immune responses, that can be activated by different stimulus [250,251]. The antibodies, by the formation of antigen-antibody complex, are responsible for activating the classical complement pathway that leads to the formation of C3 convertase that cleaves C3 in C3b, that after being deposited on bacterial membrane, can mediate opsonophagocytosis or by contributing for the formation of C5, leads to bacterial killing via the membrane attack complex (MAC) [250,252].

Methods

In summary, *B. pertussis* B1917 were previously grown in THIJS medium, was defrosted and incubated at 37°C for 30 minutes. BP1917 were diluted in dilution 12 wells plates with bactericidal assay buffer (BAB), composed by 2.5 gr of bovine serum albumin in 500 ml Hank's balanced salt solution.

For a starting dilution of 1:2, 20 μl of heat inactivated serum from the first two columns was added. After that step, seven serial dilutions were performed. In the last column, 5μl of test serum was added. Absorbance of bacteria at OD_{600nm} was read and diluted to $8x10^4$ CFU/ml using BAB. For the first 10 columns, 10 μl of an active complement at the dilution of 1:2.5 was added, giving a final concentration in the well of 10%. At the same dilution as previously, heat inactivated complemented (human IgG and IgM depleted plasma) (10 μl) was added to the last two columns to give a final concentration in the well of 10%. For the final step of the plate preparation, 10μl of the working solution of the bacteria was added to each well. From column 11 a sample of 10μl of the mixture was plated in *Bordetella* selective agar plates using the tilt method.

The plate was incubated in the shaking incubator for two hours at 37°C at 900rpm. After the incubation period, 10μl of each well was plated in *Bordetella* selective agar plates using tilt method and left upright for approximately 10 minutes before inverting. The plates were incubated for five days at 35°C in a humidified atmosphere. After the incubation period, the colonies present in the plate were counted using a Sorcerer Colony Counter. An interpolated titre was calculated, by using 50% survival compared with complement only control using the formula below (Figure 2.2).

$$y = y_1 + (x-x_1) \quad \frac{y_2-y_1}{x_2-x_1}$$

Figure 2.2 Formula to calculate interpolated titre in the SBA

2.3.3.2 Opsonophagocytosis assay (OPA)

Background

As previously described, antibodies can activate the complement classical pathway that leads to the formation of C3 convertase that cleaves C3 in C3b, that after being deposited on bacterial membrane, mediates opsonophagocytosis [250,252]. The principle of these assays is to replicate the opsonophagocytosis process where antibodies, like pertussis specific antibodies, work as an opsonin that marks a pathogen to be killed by phagocytes.

Methods

Before performing this assay, blood from volunteers need to be pre-prepared for isolation of polymorphonuclear neutrophils (PMN).

In a 96 well U bottom plate, 10μl of participants serum was added in duplicate. On the same plate three controls in triplicate were incorporated: well with only cells, a well with cell and bacteria only and a well with bacteria and complement only and 10μl of glucose buffer with magnesium and calcium (GBMC).

Vials with *B. pertussis* B1917 previously grown in the same conditions as described for the SBA assay were defrosted and after read at an absorbance of OD_{600nm} and diluted with GBMC to achieve an OD_{600nm} of 0.06. To each well (except the cell only control wells that 40 μl of GBMC was added), 40μl of *B. pertussis* was

added. The plates were incubated for 50 minutes in the shaking incubator for 37°C at 900rpm. To all wells, except the cell only well and the bacteria and cell well that 5μl of GBMC was added, 5μl complement was added to the wells and a second incubation cycle in the shaking incubator was done at the same condition as previous cycle. After incubation the pre-prepared fresh cells were added to all wells (50μl) and a new incubation cycle was performed with the same settings. In the final step after incubation, 105μl of formaldehyde in PBS was added to all wells to give a final concentration in the well of 0.5% before incubating at room temperature for 30 minutes, protected from the light. The plate was acquired on Intellicyt iQue flow cytometer.

2.3.3.3 Complement deposition assay (CDA)

Background

The CDA is a duplexed assay that by using the principles of complement pathway after activation, measures antibody-mediated complement C3b/iC3b and C5b-9 deposition onto the surface of *B. pertussis* by flow cytometry, as a surrogate for the potential opsonophagocytosis and bactericidal activity, respectively, mediated by antibodies via complement [253].

Methods

The human complement source was from volunteers that donated blood after screening to identify the participants lacking intrinsic cross-reactive antibodies against the test strain. From the whole blood, plasma was separated, and IgG and IgM depleted.

In summary, 5µl of each participant heat inactivated sample of serum or plasma (56 °C, 30 minutes) were added to a U bottom 96 well plate in duplicate followed by 5µl of blocking buffer (2% BSA PBS). To that mixture 5µl of IgG-IgM-depleted human plasma were added.

Pre-labelled B1917 diluted bacteria were defrosted and diluted in blocking buffer to achieve an OD_{600}=0.1 before adding 90µl to each well.

For each assay plate, three controls were also considered: B1917 only in duplicate wells, B1917 and conjugate (C3c-FITC and C5b-9-AF647 antibodies diluted in the assay buffer) and a B1917, conjugate and complement.

The plate was incubated for 45 minutes at 37°C in the shaking incubator (900 rpm). After incubation the plates were centrifuged at 3060g for five minutes and the pellets resuspended in blocking buffer. After a second centrifuging cycle the wells with participant samples were resuspended with an anti-C3c FITC and anti-C5b-9 AF647 conjugated mix. The bacteria only controls were resuspended with blocking buffer only. The plate was incubated at room temperature in the dark for 30 minutes. After incubation, the plate was centrifuged at 3060 g for five minutes and after a first wash, pellets were resuspended in 2% formaldehyde PBS. After a second one-hour incubation period and centrifuging of the plate the pellets were resuspended with a 1:5000 dilution LIVE/DEAD™ fixable violet stain in PBS. After a final incubation step for 30 minutes at 4°C, the plate was centrifuged twice, and pellets resuspended in 200µl PBS after the supernatant was removed. After second resuspension cells were analysed in the flow cytometer (BD LRSFortessa™ flow cytometer). The results were expressed as medium fluorescence intensity minus complement background (MFI-C'). Figure 2.3 illustrates the steps of CDA.

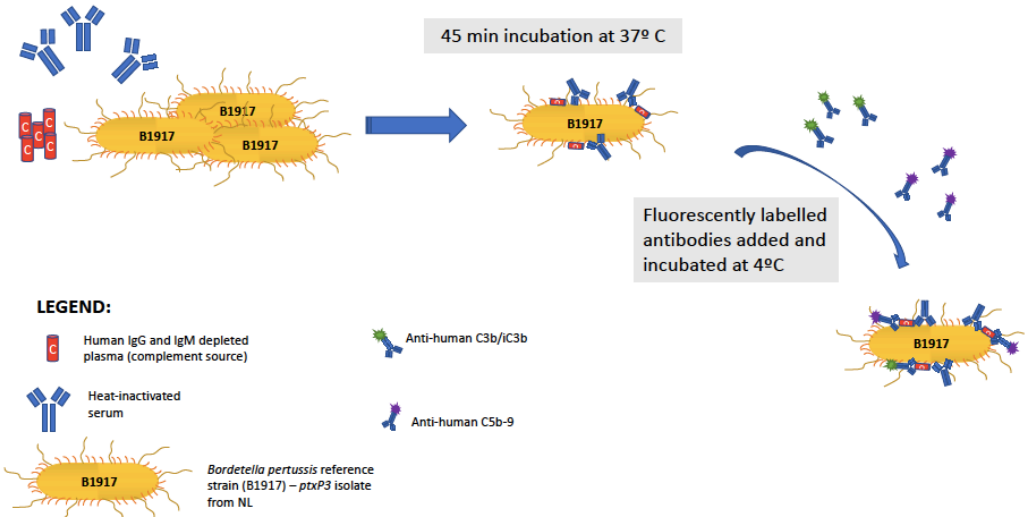


Figure 2.3 Schematic description of the steps to perform the complement deposition assay

The flow-cytometry analysis of the samples from one of the studies (**Infants_African_wP)** showed an unusual staining pattern, suspected to be due to contamination of the sera. A full description of the evaluation of this finding is described in Supplementary data from Chapter 2.

2.3.3.4 Bacterial adherence assay (BAI)

Background

It is expected that the presence of mucosal antibodies prevents the attachment of pathogens, in this case, *B. pertussis* to the respiratory epithelium and prevents the initiation of the colonisation and infection process [254].

A novel high-throughput BAI for *B. pertussis* was first described by *Zanaboni et al* [254]. This assay aimed to understand the role of pertussis specific antibodies in the adherence of labelled *B. pertussis* to a respiratory cell line, by quantifying that adherence measured by fluorescence [254]. BAI was applied to a set of samples from

the biobank studies (Infants_African_wP, Infants_African_aP, Children_UK_wP and Adolescent_UK_wP) and the samples selected from different studies were randomised all together.

Methods

Before the performance of actual assay, two fundamental steps needed to be completed: labelling of *B. pertussis* and culture of A549 cells.

B. pertussis strain B1917, a strain representative of the ptxP3-ptxA1 lineage [48,255], were plated in warm selective Oxoid X10 Agar plates (Thermo Fisher Scientific, Waltham, MA, USA) after being thawed from frozen vials and incubated at 37°C during 3 to 4 days to allow growth. After the incubation period, the bacteria were collected, measured at an optical density of 600 (OD_{600}) and suspended into a Thalen-IJssel (THIJS) medium for incubation overnight at 37°C in a shaking incubator. After incubation, the bacterial solution had serial measures at OD_{600} and the B1917 resuspend in PBS (Lonza, Basel, Switzerland) to achieve a final OD_{600} of 0.5. To label the B1917, the solution previously prepared was incubated for 30 minutes at room temperature with 1M $NaHCO_3$ (Sigma-Aldrich, St Louis, MO, USA) and 10mg/ml of OregonGreen 488 Carboxylic Acid Succinimidyl Ester, 6 isome (Molecular Probes, Life Technologies, Carlsbad, CA, USA). The B1917 solution was centrifuged and resuspended first in PBS and secondly in a PBS/10% DMSO (Merck, Darmstadt, Germany) to a final solution at an OD_{600} of 4 for storage at -80 °C. Before storage the labelling of the bacteria was checked in TECAN Spark 10M reader (Tecan, Mannedorf, Switzerland).

In this assay cell line A549 (ATCC®, Manassas, VA, USA) derived from human epithelial alveolar basal adenocarcinoma and representative of alveolar type II

pneumocytes in the human lung was used [256]. These adherent cells are able to produce a monolayer.

A549 cells were maintained in DMEM (Invitrogen, Carlsbad, CA, USA) /10% FCS (Greiner Bio-one, Kremsmunster, Austria) medium supplemented with antibiotics (Penicillin- Streptomycin) in a cell culture flask, incubated at 37 °C and 5% of CO_2 and seeded for use in the assay when achieved around 80% confluency. Cells were washed and resuspended in DMEM/10%/10%FCS to a concentration of $2.5x10^5$ cells/ml and plated in a black 96 well with flat bottom (BD Falcon, Franklin Lakes, NJ, USA) at a concentration of $2.5x10^4$ cells/ml per well. The plates were incubated overnight at a temperature of 37°C and 5% of CO_2 to be used in the next day under the condition that the monolayer covered the totality of the bottom of the well.

The first step of the BAI assay was to defrost the bacteria previously labelled.

Fluorescence intensity of the defrosted bacteria after being serial diluted was measured with TECAN Spark 10M reader to determine the gain (90% of maximum signal) to be used at the final read out.

The pre-labelled bacteria were diluted to a 1:20 concentration with DMEM/1% FCS and 55µl added to each well of a round bottom 96 well plate.

In a separate plate, participant samples and a control sample (NIBS 06/140) were serial diluted eleven times in DMEM/1% FCS, with maximum of seven samples and a control sample per plate. Each sample was previously heat inactivated (56°C, for 30 minutes).

After serial diluting samples, 55µl of each dilution of each participant sample was added to the pre-labelled bacteria plate. For each participant sample, a well with labelled bacteria with medium without any serum was used to determine the maximum bacterial adherence and fluorescence. The plate was incubated in the dark, for one

hour at 225rpm at 37°C. After the incubation period, 100µl of each well were transferred to the A549 cell plate after the cells were washed with PBS and all solution was removed. The plate was centrifuged for 5 minutes (500g) and incubated for one hour at 37°C and 5% of CO_2. After incubation period the wells were washed three times with PBS. For measurement of fluorescence, 100µl of PBS were added to the dried wells and the plate read with the TECAN Spark 10M reader, using 485/530 filters for Alexa488 (Figure 2.4).

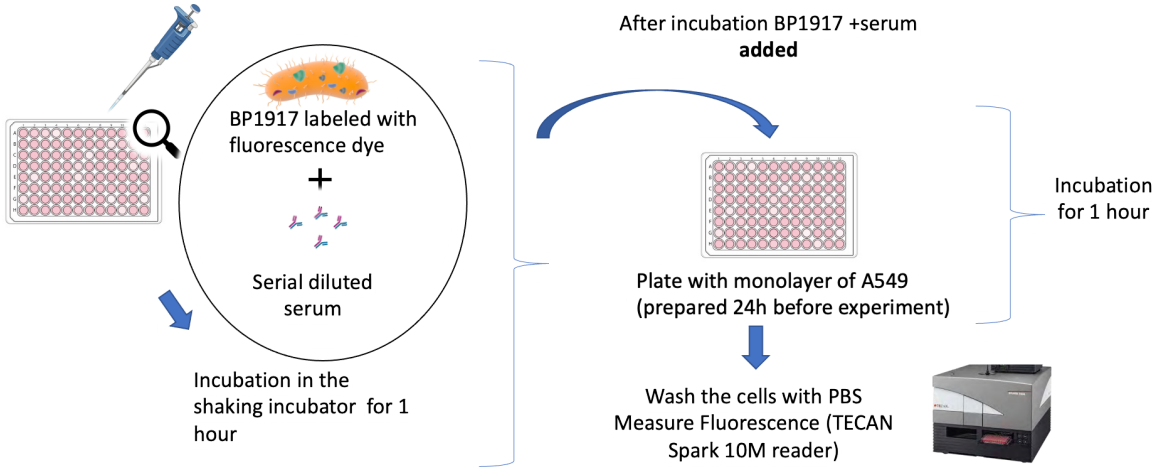


Figure 2.4 Summary of the steps to perform the bacterial adherence assay

Legend: BP1917: *B. pertussis* 1917 strain; PBS: Phosphate buffered saline

After reading the samples in the TECAN Spark 10M reader, it was calculated the half maximal effective concentration (EC50) of each sample, using a four-parameter logistic curve (4PL) calculated in GraphPad® (version 9), using the following formula:

Y=Bottom + (Top-Bottom)/(1+10^((LogEC50-X)*HillSlope))

The "HillSlope" represented the steepness of the curve and the "Top" and "Bottom" the plateaus of the units of the Y axis. When the EC50 were calculated prior to the analysis of these results in different populations, in a group of samples from

each group was not possible to determine with accuracy the EC50 of those samples, since a higher number of serial dilutions needed to fit the curve (Table 2.3). For the effect of this thesis only the samples where it was possible to calculate EC50 without ambiguity were included for final analysis.

Table 2.3 Summary of the studies used to perform the BAI assay and results of the calculations of the EC50 using the pre-determined assay serial dilutions

Study	N	Ambiguous EC50	Not converged	R squared >0.7
Infants_African_wP	94	24 (25.5%)	1	79 (84%)
Infants_African_aP (6 month of age)	54	33 (61.1%)	1	34 (63%)
Infants_African_aP (12 month of age)	67	15 (22.4%)	0	67 (100%)
Children_UK_wP	41	12 (29.2%)	0	33 (80%)
Adolescent_UK_wP	63	14 (22%)	0	50% (79.3%)
NIBS serum (positive control)	52	9 (17%)	0	50 (96.2%)

Legend: EC50: half maximum effective concentration

2.3.4 ELISpot assay: *ex-vivo* and MBCs

Background

The ELISpot assay is a sensitive immunoassay that enumerates ASC, quantified by the binding of a specific antibody produced by a single cell to specific antigens on a coated membrane plate. This binding is revealed after a secondary antibody conjugates with an enzyme, binds to the antibody of interest and reacts with

a substract that precipitates after a chemical reaction and forms a visible, coloured spot [257].

Plasma cells frequencies seven days after booster immunisation (*ex-vivo* ELISpot) allowed to estimate the magnitude of the immediate immune response. MBCs allowed to determine long-term responses to booster immunisations and the kinetics of those responses.

Methods

To perform the ELISpot assays, 96 wells ELISpot plates (Millipore, Cork, Ireland) were pre-treated with 70% ethanol before coating in order to transform the porous hydrophobic structure of the membrane in to hydrophilic, allowing a better capture of the coating antigens. The plates were coated with 50μl of 10μg/ml PT (GSK, Brentford, Middlesex, UK); 5μg/ml of FHA (Sanofi-Pasteur, Lyon, France); 5μg/ml of Prn (GSK, Brentford, Middlesex, UK); 2.5μg/ml of FIM 2/3 (Sanofi-Pasteur; Lyon, France); 3.5 μg/ml of TTx (GSK, Brentford, Middlesex, UK) and 10μl/ml of goat-anti human IgG (Alkaline Phosphatase (AP) anti-goat anti-human IgG, Southern Biotech, Birmingham, USA). For the negative control (blanc wells) 50μl of PBS (Gibco, Life Technology Corporation, Grand Island, NE, USA) were used. Plates were sealed and incubated overnight or for a maximum period of five days at a temperature of 4°C in a box with wet tissue to minimise dryness of the wells.

For the *ex-vivo* ELISpot, fresh PBMC's isolated from whole blood at D7 post-booster (described in section 2.2.1.5) were directly plated for the ELISpot assay. Details of ELISpot assay optimisation are presented in Supplementary data from Chapter 2.

For the MBCs ELISpot, we used frozen PBMC's that were previously isolated at D0, D28 and D365 after booster immunisation. As a rule, cells of the three time-points of each participant, were plated in the same ELISpot plate (Supplementary figure 4). To try to mitigate potential batch to batch variation, participants ID were randomised to determine the order of the assay. To avoid further delays on the completion of this assay, the randomisation was done for the paediatric cohorts separately and the adults' cohorts, considering that Cohort C and D completed the study earlier. Despite this fact, halfway through the assay, an overlap between paediatric and adults' samples occurred, minimising potential batch to batch variation based on age.

The frozen PBMC's were first cultured and stimulated to induce the proliferation and differentiation of a small number of resting MBCs in ASC (plasmablasts). First, frozen PBMC were thawed using a thawing media (TM) composed by RPMI 1640 with GlutaMax with HEPES (Gibco, Life Technology Corporation, Grand Island, NE, USA), 10% fetal bovine serum heat inactivated (Sigma Aldrich, St. Louis, MO, USA), penicillin/streptomycin (Sigma Aldrich, St. Louis, MO, USA), essential medium non-essential amino acids 100x (Gibco, Life Technology Corporation, Grand Island, NE, USA), sodium pyruvate 100mM (Gibco, Life Technology Corporation, Grand Island, NE, USA) and 2-mercapto-ethanol 50mM (Gibco, Life Technology Corporation, Grand Island, NE, USA) to which benzonase nuclease was added (Millipore Corporation, Hyward, CA USA). Two centrifuging cycles (400g for 10 minutes) were performed before resuspending the cells in complete medium (CM) for cell count using the Muse™ cell analyser (Millipore Corporation, Hyward, CA, USA, currently designated Luminex Corporation, Austin, TX, USA) and the Muse® count and viability kit (Luminex Corporation, Austin, TX, USA). The CM was composed by AIM V albumax,

gentamycin, streptomycin and L-glutamine (Gibco, Life Technology Corporation, Grand Island, NE, USA), 10% fetal bovine serum heat inactivated (Sigma Aldrich, St. Louis, MO, USA) and 2-mercapto-ethanol 50mM (Gibco, Life Technology Corporation, Grand Island, NE, USA).

After counting, cells were resuspended in CM to a concentration of $2x10^6$ cells/ml. Cells were plated at a concentration of $2x10^5$ cells/well and polyclonal stimulated with a mixture composed by CM, *Staphylococcus aureus* Cowan strain (CPG – Invivogen, San Diego, CA, USA) 1mg/ml at a dilution of 1:166.7; RhIL-2 100 µg/ml at a dilution of 1:5000 (R&D Systems, Minneapolis, MN, USA) and RhIL-10 100 µg/ml at a dilution of 1:5000 (BD Biosciences, San Jose, CA, USA) and incubated at 37°C in 5% carbon dioxide and 100% humidity for five days.

After culture, the cells were collected from the plate and centrifuged at 400g for 10 minutes, resuspended in CM for a second washing step (400g for 10 minutes). The cell pellet was resuspended in CM for cell counting using Muse™ cell analyser.

The ELISpot assay methodology was the same for MBCs and *ex-vivo* ELISpot (Figure 2.5).

Before adding the cells to the ELISpot plate, the wells were blocked two hours before platting the cells with CM. By the use of a medium containing protein, free sites of membrane surface after coating are blocked, reducing background because of non-specific binding [257].

To the pre-coated, blocked plates, 100µl of cells at a concentration of $2x10^6$ cells/ml were added to the neat wells. Several dilutions with CM to achieve cell concentrations according to plate layout, were performed (Supplementary figure 4). The ELISpot plate was incubated overnight at 37°C, 5% carbon dioxide and 100% humidity in a box with wet tissue. In the next day, cells were washed from the plate

using washing buffer composed by PBS (Gibco, Life Technology Corporation, Grand Island, NE, USA) and 0.05% Tween 20 (Sigma-Aldrich, St. Louis, MO, USA). To allow the lysis of potential remaining cells, distilled water (Baxter, Deerfield, IL, USA) was added for five minutes to the plate before the last washes. After the last wash, 50µl of conjugate buffer composed by PBS, goat serum (LCN biomedicals, Stevenage, UK), Alkaline phosphatase (ALP) anti-goat anti human IgG (ex-vivo: Sera Care Life Sciences, Milford, CT, USA; MBCs ELISpot: Calbiochem®, Sigma-Aldrich, St Louis, MO, USA) and Tween 20 was added to each well, allowing a secondary antibody to attach to the antigen-antibody specific complex previously formed. The plate was incubated during two hours at 37°C, 5% carbon dioxide and 100% humidity in a box with wet tissue. After the incubation period, the wells were firstly washed four times with washing buffer and secondly three times with PBS, before adding 5-bromo-4-chloro-3-indolyl phosphate (BCIP) (Sigma-Aldrich, St Louis, MO, USA), a colorimetric substrate used for the detection of ALP labelled substances. The final step of the ELISpot was the enzymatic reaction between the ALP in the secondary antibody and BCIP, revealing the antigen-antibody binding in a dark-blue indigo colour spot. The enzymatic reaction was stopped with PBS and tap water and the plate dried overnight. Plates were read in automated ELISpot reader (Advance Imaging Devices GmbH, Straßberg, Germany) after specific settings were pre-defined for each specific antigen.

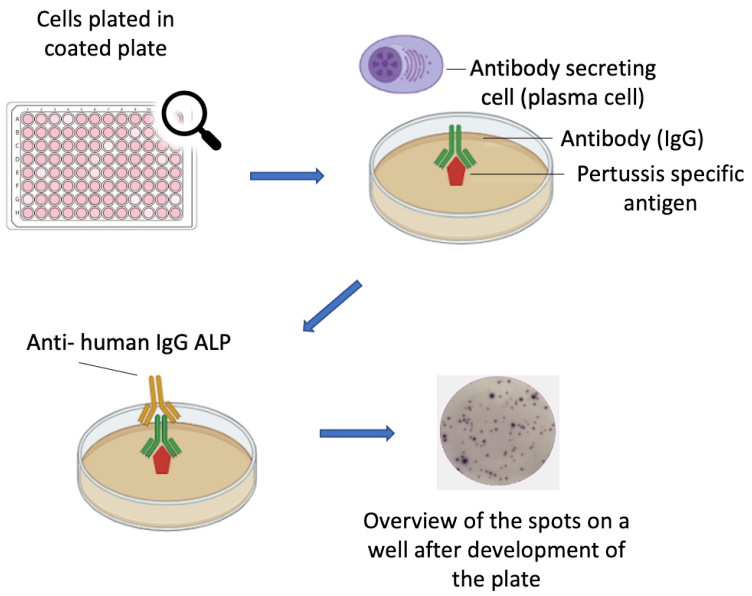


Figure 2.5 Schematic figure of BERT ELISpot assay

Legend: ALP: Alkaline Phosphatase

2.3.5 Rapid whole blood T cell assay

Background

Previous animal models have shown that $CD4^+$ T lymphocytes have a relevant role in protection against pertussis disease and colonisation [197,202,258]. Insights of the animal models, particularly in the baboon models, showed that wP induced a type of Th1/Th17 responses, that stimulated the secretion of IFN-γ and IL-2, and aP induced a mixed Th1/Th2 response [194,197]. Those findings were also described in adults recently boosted with an aP, but there is still a gap of knowledge on the behaviour of $CD4^+$ T lymphocytes in paediatric populations [259].

A whole blood T cell assay was developed as part of the work package 5A, task 5.7 and 5.12 of the Periscope project. This assay allowed the detection of specific T cell cytokines produced against *B. pertussis* antigens. At the original assay two read-outs were performed immediately after harvesting the supernatant and the cells after

24 hours of whole blood culture, without any intermediate freezing step, and all performed in the same assay timing [260]. The lack of available facilities, qualified researchers for this time-consuming technique in a regular basis, increased the need to develop a simpler technique, that could be performed for large sample sets and by all research assistants.

The variation of the whole blood T cell assay was the rapid whole blood T cell assay. The initial objectives of the pilot study were to determine the best assay conditions and timepoints, including the duration of the culture, need to use co-stimulants, minimal panel of cytokines to test and ideal day to study the effect of booster immunisation.

BERT study participant whole blood samples, from Oxford and Finland (Turku) were used for the optimisation process. In this section, the steps of the optimisation process performed at OVG laboratory with BERT UK samples are described. The samples were selected using a convenience method, based on the participants that were booked for the days that the assay was set up and with enough blood volume to allow all other programmed assays. The laboratory testing of the supernatants was performed in Sanofi Pasteur's facilities in France by Pascal Blanc.

Methods

Fresh whole blood collected at the Heparin-Na tube was transferred to a falcon tube and diluted 1:4 and/or 1:2 with RPMI-1640 with GlutaMax medium (Gibco, Life Technology Corporation, Grand Island, NE, USA) with penicillin-streptomycin (Sigma Aldrich, St. Louis, MO, USA) and 10% FBS (Sigma Aldrich, St. Louis, MO, USA). In a separate plate, pertussis specific antigens were diluted in the RPMI medium to a final concentration of 5 μg/ml PT mutant (List Biological laboratories, Campbell, CA, USA)

5µg/ml FHA (Sanofi Pasteur, Lyon, France) and 10µg/ml of Bp lysate used after HI at 80°C for 10 minutes (Quality Biological, Gaithersburg, MD, USA).

Co-stimulation was a mixture of 1µg/ml purified Mouse Anti-Human CD28 (clone L293) (BD Biosciences, San Jose, CA, USA) and 1µg/ml purified Mouse Anti-Human CD49d (clone L25) (BD Biosciences, San Jose, CA, USA) in RPMI medium.

To a 48 wells cell culture plate, 50µl (1:2 blood dilution) or 100µl (1:4 blood dilution) of co-stimulant or RPMI medium was added, followed by the same volume of the antigen of interest and by the diluted blood (1:2: 300µl and 1:4: 600µl). For each condition, a negative control well (mock) was present where the antigen of interest was replaced by RPMI medium.

The plates were incubated at 37°C and 5% of CO_2, for a period of 24, 48 or 72 hours. After the incubation period, the plates were centrifuged at 500g for 10 minutes and supernatants collected to individual vials in micronic racks (Micronic, Greiner Bio-one, Kremsmunster, Austria) and stored at -80°C until further analysis. The supernatants were analysed at Sanofi Pasteur facilities in France (Lyon), using a customised 'Human Th-cell lineage indicator cytokine kit (Luminex® Millipore Corporation, Hayward, CA, USA) for multiplexed measurement of human cytokines. After a first analysis of the Turku samples, with a total of 15 cytokines tested, it was determined that the final cytokine panel to be used in the UK samples was IL-17A, IL-17F, IFN-γ, IL-13, IL-5, IL-10 and IL-2. The concentration of each cytokine per supernatant was determined based on the interpolation of the standard curve of the kit in pg/ml. After receiving the results from Sanofi Pasteur, the author performed the final statistical analysis.

2.4 STATISTICAL ANALYSIS

In the BERT study a descriptive analysis of the demographic data of the study participants was performed, including evaluation of sex, age at recruitment and previous pertussis immunisation information, including total number of vaccine doses and time since the last immunisation at time of recruitment.

For the evaluation of immunogenicity geometric mean concentrations (GMC) and 95% CI were calculated of pertussis specific antibodies (PT, FHA, Prn, FIM 2/3 IgG and IgA) at different time points, D0, D28 and D365 post-booster for all individuals and also D7 and D14 for a sub-group of individuals. GMC were calculated using *ameans* in Stata 16.1 version. This statistical test allows calculation of the GMC by calculating the exponential of the mean of the log transformed of antibody levels measurements. To evaluate the magnitude of increase of antibody levels median fold change (FC) and interquartile (IQR) range was determined between D0 and D28, D0 and D365 and D28 and D365. In the subgroups that D7 and D14 antibody levels were determined, median FC (IQR) was also calculated between D0 and those two timepoints.

MBCs frequency was adjusted to and expressed as ASC/1x10^5 PBMC. MBCs geometric mean (GM) frequencies, 95% CI were calculated using the same methods as for immunogenicity.

For the BERT study a mixed effect model was used to explore the effect of specific covariables (fixed effects) in the outcome variables like pertussis specific antibody levels (PT IgG and IgA, FHA IgG and IgA, Prn IgG and IgA, FIM 2/3 IgG and IgA) and cellular data (MBCs). This analysis was performed unadjusted and adjusted to gender (male and female). For this evaluation the outcome variables were log 10 transformed before added to the model. The fixed effects determined for the model

were the study cohort (A, B, C and D), representing different age groups (children: 7-10 years of age; adolescents: 11-15 years of age; adults: 20-35 years of age and elderly: 60-70 years of age) and different immunisation backgrounds (cohort A and B: aP, cohort C: wP, cohort D: wP/natural infection) and visit timepoints (D0, D28 and D365 after pertussis booster immunisation). The random effect was the participant study identification number (ID). An analysis using as fixed effect primary immunisation background only (Cohorts A and B: aP primed and Cohort C and D: wP primed/natural infection) was also performed. The p values reported were 2-sided.

SBA GMTs (95% CI) were calculated using the same method described previously and median FC (IQR) were also calculated.

OPA, CDA, BAI and plasma cells medians and IQR were calculated for the available timepoints (D0 and D28 post-booster) and to determine if statistically significant differences were identified a non-parametric Wilcoxon paired rank test was performed. Kruskal-Wallis was used to compare the differences of four cohorts (A, B, C and D) for the same assay.

To compare the antibody levels between the BERT populations and the wP primed biobank UK population (Children_UK_wP and Adolescent_UK_wP-wP) an unpaired Welch's t-test was performed.

Before determining correlation between antibody levels of different sub-classes (IgA and IgG) and antibody functionality, MBCs, plasma cells at different timepoints, data results were log transformed (log10). Correlation was determined using non-parametric spearman correlation coefficient, rho values and p values reported, and correlations represented graphically. The evaluation of these correlations was performed in the overall population, per cohort and per immunisation background, with

cohort A and B being analysed together and cohorts C and D, representing wP priming or natural infection, being analysed together.

Regarding the T cell whole blood assay, a descriptive analysis of the cytokines IL-17A, IL-17F, IFN-γ, IL-13, IL-5, IL-10 and IL-2 produced against PT and FHA was performed, in Stata 16.1 version. Median and IQR were evaluated, and the non-parametric Wilcoxon paired rank test was used to compare the cytokine levels from the same participant at the same timepoint with different incubation periods (48h and 72h), to determine if statistically significant differences were identified a p value of 0.05 was considered statistically significant. The analysis and graphs were performed using STATA 16.1/SE and Prism 8.

The same methods were applied to evaluate the immunogenicity and functional antibody assays results, including PT IgG avidity of the biobank studies.

For all the analysis a p-value of 0.05 was considered statistically significant. The analysis and graphs were performed using STATA 13.1 and 16.1/SE and Prism 8. The figures were produced in combination using Microsoft® power point 2021 and Biorender at https://biorender.com/.

B. REACTOGENICITY STUDIES

2.5 GENERAL DESCRIPTION OF THE STUDIES DESIGN AND STUDIES PARTICIPANTS

The wP is considered relatively reactogenic and in the 1970's was associated with a decrease in vaccine acceptance leading to the switch to aP in high income countries [109,113,159,261].

It was originally planned to study the reactogenicity of wP versus aP, but this was not possible within the time frame of the DPhil. For the purpose of this thesis, in order to evaluate fever rates and dynamics after immunisation using a continuous temperature monitoring method (iButton®), the author chose a vaccine that is currently used in the UK and also considered to be highly reactogenic: 4-component capsular group B meningococcal vaccine (4CMenB).

Two infant randomised clinical trials were selected, both performed at OVG:

EUCLIDS study [1,2]: This study, entitled "*Towards improved meningococcal vaccines: a randomised, descriptive, open label study, exploring the relationship between gene expression signatures with reactogenicity and immunogenicity following vaccination with serogroup B meningococcal vaccine (4CMenB)*" (REC reference: 14/SC/0077) was funded by European Commission FP7 grant.

This was a single-centre, open label clinical trial that recruited healthy Caucasian infants' aged 8 to 12 weeks. The infants were randomised in two groups, and then allocated to one of four sub-groups. The sub-groups were only relevant for the primary endpoint of the study (kinetics of global gene expression in whole blood in this population) and not an object of this analysis.

The study design and timings of vaccine administration and temperature collection is described in Figure 2.6. Temperature using an axillary thermometer was measured after each immunisation visit at four- and eight-hours post-vaccination on the first day and once a day in the following six days or on demand.

The measured values and time of temperature collection were recorded in electronic diary by the parents. The iButton® was only applied at the four- and 12-months immunisations.

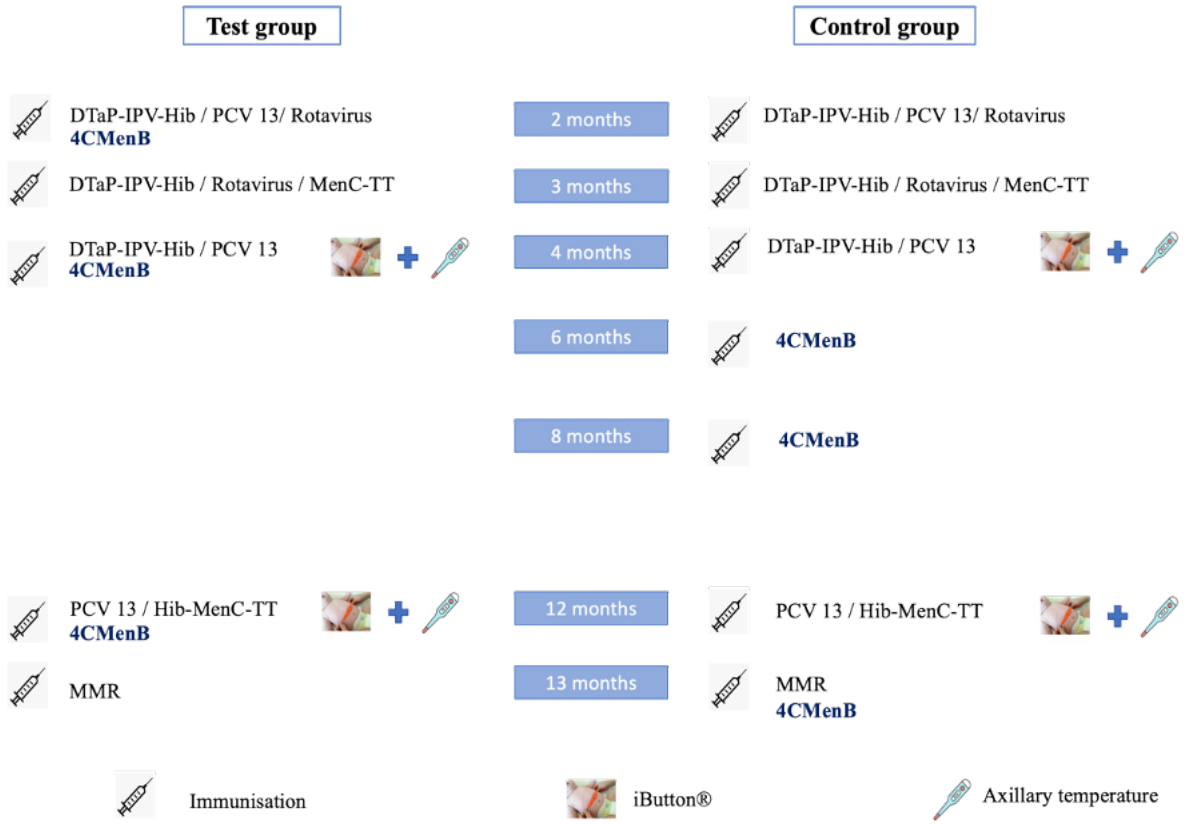


Figure 2.6 EUCLIDS study immunisation schedule per group and temperature measurement timepoints

Legend: 4CMenB – 4-component Capsular Group B Meningococcal vaccine (Bexsero® GlaxoSmithKline); DTaP-IPV-Hib – Diphtheria, tetanus, acellular pertussis, inactivated polio vaccine and *Haemophilus influenza* type B vaccine (Pediacel®, Sanofi Pasteur); Hib-MenC-TT - *Haemophilus influenza* type B, Meningococcal Capsular Group C tetanus toxoid vaccine (Menitorix®, GlaxoSmithKline); MenC-TT – Meningococcal group C tetanus toxoid vaccine (NeisVac-C®, Baxter Vaccines); MMR – Mumps, measles and rubella (Priorix®, GlaxoSmithKline); PCV 13 – Pneumococcal conjugated vaccine 13 (Prevenar-13®, Pfizer); Rotavirus vaccine (Rotarix®, GlaxoSmithKline).

Paracetamol and ibuprofen were given on demand, whenever the infants had fever or pain/discomfort.

Sched 3 study [3,262]: The sched 3 study ("*Assessment of post booster antibody responses in UK infants given a reduced priming schedule of meningococcal serogroup B and 13 valent pneumococcal conjugate vaccines*"), was a joint collaboration between OVG and PHE funded by the UK Department of Health and Bill and Melinda Gates Foundation. This study was a randomised multicentre clinical trial, open labelled, in infants aged between 7 and 12 weeks of age. The infants were randomly allocated into two groups to receive either 13 valent pneumococcal vaccine (PCV13; Prevenar 13®, Pfizer, New York, NY, USA) according to the UK national immunisation schedule at two, four and 12 months of age or to receive a reduced schedule at three and 12 months (Figure 2.7).

In this population axillary temperature was measured by the parents/guardians for a period of seven days and recorded in a paper diary in all immunisation time-points. It was requested in the first 24 hours to record temperatures at around four and eight, 20 and 24 hours after immunisation and once a day in the following days. At the time of this study, PHE, after advice from the Joint Committee on Vaccination and Immunisation (JCVI), recommended the use of three doses of paracetamol in the first 24 hours, four to six hours apart [263,264]. In this study the recommended paracetamol doses were not recorded by the parents and it was assumed by the study investigators that they were administered.

Continuous temperature measurements were recorded at all immunisation timepoints.

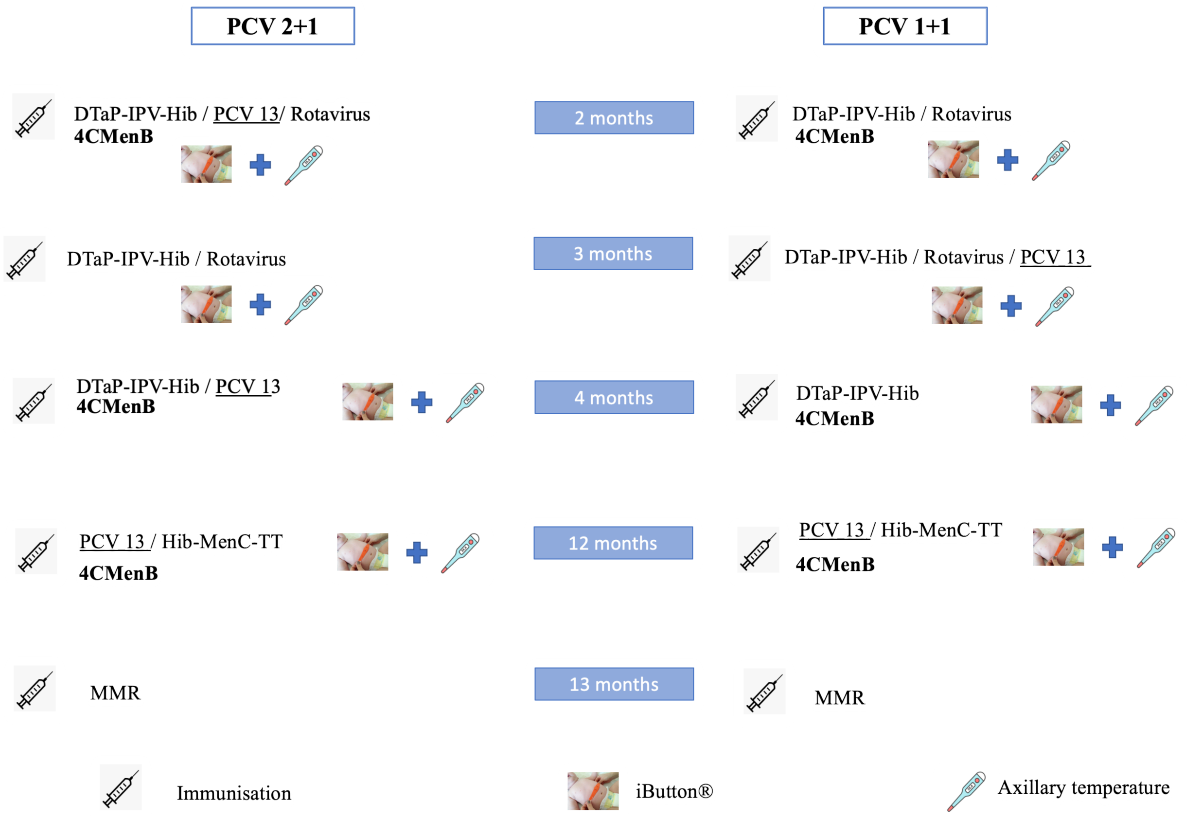


Figure 2.7 Sched 3 study immunisation schedule per group and temperature measurement timepoints

Legend: 4CMenB – 4-component Capsular Group B Meningococcal vaccine (Bexsero® GlaxoSmithKline); DTaP-IPV-Hib – Diphtheria, tetanus, acellular pertussis, inactivated polio vaccine and *Haemophilus influenza* type B vaccine (Infanrix-IPV-Hib®, Sanofi Pasteur); Hib-MenC-TT - *Haemophilus influenza* type B, Meningococcal Capsular Group C tetanus toxoid vaccine (Menitorix®, GlaxoSmithKline); MMR – Mumps, measles and rubella (MMRVAXPRO®, MSD vaccines); PCV 13 – Pneumococcal conjugated vaccine 13 (Prevenar-13®, Pfizer); Rotavirus vaccine (Rotarix®, GlaxoSmithKline).

Reactogenicity studies vaccine:

Each dose 4CMenC vaccine [265] was composed by 50µg of recombinant Men B NHBA fusion protein, 50µg of recombinant Men B NadA protein, 50µg of

recombinant Men B fHBp fusion protein and 25µg of OMV from Men B NZ98/254 strain (total amount of PorA P1.4), absorbed on 0.5mg aluminium hydroxide.

2.6 IBUTTON®: DEVICE DESCRIPTION AND RELATED STUDY PROCEDURES

Background

The standard methods to measure temperature after immunisation are the intermittent methods, like axillary temperature, with pre-defined timings by the investigators. Those have the potential to miss fever episodes and not to capture the dynamics of those episodes. Reactogenicity can have implications in vaccine acceptance and consequently vaccine coverage [266-268].

Methods

The continuous temperature method was applied simultaneously with a conventional method (axillary temperature).

The device of choice for continuous temperature monitoring was the Thermochron iButton® DS1921H (Maxim Integrated Products, Inc, San Jose, CA), which had been previously used in other human studies, for purpose of validation and comparison with other methods [269-272] or with clinical applications including circadian rhythm, medical conditions and surgical procedures [273-275].

The iButton® DS1921H is a small wireless device that was designed to allow the measurement of temperatures within the human range (+15°C to +46°C), in a non-invasive way and has the ability to record and storage the temperatures in a memory section for posterior downloading [270,272,276]. The general functional

characteristics are: the device dimensions are maximum diameter of 17.35 mm and a depth of 5.89 mm with 1-Wire transmitter/receiver; accuracy of ±1°C and a resolution of 0.125°C; temperatures can be collected in a sample rate between 1 minute to 255 minutes apart, for a maximum total of 2048 minutes or temperature values, with a lithium battery and a life shelf of around five years, but conditional to use and conditions of storage [276]. The device manufactured in a stainless steel package resistance to the environmental hazards. In a study from *van Marken Linchtenbelt et al* it was shown that the average time that the iButton® took to detect a change in temperature (iButton® response time) was 19 seconds [272].

In both studies, the iButton® was applied on the infant abdomen, secured by a latex free bandage (Figure 2.8) immediately after immunisation at the specific timepoints. The device was programmed by the study team to collect one temperature per minute and the parents were instructed to remove the device 24 hours after immunisation procedure. A diary chart was given to parents to record the periods that the iButton® was removed and why, and if any events were identified, like misplacement of the device or an allergic reaction. During this period the parents were asked to record axillary temperatures in pre-defined timings and on demand in case the infant had any suggestive manifestation of fever.

Due to the small dimensions of the device, to reduce the risk of choking or other hazards, the iButton® was mounted and secured in a structure similar to a wrist band with a lock ring (DS9093RA) [276] (Figure 2.8).

For effect of the analysis, temperatures recorded below 35°C were considered non-physiological and were excluded from the analysis. Fever was defined as at least one temperature ≥38°C recorded by any of the used devices, as described by the Brighton Collaboration Fever working group [277].

A



iButton® device and security strap

B



Application of the iButton® on the infant abdomen with latex free elastic bandage

Figure 2.8 iButton®: characteristics of device and application on the infant abdomen

2.7 STATISTICAL ANALYSIS

A descriptive and comparative analysis of intermittent temperatures and the iButton® temperature data in the first 24 hours after immunisation was performed. A comparison between the fever rates in different study groups was performed using a Chi-squared test and two-sided t-test. Time for the first fever, defined as the first episode of temperature ≥38°C identified by the iButton® was calculated by a Kaplan-Meier survival analysis. For this analysis the statistical significance differences between study groups were calculated using a log-rank test and represented by failure curves.

Differences between the time for the first fever between infants that had at least a fever episode were calculated after normalisation (log10) using a one-way repeated measures ANOVA. To determine if the temperature measurements between the two used methods (continuous and intermittent) were concordant, the exact time (hours and minutes) of the axillary temperature that was recorded by the parents was

compared with the temperature recorded at the same time by the iButton®. This concordance correlation was calculated using the Lin coefficient and represented by Bland-Altman plots.

The data were analysed in STATA version 13.1.

CHAPTER 3

SEROLOGIC RESPONSES TO PERTUSSIS IN DIFFERENT AGE GROUPS AND WITH DIFFERENT IMMUNISATION BACKGROUNDS

3.1 BACKGROUND

Routine immunisation programmes have been implemented worldwide to prevent *B. pertussis* infections, but despite high vaccination coverages the bacterium continues to circulate and in vulnerable groups can cause severe disease [278]. The current implemented pertussis schedules are different around the world and the number of booster doses and timings of administration are highly variable [163].

A meta-analysis of 17 studies in adolescents and adults, showed that a booster dose of aP was associated with an increase in the pertussis specific antibody levels from baseline to D28 and D365 after immunisation, with protective rates of 88.9% [279]. The pertussis specific antibody with the highest increase since baseline to one-month post-immunisation was FHA IgG followed by Prn IgG. A waning of antibody levels from D28 post-immunisation to D365 was identified in all evaluated trials [279]. This strategy, when considering both adolescents and older groups was considered to be break-even in costs, but particularly cost-effective if applied only to the adolescent population, preventing in the USA an estimated maximum of 1.8 million cases [280]. In a study by *Jahnmatz et al*, that evaluated the effect of a fifth dose of an aP in an adolescent population, an increase in antibody levels after a booster dose was identified with association to evidence of long-term protection, represented by the increase in MBCs frequencies [281]. The pre-school booster vaccine was associated

with protection, but that protection can be limited in time. In a study by *Wang et al,* after more than seven years since the last booster dose, the risk of pertussis disease was more than three times when compared with individuals vaccinated before that period [282].

There is a lack of studies aiming to directly compare individuals from different age groups and with different immunisation backgrounds to determine the differences of the effect of a booster aP in serologic responses and antibody quality before and after a controlled intervention. To evaluate those differences, we need to rely on studies focusing on the differences of the serologic responses after natural infection in different age groups. A Dutch study using a bi-exponential decay model, evaluated the antibody responses and their decay after pertussis disease in individuals from infants to elderly, in a total of 289 cases [283]. The antibody that waned quickly in all age groups was PT IgG, but no effect of age on antibody levels or antibody waning was identified in general for IgG pertussis specific antibodies. The effect of age was described for IgA pertussis specific antibodies, with a waning being more significant in younger age groups and less in the elderly, with older age groups showing consistently higher antibody levels [283]. Some of these findings were also previously reported in seroprevalence studies.

Seroprevalence studies are designed to measure specific antibody levels in a selected population. A seroprevalence study from Japan [284], by *Konda et al*, evaluated the levels of PT IgG and FHA IgG in a healthy population aged 0 to 70 years of age in 1994. In this population, individuals aged 45 years or more were assumed not to be immunised. The age range with higher proportion of individuals with positive PT IgG titres (>1EU/ml) were 1 to 4 years old and 16 to 19 years old and for FHA were 9 to 15 years and 3 to 5 years old.

IgA levels increased with age, representing a cumulative exposure to *B. pertussis*, [285], as previously described by *van Twillert et al* [283]. This finding was also described in early studies, when only wP were widely distributed in NIP [286].

Although current studies commonly evaluate the responses to pertussis antigens contained in the aP, the most common selected antigen is PT IgG. The main advantage of PT specific antibodies, is that this antigen is specific for *B. pertussis*, not been shared by other *Bordetella* species as FHA and Prn [22,287]. Also, it is considered by some authors as a key protective element against disease [288], as demonstrated by *Sato et al* mouse model used for intracerebral or aerosol challenge studies with *B. pertussis* [289]. The underlying mechanism of protection is not fully understood.

Other pertussis antigens have been relevant elements for protection against pertussis. According to a study by *Hellwig et al,* Prn IgG was the only antigen able to induce opsonic antibodies, with the anti-Prn IgG antibodies showing a fundamental role in phagocytosis [290].

Understanding antibody functionality is the key to further understand the mechanisms of protection against disease. Complement mediated killing, antibody-dependent is one of the mechanisms of protection against *B. pertussis* infection [291]. The LOS was described to induce bactericidal activity by the production of IgG3 and IgM [291]. Novel functional antibodies assays, like SBA, OPA and surrogate CDA, can help to better explain this mechanism, on exploring how and what pertussis specific antibodies can mediate complement killing and what pathways are involved. A study of opsonophagocytic activity associated with immunisation against pertussis was performed in the early 2000's, showing a significant and strong correlation between OPA and FHA IgG (r=0.79) followed by PT IgG (r=0.68), but the correlations with Prn

IgG were not tested [292]. Another study evaluating serum bactericidal activity in 34 adults after a pertussis booster dose, showed a correlating with Prn IgG [293].

The main advantage and novelty of a study like the BERT is the use of the same vaccine intervention and assays in populations with different ages and different immunisation backgrounds, allowing a direct comparison of their immune responses.

3.2 BERT STUDY: UK STUDY POPULATION

3.2.1 BERT study population and demographic data

A full overview of the BERT study design is described in CHAPTER 2, section 2.2.1.

A total of 131 participants, divided by four cohorts were enrolled in the UK as part of the BERT study. This number was higher than the programmed 122 participants, since according to the study protocol it was possible to recruit extra-participants in case of failed D28 blood collection (primary endpoint) or in case of participant withdraw, if recruitment was still open.

The CONSORT diagram is described in Figure 3.1.

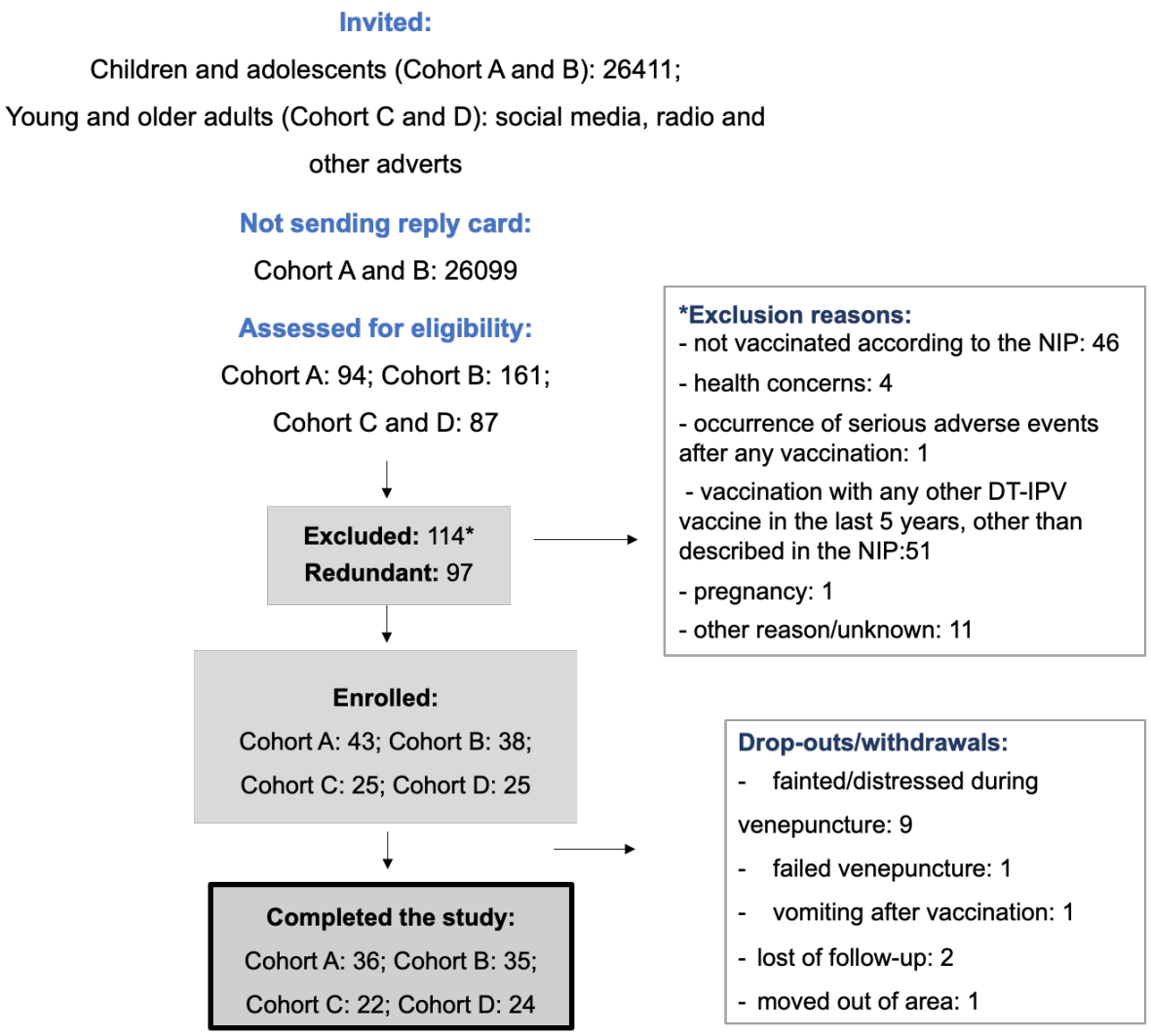


Figure 3.1 CONSORT diagram BERT study-UK

A total of 117 participants, out of the planned 122, had the final study visit, but only 115 blood samples were collected. The primary endpoint visit (D28) was performed in 121 participants (1 participant withdraw) and 119 participants had a successful blood draw. Table 3.1 describes the demographic characteristics and immunisation background of the study participants that were initially recruited for this study.

Detailed information about the immunisation background was available for the majority of individuals from cohort A and B but lacking for cohort C and D. Considering

that only individuals who had received immunisations in the UK were included in the study, the use of the DOB to determine the immunisation background (aP versus wP) was considered to be accurate. In this study the younger cohorts (A and B) were primary immunised with aP and the older cohorts (C and D) were considered to be primary immunised with wP (cohort C or residual number of individuals from cohort D) or did not received any primary immunisations (cohort D).

Table 3.1 Demographic characteristics of the participants of the BERT study

Characteristics		Parameters/ categories	Children Cohort A N: 43	Adolescents Cohort B N:38	Adults Cohort C N:25	Elderly Cohort D N:25
Sex		Male	24 (55.8%)	20 (52.6%)	8 (32.0%)	12 (48.0%)
		Female	19 (44.2%)	18 (47.4%)	17 (68.0%)	13 (52.0%)
Age at recruitment (years)		Median	9.5	12.8	25.8	66.2
		(Min-Max)	(7.4-10.8)	(11.0-14.3)	(20.3-34.5)	(61.1-70.8)
Previous pertussis immunisation (imms)	Primary imms		acellular	acellular	whole cell	None or whole cell
	Total number of pertussis vaccine doses	1 dose	0	0	1	0
		3 doses	3	0	8	1
		4 doses	32	31	1	0
		5 doses	0	1	0	0
		7 doses	0	0	1	0
		Not available	8	6	14	24
	Time since last pertussis imms	Data available (N)	34	31	11	1
		Median	6.3	9.3	21.6	-
		(Min-Max) years	(4.1-9.3)	(7.3-12.0)	(5.4-32.0)	

During the study period two SAE were reported in the same participant (cohort D), although no relation with vaccine administration was considered. The two SAE were: Takotsubo cardiomyopathy and ductal carcinoma in situ in the left breast. The AEs are described in Table 3.2.

Table 3.2 List of adverse events in the BERT study population per cohort

	Total number participants with adverse events (AEs)	AEs probably related to immunisation (imms) (symptoms/diagnosis)	AEs probably not related to imms (symptoms/diagnosis)	Needed medical evaluation
Cohort A **Children**	6	Dizziness (1) Vomiting+Fever (1)	URI (2) Arm fracture (1) Rhinitis/eczema (1)	2
Cohort B **Adolescents**	3	-	URI (2) Vomiting (1)	0
Cohort C **Adults**	7	Pain injection site (4) Swelling injection site (2) Headache (3) Arthralgia (2) Abdominal pain (1) Flu-like symptoms (1)	-	0
Cohort D **Elderly**	8	Pain injection site (2) Flu-like symptoms (2) Sleepiness/fatigue (2) Myalgias (1)	Insect bite (1) Asthma (1) Epistaxis (1)	1

Legend: URI: Upper respiratory infection

3.3 EVALUATION OF IMMUNOGENICITY OF AN ACELLULAR PERTUSSIS VACCINE IN THE BERT POPULATION

3.3.1 IgG pertussis specific antibodies

In the BERT study, for all cohorts, pertussis specific antibody levels (IgA and IgG PT, FHA, Prn and FIM 2/3) were determined at D0.

For all individuals it was determined the antibody levels at D28 and D365 after immunisation. In a sub-group of individuals IgG antibody levels at D7 and D14 after immunisations were also evaluated as the speed of antibody response post-booster dose is a further aspect of immunological memory that may be relevant in terms of preventing disease following infection.

The GMC IgG baseline antibodies levels, except for FIM 2/3 (not included in the vaccine), were lower in all cohorts when compared with post-intervention timepoints. An increase from D0 to D28, with a decay from D28 do D365 after booster was observed (Figure 3.2).

Considering only the antigens contained in the booster immunisation, the pertussis specific antibody concentrations at D0 were generally highest for FHA/Prn and lower for PT IgG, a pattern maintained at D28 and D365 (Figure 3.2).

The results per pertussis specific antigens and interactions between timepoints and groups are presented below. For the purpose of this chapter, the results of the mixed effects models reported are from the analysis adjusted to gender.

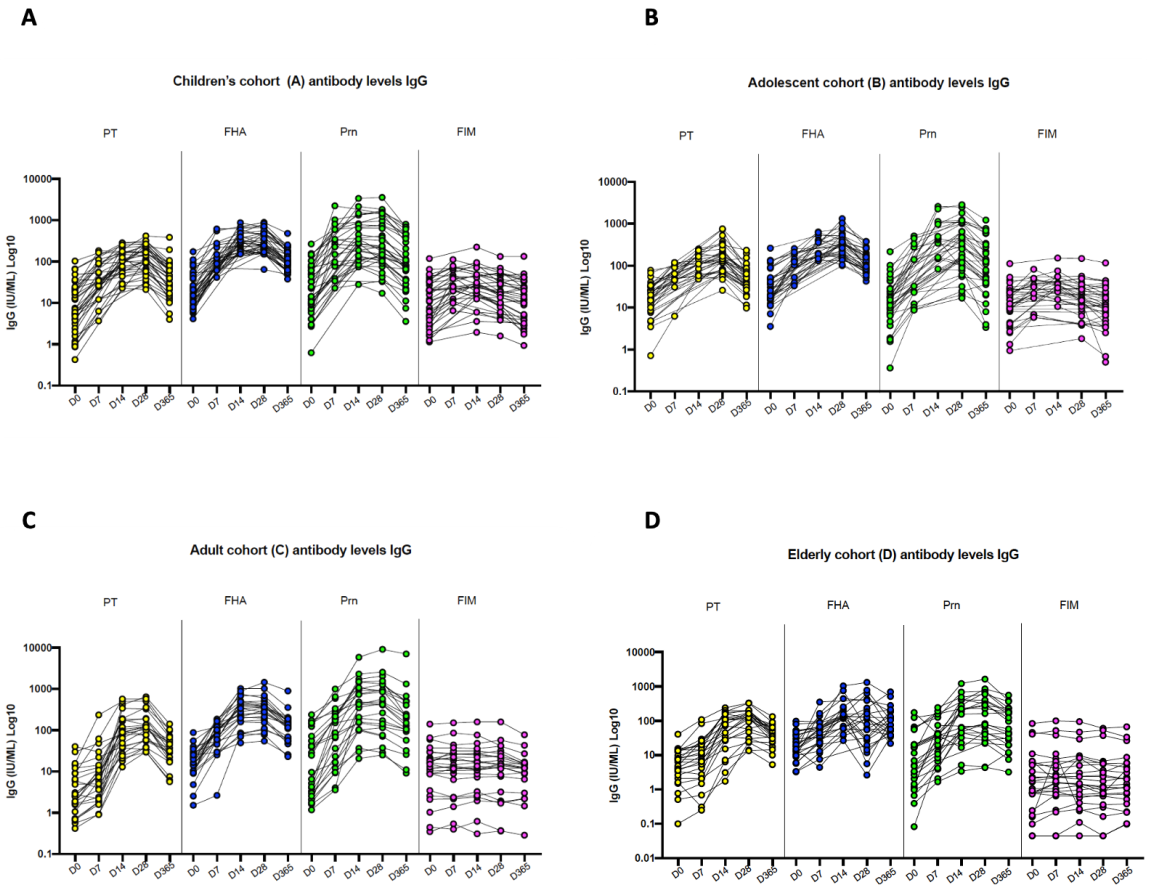


Figure 3.2 Kinetics of pertussis specific IgG antibody levels (PT, FHA, Prn, FIM 2/3) at different timepoints for the BERT study, per cohort (IU/ml)

a. PT IgG

In the older cohorts (C and D), at D0, no individuals had antibody levels over 50 IU/ml, showing potentially no recent contacts with the bacteria. In the younger age groups (A and B), six individuals showed levels above 50 IU/ml.

The PT IgG D0 levels were low for all cohorts (Table 3.3). However, there were differences between cohorts: adolescents had significantly higher levels when compared with other cohorts, but no significance differences were found between the elderly (D) and both adults (C) and children (A).

In a subgroup of the population, antibodies were measured at D7 and D14. At D7, the youngest cohorts (A and B) had a markedly higher increase, when compared with older cohorts (fold change median (FC) and interquartile range [IQR] from D0 to D7: cohort A: 4.1 [2.5-6.3]; cohort B: 3.0 [2.2-4.3]; cohort C: 1.9 [1.4-3.2]; cohort D: 1.5 [1.1-2.2]). Between D0 and D14 this increase was steeper in the adults (FC median [IQR] from D0 to D14: Cohort A: 17.6 [4.9-49.8]; Cohort B: 6.2 [4.5-12.7]; Cohort C: 24.9 [14.8-63.5]; Cohort D: 13.5 [5.9-30.1]) (Figure 3.3).

Twenty-eight days post-booster there was no longer any significant differences between cohorts although there was a trend towards the elderly cohort (D), who had the lowest GMC when compared with adolescents (B) *(p=0.058)* (Table 3.3).

A waning of the antibody levels was seen for all cohorts from D28 to D365 (Table 3.3). Of interest, despite less marked initial antibody responses, this waning was slower for the elderly (D) than the other cohorts.

By D365, whether from better response to boosting (cohort C) or from slower antibody waning (cohort D), there were no longer significant differences between cohorts seen at baseline (Figure 3.3). Overall, the highest FC were seen in the wP primed adult cohort (C) between both D0/D28 and D0/D365 (Figure 3.3).

Table 3.3 Baseline and post-booster pertussis toxin (PT) IgG specific antigen geometric mean concentration (GMC) after vaccination with Boostrix® in the BERT study population and per cohort

Antigen	Timepoint		Cohort A	Cohort B	Cohort C	Cohort D	All cohorts
PT (IgG)	D0	N	36	35	25	25	121
IU/ml		GMC	7.3	16.4	3.2	4.6	7.1
		95% CI	4.5, 11.7	11.9, 22.6	1.9, 5.4	2.7, 7.8	5.5, 9.0
	D7	N	16	12	25	25	78
		GMC	40.6	55.0	6.9	7.2	13.9
		95% CI	22.5, 73.3	32.7, 92.7	3.9, 12.2	3.8, 13.5	9.7, 19.8
	D14	N	19	12	25	21	77
		GMC	92.7	124.9	84.6	47.0	78.3
		95% CI	61.4, 139.9	86.6, 180.2	54.7, 130.8	24.2, 91.0	60.8, 100.9
	D28	N	34	35	25	25	119
		GMC	113.0	139.4	111.8	86.4	113.3
		95% CI	85.2,150.0	107.4-180.9	76.6-163.0	58.8-126.8	97.1-132.3
	D365	N	34	35	22	24	115
		GMC	35.6	49.2	32.5	37.6	39.1
		95% CI	25.4, 49.8	38.5, 62.8	22.1, 48.0	27.6, 51.2	33.5, 45.6

Legend: 95% CI: 95% confidence interval

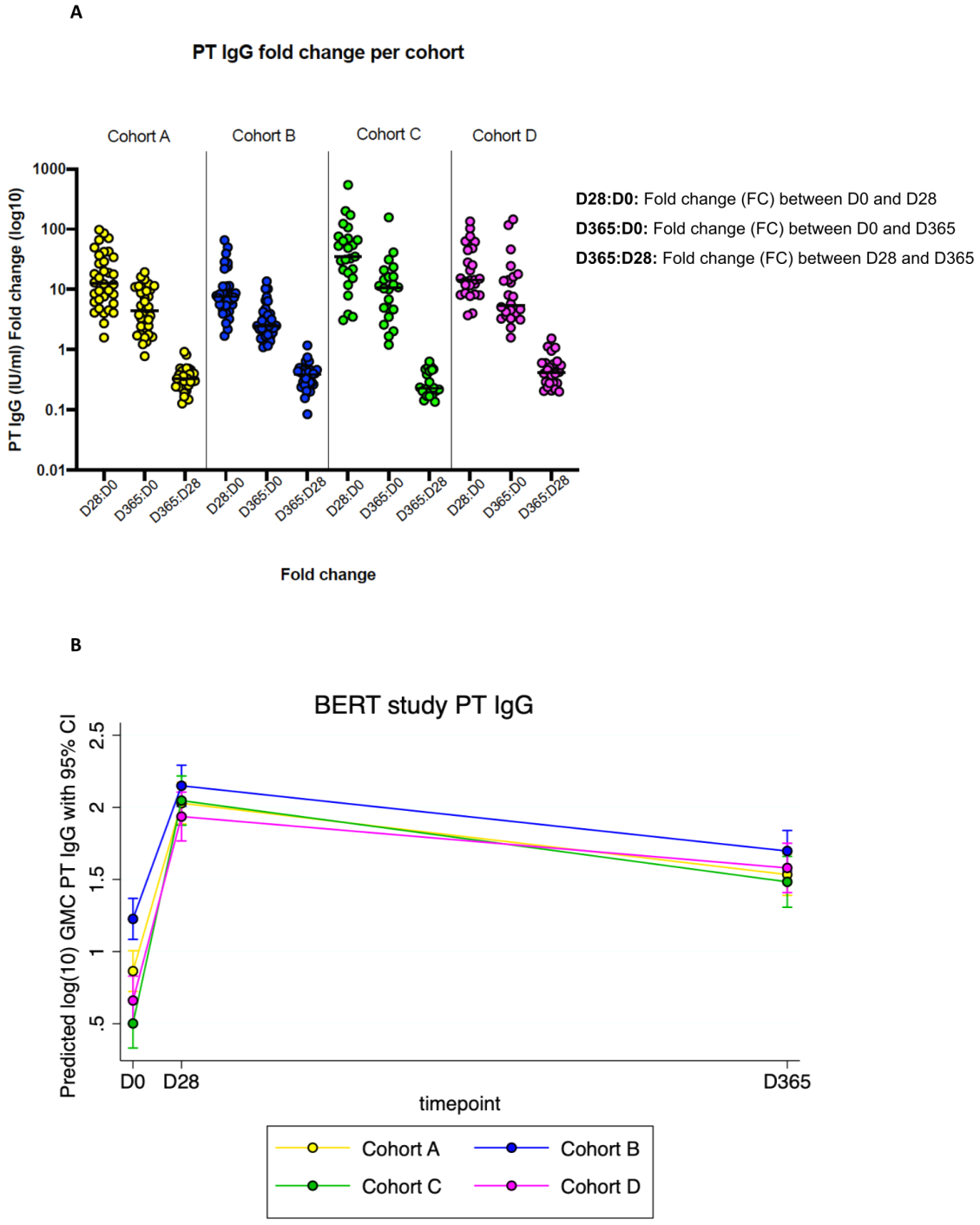


Figure 3.3 Pertussis toxin (PT) IgG antibody levels in the BERT study population

A: Dot plot with fold changes (FC) and median; **B:** Predicted log(10) GMC PT IgG and 95% CI, per cohort

b. FHA IgG

For most participants FHA IgG pertussis specific antibody for the overall BERT population had the highest D0 levels (Table 3.4). When considering the three main timepoints (D0, D28 and D365), the adolescent cohort (B), similar to PT IgG, had a significantly higher FHA IgG GMC than any other cohorts, which were not significantly different from each other (Figure 3.4).

In the subgroup of individuals that had antibody levels at D7 and/or D14, the quickest increase within the first week after immunisation was found in the youngest cohort (A), followed by adult cohort (C) (FC median [IQR] cohort A: 5.4 [3.1-8.3]; cohort B: 3.4 [1.9-8.1]; cohort C:4.1 [2.5-7.2], cohort D: 2.3 [1.3-3.5]). When considering the increase from D0 to D14, this increase continued to be the highest in cohort A (cohort A: 24.5 [12.5-47.7]; cohort B: 7.1 [4.9-15.0]; cohort C:18.0 [7.9-35.3], cohort D: 6.0 [2.7-12.2]) (Figure 3.4).

The GMC peak was achieved at D28 for all cohorts, except for the youngest individuals (A), that strikingly achieved the peak at D14. At this point the elderly (D) had significantly lower GMCs than all other cohorts (cohort A vs cohort D *p=0.02,* cohort B vs cohort D *p=0.01,* cohort C vs cohort D *p=0.04)* (Figure 3.4), but these differences were no longer apparent by D365.

A waning of antibody levels from D28 to D365 was identified, although the GMCs never reached as lower levels as in D0 (Table 3.4).

Table 3.4 Baseline and post-booster filamentous haemagglutinin (FHA) IgG specific antigen geometric mean concentration (GMC) after vaccination with Boostrix® in the BERT study population and per cohort

Antigen	Timepoint		Cohort A	Cohort B	Cohort C	Cohort D	All cohorts
FHA (IgG) IU/ml	D0	N	36	35	25	25	121
		GMC	19.7	29.5	15.5	18.0	20.7
		95% CI	14.3, 27.1	21.5, 40.4	10.7, 22.4	12.4, 26.2	17.4, 24.5
	D7	N	16	12	25	25	78
		GMC	135.7	108.8	68.1	41.8	72.1
		95% CI	89.9, 204.9	72.1, 164.0	46.8, 98.9	28.0, 62.4	58.0, 89.6
	D14	N	19	12	25	21	77
		GMC	318.7	286.4	262.8	130.4	230.7
		95% CI	250.5, 405.4	198.9, 412.4	186.7, 370.0	85.1, 199.8	191.3, 278.4
	D28	N	34	35	25	25	119
		GMC	289.7	291.0	275.4	173.6	257.7
		95% CI	235.4, 356.5	233.7, 362.3	198.2, 382.6	115.8, 260.3	224.4, 296.1
	D365	N	34	35	22	24	115
		GMC	110.7	123.6	114.9	90.7	110.6
		95% CI	90.5, 135.3	101.0, 151.3	79.8, 165.5	62.8, 131.0	97.2, 125.8

Legend: 95% CI: 95% confidence interval

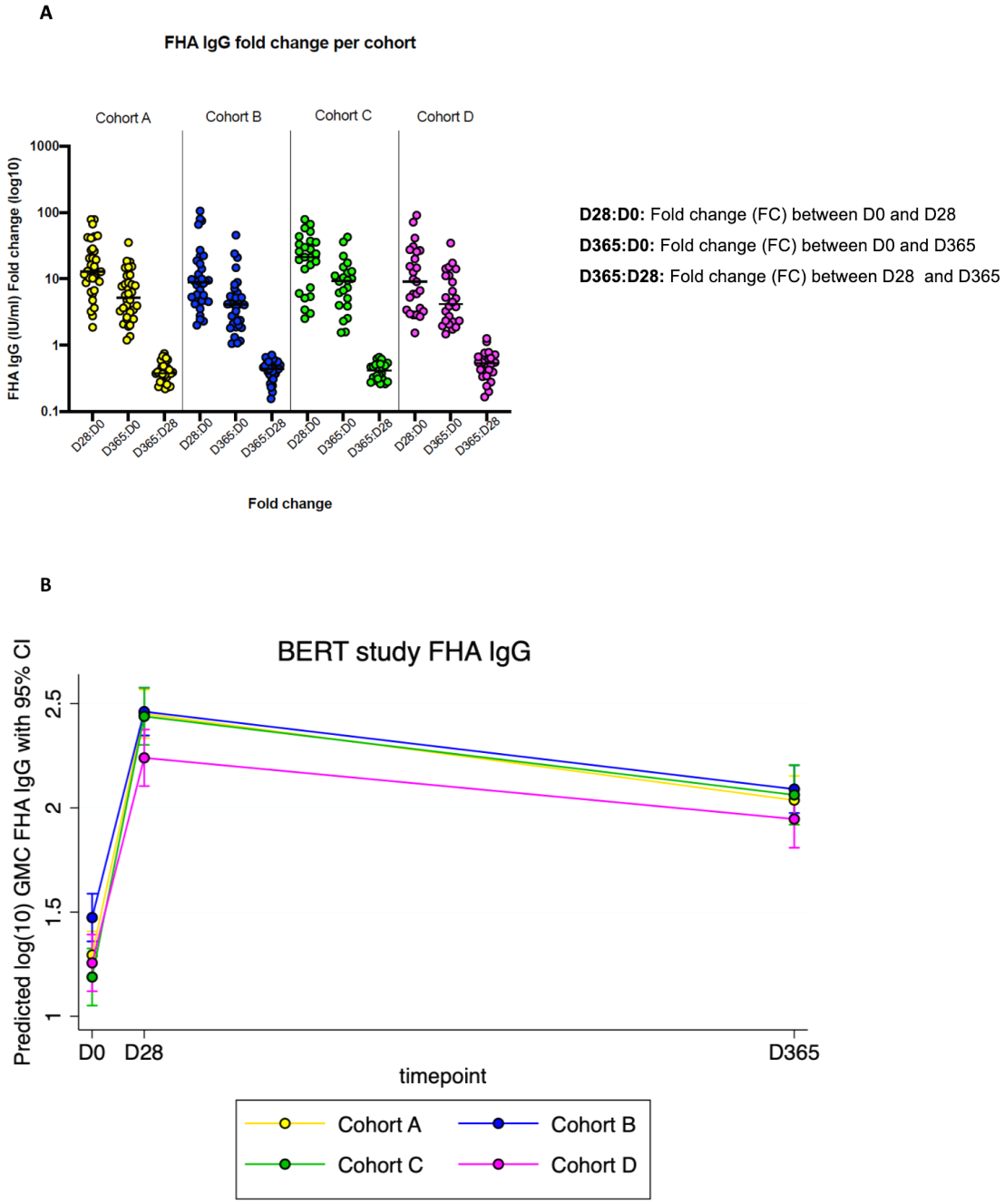


Figure 3.4 Filamentous haemagglutinin (FHA) IgG antibody levels in the BERT study population

A: Dot plot with fold changes (FC) and median; **B**: Predicted log(10) GMC FHA IgG and 95% CI, per cohort

c. Prn IgG

Prn IgG antibody responses had a similar kinetics to FHA IgG in the BERT study population and was the pertussis specific antibody with some of the highest GMCs, except in the elderly population (D).

At D0, the elderly cohort (D) had a significant lower GMC compared to all the other cohorts (cohort A *vs* cohort D *p=0.002,* cohort B *vs* cohort D *p=0.03,* cohort C *vs* cohort D *p=0.05)*, although no significant differences were identified between the other cohorts.

In terms of early responses, by D7, the children (A) had the highest FC increase in the first week (FC cohort A: 8.0 [3.9-18.2]; cohort B: 5.5 [3.9- 11.8]; cohort C:4.4 [2.5-8.1], cohort D: 2.6 [2.0-5.9]). Notably, the Prn GMC achieved the peak in the two youngest cohorts (A and B) at D14 and then waned by D28, but for the older cohorts that peak was achieved at D28. Overall across different antigen specific responses the increase in antibody levels after booster immunisation was the highest for this antibody, with a median FC from baseline to D28 ranging from 13.1 to 21.8 (Table 3.5 and Figure 3.5).

At D28 after booster immunisation the three younger cohorts had no significant differences between them, but the elderly cohort (D) had a significantly lower GMC (cohort A vs cohort D *p=0.03,* cohort B vs cohort D *p=0.03,* cohort C vs cohort D *p=0.004)*. By D365 adults (C) had the highest GMC, but only statistically significant when compared to the elderly (D) (*p=0.02*). Except for this significant difference at this timepoint no other significant differences between cohorts were identified (Figure 3.5).

Similar to other pertussis specific antibodies, statistically significant differences were identified between the three timepoints (*p<0.0001*), with higher levels at D365 in comparison with baseline. Despite the lower initial antibody responses in the elderly

(D) and the adults (C), there was a significantly slower rate of subsequent decline. In the elderly, this was a similar pattern seen to that in PT IgG data and of interest in relation to differences in the quality of immunological memory that might be induced by differences in original priming (Figure 3.5).

Table 3.5 Baseline and post-booster pertactin (Prn) IgG specific antigen geometric mean concentration (GMC) after vaccination with Boostrix® in the BERT study population and per cohort

Antigen	Timepoint		Cohort A	Cohort B	Cohort C	Cohort D	All cohorts
Prn (IgG)	D0	N	36	35	25	25	121
IU/ml		GMC	20.8	13.6	13.9	6.1	13.2
		95% CI	12.9, 33.5	8.8, 21.1	6.7, 28.7	2.8, 13.5	9.9, 17.6
	D7	N	16	12	25	25	78
		GMC	208.5	53.1	73.4	24.3	60.7
		95% CI	103.1, 421.9	18.7, 150.5	37.7, 143.0	13.6, 43.2	41.8, 88.2
	D14	N	19	12	25	21	77
		GMC	343.8	522.8	361.9	97.8	264.8
		95% CI	187.2, 631.2	263.0, 1039.2	201.1, 651.3	48.0, 199.3	188.8, 371.6
	D28	N	34	35	25	25	119
		GMC	256.2	259.7	375.0	111.4	233.9
		95% CI	160.2, 409.7	161.5, 417.6	201.8, 696.9	57.9, 214.1	178.8, 306.1
	D365	N	34	35	22	24	115
		GMC	93.5	96.8	166.1	66.0	98.1
		95% CI	57.7, 151.6	57.8, 162.1	84.2, 327.5	36.9,118.2	74.9, 128.4

Legend: 95% CI: 95% confidence intervals

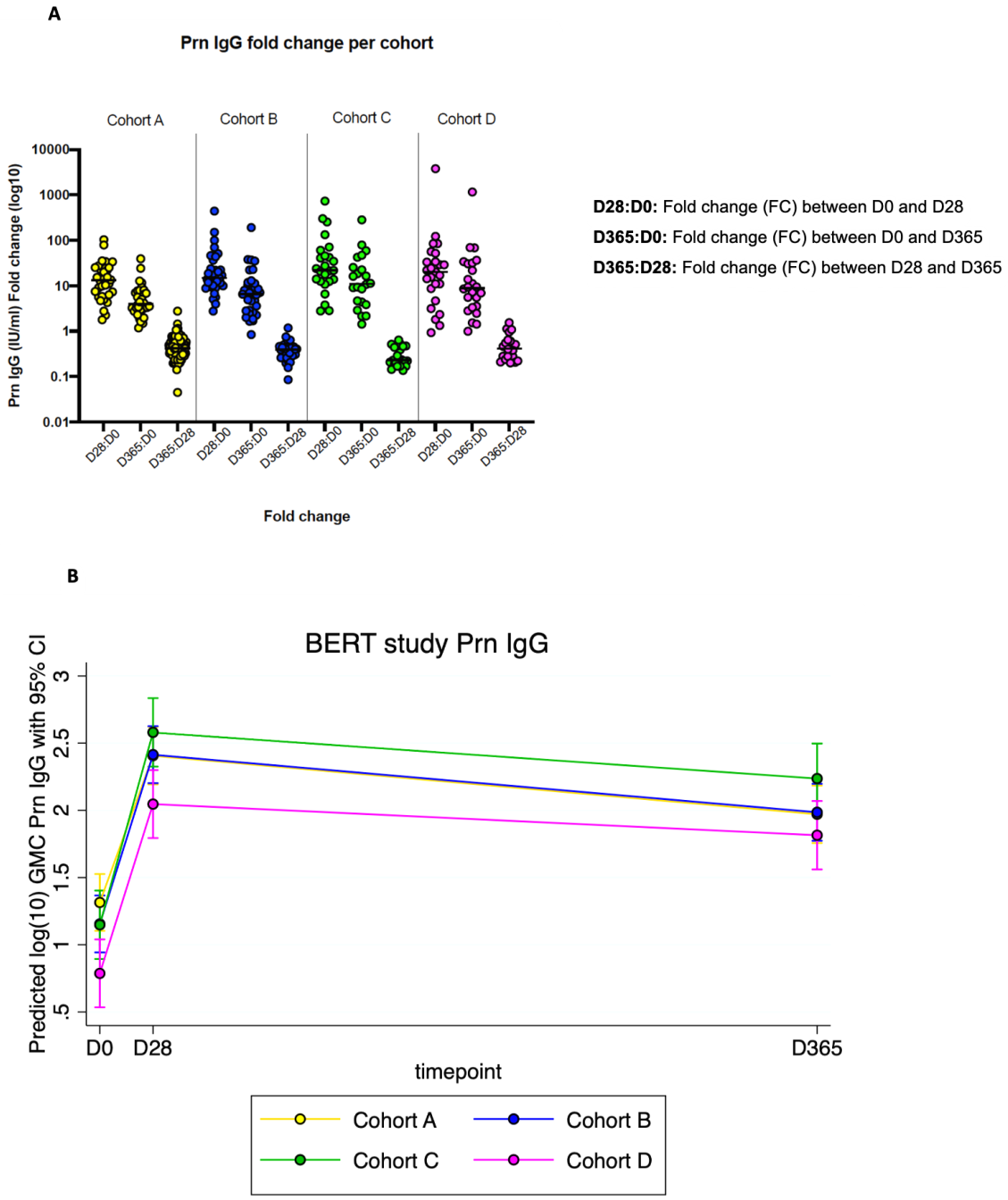


Figure 3.5 Pertactin (Prn) IgG antibody levels in the BERT study population

A: Dot plot with fold changes (FC) and median; **B:** Predicted log(10) GMC Prn IgG and 95% CI, per cohort

d. FIM 2/3 IgG

FIM 2/3 is not part of the components of the vaccine given to the study participants. The elderly (D), for all timepoints, had always the lowest GMCs and cohort B had the highest GMC (Table 3.6 and Figure 3.6).

Table 3.6 Baseline and post-booster fimbriae (FIM) 2/3 IgG specific antigen geometric mean concentration (GMC) after vaccination with Boostrix® in the BERT study population and per cohort

Antigen	Timepoint		Cohort A	Cohort B	Cohort C	Cohort D	All cohorts
FIM 2/3 (IgG) IU/ml	D0	N	36	35	25	25	121
		GMC	9.8	12.0	10.5	1.5	7.2
		95% CI	6.4, 14.9	8.4, 17.2	5.7, 19.3	0.7, 3.6	5.4, 9.6
	D7	N	16	12	25	25	78
		GMC	28.0	19.1	10.9	2.0	8.3
		95% CI	18.1, 43.4	10.5, 34.7	6.0, 19.8	0.9, 4.3	5.6, 12.4
	D14	N	19	12	25	21	77
		GMC	25.7	36.2	11.1	1.9	10.1
		95% CI	14.8, 44.6	23.1, 56.7	6.2, 20.1	0.8, 4.6	6.7, 15.3
	D28	N	34	35	25	25	119
		GMC	17.0	15.5	10.6	1.7	9.2
		95% CI	12.2, 23.8	11.3, 21.3	5.8, 19.5	0.8, 3.5	6.9, 12.2
	D365	N	34	35	22	24	115
		GMC	11.2	11.0	8.9	1.8	7.3
		95% CI	7.5, 16.9	7.5, 16.3	5.2, 15.3	0.9, 3.7	5.5, 9.6

Legend: 95% CI: 95% confidence interval

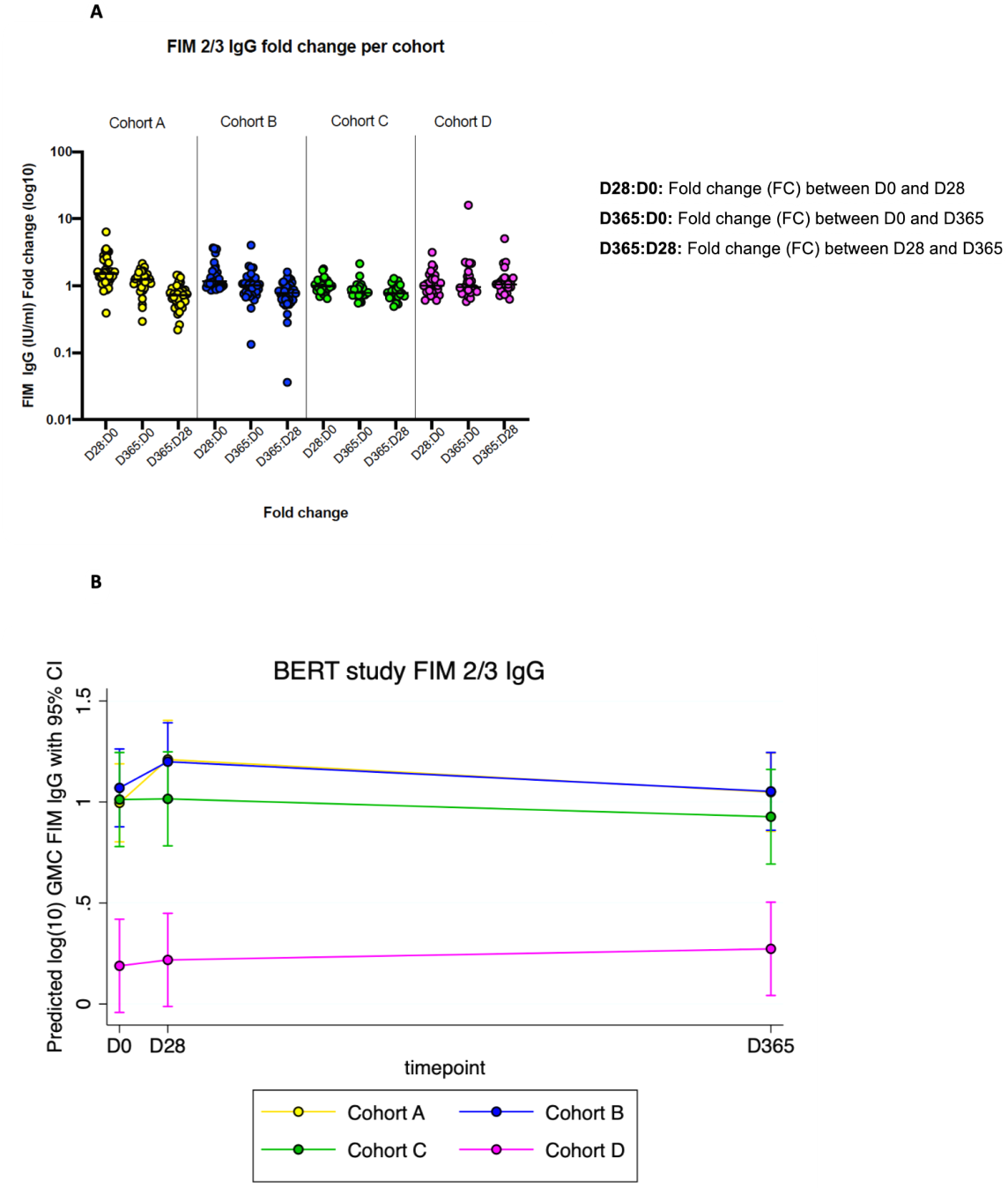


Figure 3.6 Fimbriae (FIM) 2/3 IgG antibody levels in the BERT study population

A: Dot plot with fold changes (FC) and median; **B:** Predicted log(10) GMC FIM 2/3 IgG and 95% CI per cohort

3.3.2 IgA pertussis specific antibodies

The IgA levels were measured at D0, D28 and D365 for PT, FHA, Prn and FIM 2/3. The antigens present in the booster vaccine (PT, FHA and Prn) showed an increase from D0 to D28 and decrease from D28 to the D365 timepoint for all cohorts (Figure 3.7). In general, the GMCs for this immunoglobulin were lower than the GMC achieved for IgG.

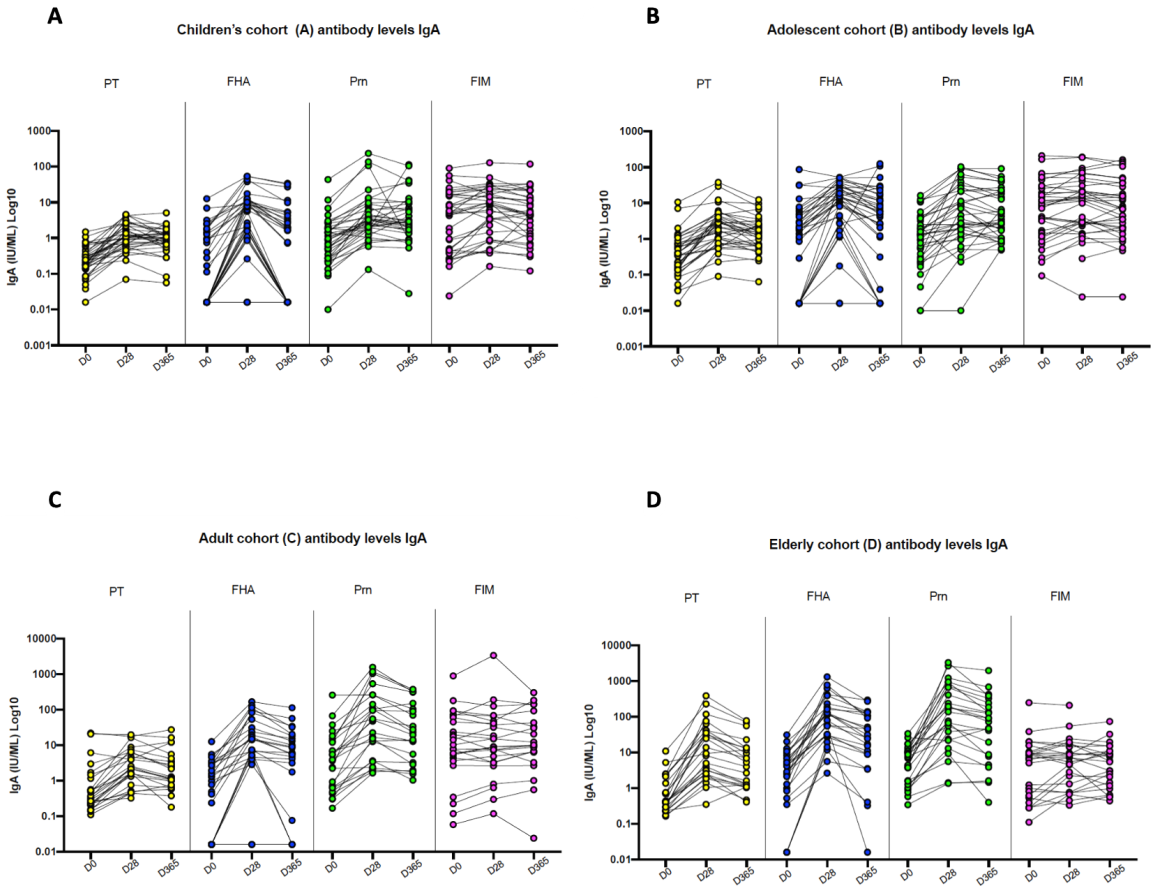


Figure 3.7 Kinetics of pertussis specific IgA antibody levels (PT, FHA, Prn, FIM2/3), at different timepoints for the BERT study, per cohort (IU/ml)

For IgA, considering the antibodies of the antigens contained in the vaccine, the older the cohort, the higher the GMC, a pattern that was consistent even one year after immunisation.

a. PT IgA

PT IgA antibody levels were in general low for all cohorts, but the oldest cohorts (C and D) had significantly higher GMCs when compared with the younger cohort (A).

At baseline no differences were found between the adjacent age groups immediately close (cohort A vs B *p=0.1*; cohort B vs C *p=0.2* and cohort C vs D *p=0.7).*

After the booster dose an increase in the GMCs was identified in all cohorts, with the highest GMCs in the elderly individuals (D) corresponding also to the cohort with the highest median FC from baseline to D28 (FC median 18.3; IQR:2.3-7.1) (Table 3.7 and Figure 3.8).

At D28, the differences between cohorts were all considered significant except the differences between adolescents (B) and adults (C), a pattern that was maintained at D365 (D28 - cohort B vs cohort C *p=0.6*; D365 - cohort B vs cohort C: *p=0.5*) (Figure 3.8). At D365 that significance was also lost between the younger age groups.

Table 3.7 Baseline and post-booster pertussis toxin (PT) IgA specific antigen geometric mean concentrations (GMC) after vaccination with Boostrix® in the BERT study population and per cohort

Antigen	Timepoint		Cohort A	Cohort B	Cohort C	Cohort D	All cohorts
PT (IgA)	D0	N	38	37	25	25	125
IU/ml		GMC	0.2	0.3	0.6	0.6	0.4
		95% CI	0.2, 0.3	0.2, 0.5	0.3, 1.1	0.4, 1.0	0.3, 0.5
	D28	N	34	35	25	25	119
		GMC	1.1	2.0	2.4	11.2	2.5
		95% CI	0.8, 1.5	1.3, 3.1	1.5, 3.9	5.3, 23.7	1.9, 3.3
	D365	N	34	35	22	24	115
		GMC	0.9	1.3	1.9	4.5	1.6
		95% CI	0.6, 1.2	0.9, 2.0	1.1, 3.4	2.3, 8.6	1.3, 2.1

Legend: 95% CI: 95% confidence interval

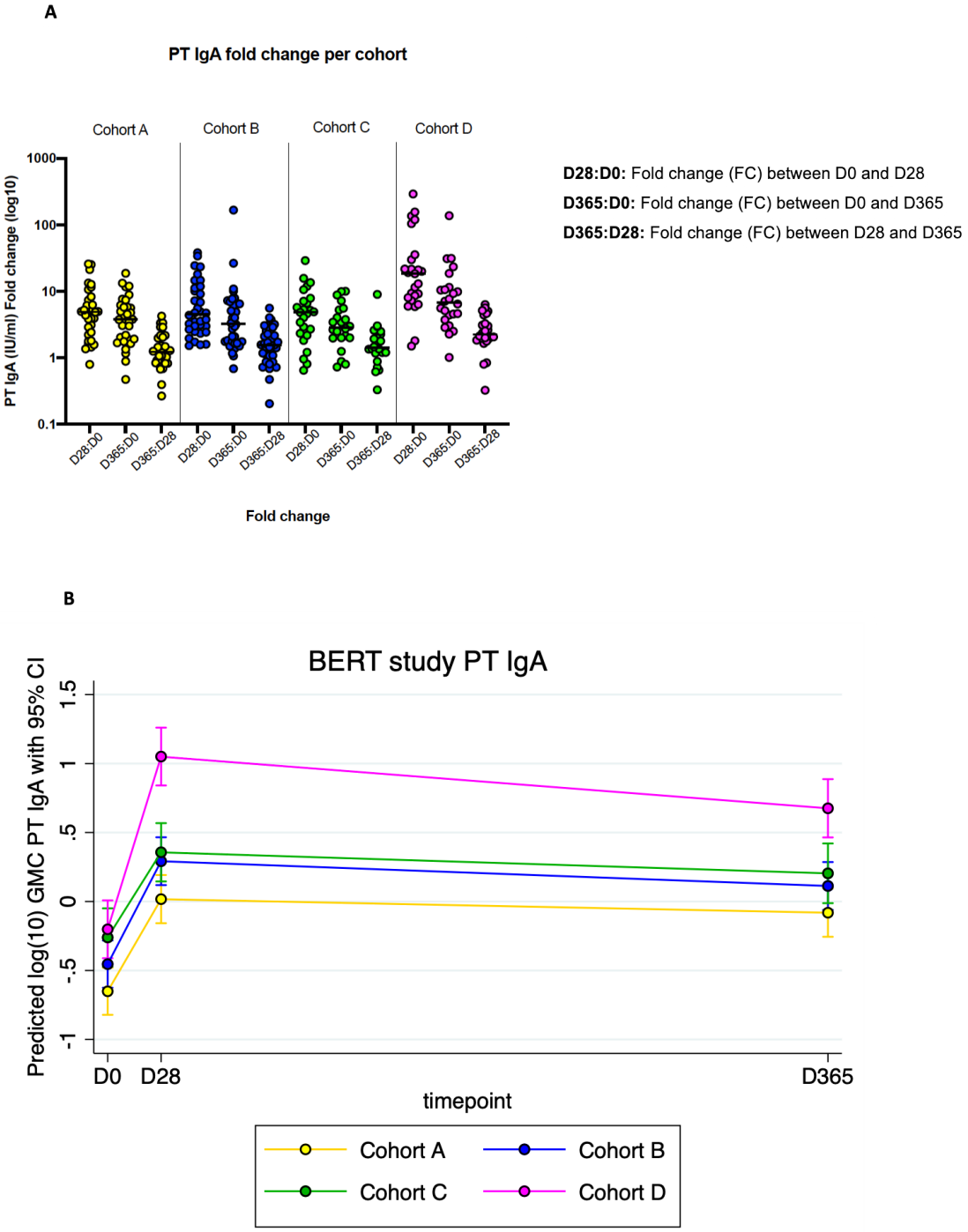


Figure 3.8 Pertussis toxin (PT) IgA antibody levels in the BERT study population

A: Dot plot with fold changes (FC) and median; **B:** Predicted log(10) GMC PT IgA and 95% CI, per cohort

b. FHA IgA

As seen in PT IgA, this pertussis specific antibody showed lower GMCs at baseline (Table 3.8), showing a significant increase in all cohorts after booster. The elderly (D) had a significantly higher FHA IgG GMCs when compared with other cohorts, although statistically significant differences were found between cohorts. At baseline (D0), with the elderly (D) showing the highest GMCs, no statistically significant differences were found between adolescents (B) and adults (C) (*p=0.7*) and between adolescents (B) and elderly (D) (*p=0.07*) (Figure 3.9).

Despite showing the lowest GMCs, the youngest cohort was the one with the highest median FC [IQR] from baseline to D28 (**A**: 32.6 [4.8,101.2]; **D:** 23.1 [11.6,43.9]) (Table 3.8).

Twenty-eight days after booster immunisation, significant differences were only identified between the elderly (D) and the other cohorts (A (*p<0.001*), B (*p<0.001)* and C (*p=0.002*)), but not between A, B and C (Figure 3.9).

At D365, the elderly (D) had the highest GMC, with statistically significant differences identified between cohorts. No differences were found between adolescent and adults (*p=0.8*), that despite different immunisation backgrounds, had closer epidemiologic exposure when compared with the other cohorts (Figure 3.9).

Table 3.8 Baseline and post-booster filamentous haemagglutinin (FHA) IgA specific antigen geometric mean concentrations (GMC) after vaccination with Boostrix® in the BERT study population and per cohort

Antigen	Timepoint		Cohort A	Cohort B	Cohort C	Cohort D	All cohorts
FHA (IgA)	D0	N	38	37	25	25	125
IU/ml		GMC	0.1	0.6	0.5	2.0	0.4
		95% CI	0.05, 0.2	0.2, 1.5	0.2, 1.3	0.8, 4.7	0.3, 0.7
	D28	N	34	35	25	25	119
		GMC	3.3	7.9	9.4	75.9	10.3
		95% CI	1.6, 6.9	4.0, 15.7	3.7, 24.1	40.2, 143.4	6.8, 15.5
	D365	N	34	35	22	24	115
		GMC	0.3	2.39	3.7	18.5	2.2
		95% CI	0.1, 0.8	0.9, 6.2	1.1, 11.8	6.9, 49.9	1.2, 3.8

Legend: 95% CI: 95% confidence interval

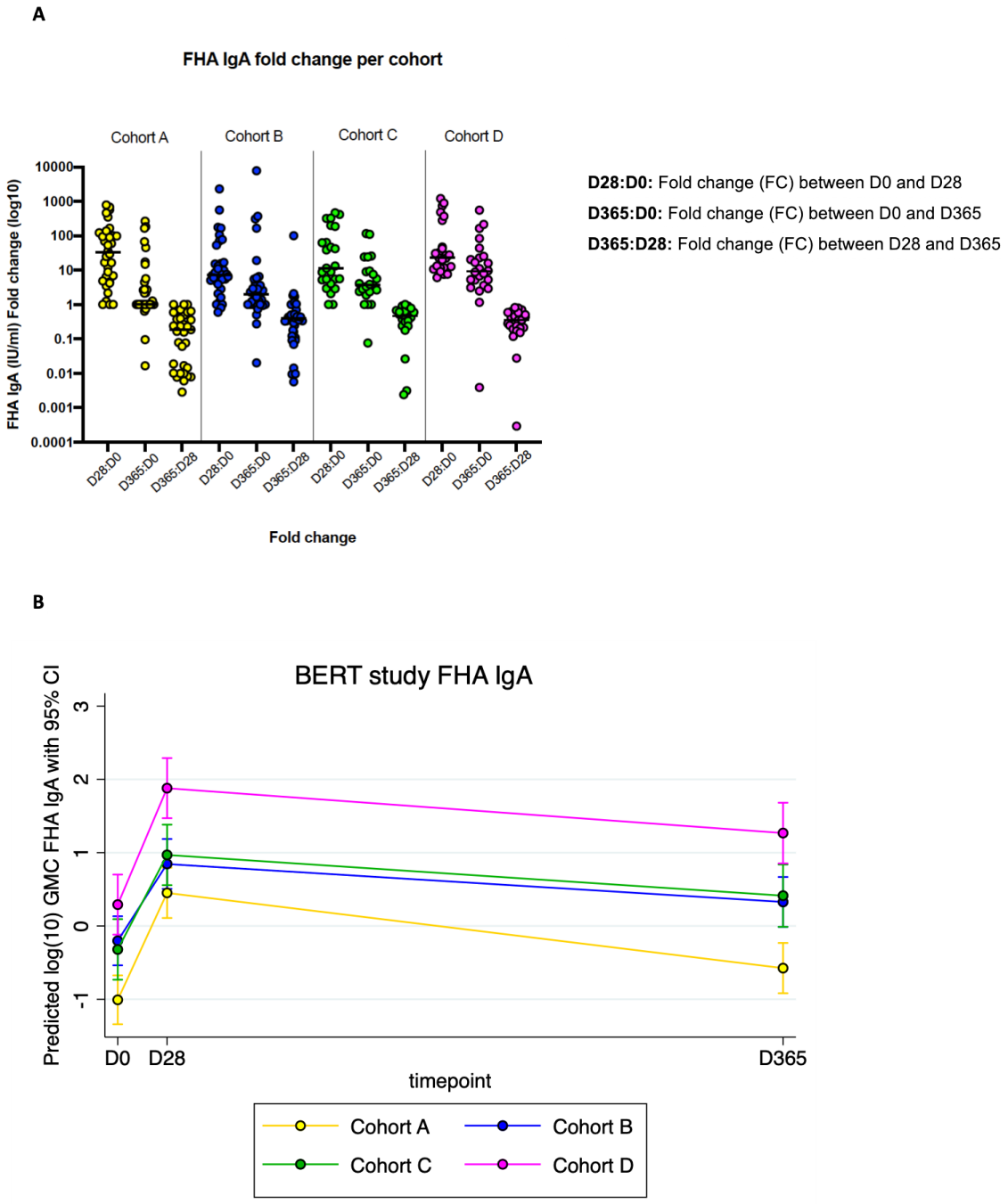


Figure 3.9 Filamentous haemagglutinin (FHA) IgA antibody levels in the BERT study population

A: Dot plot with fold changes (FC) and median; **B:** Predicted log(10) GMC FHA IgA and 95% CI, per cohort

c. Prn IgA

Prn IgA showed the highest GMCs in all cohorts, presenting with a similar pattern to FHA, with highest GMCs after booster immunisation in the older age groups (C and D) (Table 3.9).

At D0, the elderly cohort (D) and the adults (C) had significantly higher GMCs compared to the younger cohorts (A and B). When comparing the GMCs between younger cohorts (A and B) at baseline, no significant differences were identified (*p=0.8)*. The same pattern was seen between the older cohorts (C and D *p=0.5*).

After the booster dose, a statistical significance increase in the GMCs were identified from baseline to D28. For this antibody, the FC median from baseline to D28 was higher in the older cohorts (FC median, [IQR]: **A:** 4.8, [3,0,8.4]; **B:** 4.7 [1.9,8.5]; **C:** 7.9 [4.1,17.5]; **D:** 20.6 [7.4,90.4]) (Figure 3.10).

Twenty-eight days after booster immunisation, the elderly (D) had a significantly higher GMC when compared with the other cohorts. At this timepoint (D28), cohort D had the highest GMC, showing statistically significant differences between all cohorts.

The GMC pattern seen at D28 was maintained at D365, with higher levels in the elderly (D), and although differences between cohorts remained overall similar, at D365, the differences between the older cohorts (C and D) lost significance (*p=0.06*) (Figure 3.10).

Table 3.9 Baseline and post-booster pertactin (Prn) IgA specific antigen geometric mean concentrations (GMC) after vaccination with Boostrix® in the BERT study population and per cohort

Antigen	Timepoint		Cohort A	Cohort B	Cohort C	Cohort D	All cohorts
Prn (IgA)	D0	N	38	37	25	25	125
IU/ml		GMC	0.8	0.9	3.6	4.5	1.6
		95% CI	0.5, 1.3	0.5, 1.6	1.6, 7.8	2.7, 7.7	1.1, 2.2
	D28	N	34	35	25	25	119
		GMC	4.0	4.9	35.4	102.8	13.3
		95% CI	2.3, 6.8	2.5, 9.7	14.4, 87.3	42.2, 250.4	8.7, 20.3
	D365	N	34	35	22	24	115
		GMC	3.6	4.8	22.1	46.2	9.5
		95% CI	2.0, 6.4	2.8, 8.0	9.4, 52.1	18.5, 115.2	6.5, 13.8

Legend: 95% CI: 95% confidence interval

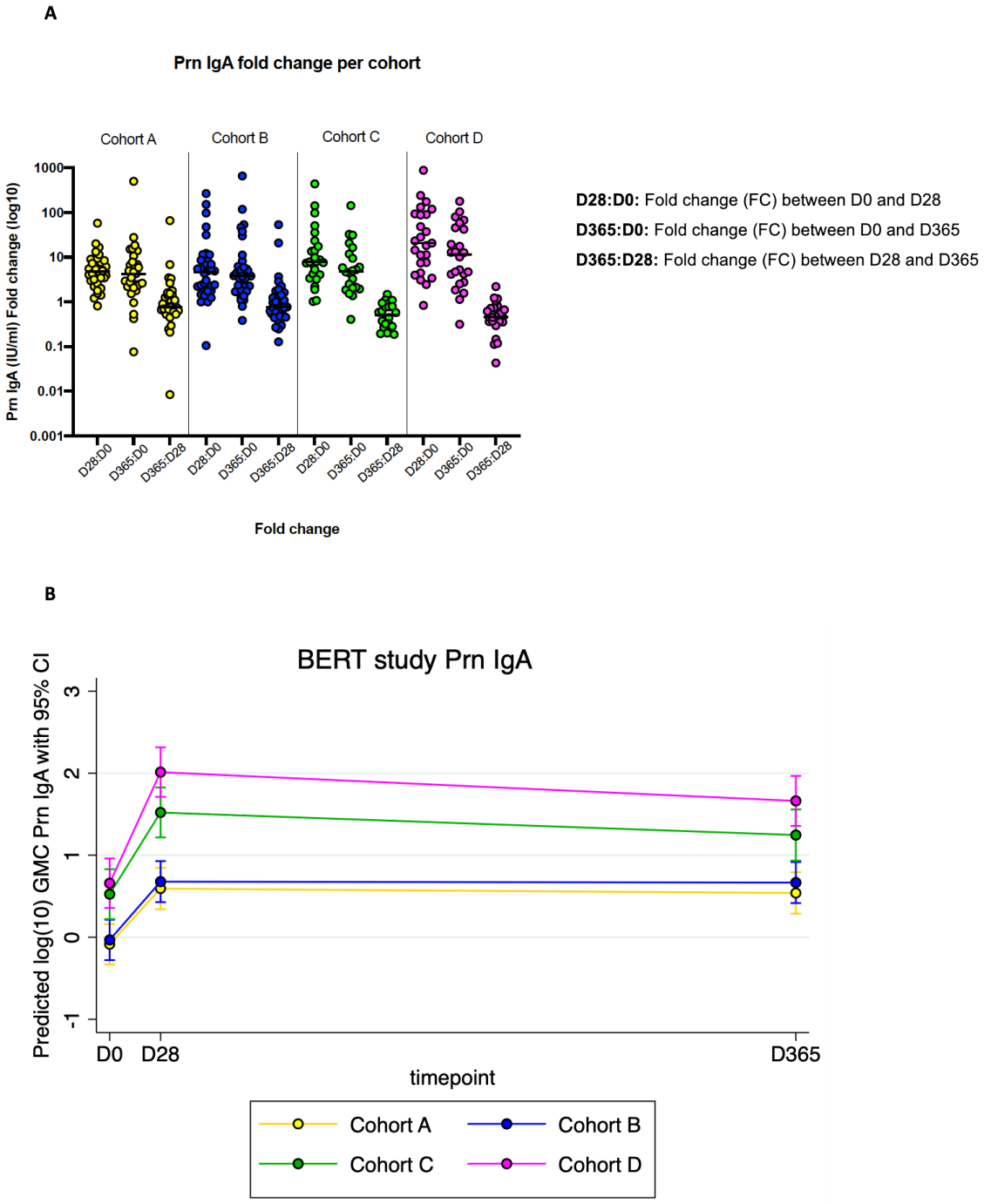


Figure 3.10 Pertactin (IgA) antibody levels in the BERT study population

A: Dot plot with fold changes (FC) and median; **B:** Predicted log(10) GMC Prn IgA and 95% CI per cohort

d. FIM 2/3 IgA

This antibody had the highest baseline GMC of all tested IgA antibodies, with highest levels seen in the adult cohort (C) at all timepoints (Table 3.10 and Figure 3.11). Of note, this antigen was not contained in the booster vaccine, and the GMC was the result of previous immunisation and epidemiologic contacts.

Table 3.10 Baseline and post-booster fimbriae (FIM) 2/3 IgA specific antigen geometric mean concentrations (GMC) after vaccination with Boostrix® in the BERT study population and per cohort

Antigen	Timepoint		Cohort A	Cohort B	Cohort C	Cohort D	All cohorts
FIM 2/3 (IgA) IU/ml	D0	N	38	37	25	25	125
		GMC	2.9	5.9	9.7	3.4	4.7
		95% CI	1.5, 5.4	3.2, 10.9	3.7, 25.4	1.6, 7.5	3.3, 6.7
	D28	N	34	35	25	25	119
		GMC	6.0	8.1	11.5	5.0	7.2
		95% CI	3.5, 10.5	4.2, 15.7	4.6, 28.3	2.6, 9.7	5.2, 10.1
	D365	N	34	35	22	24	115
		GMC	4.5	6.6	11.7	4.3	6.0
		95% CI	2.6, 7.8	3.5, 12.7	4.5, 30.4	2.4, 7.8	4.3, 8.4

Legend: 95% CI: 95% confidence interval

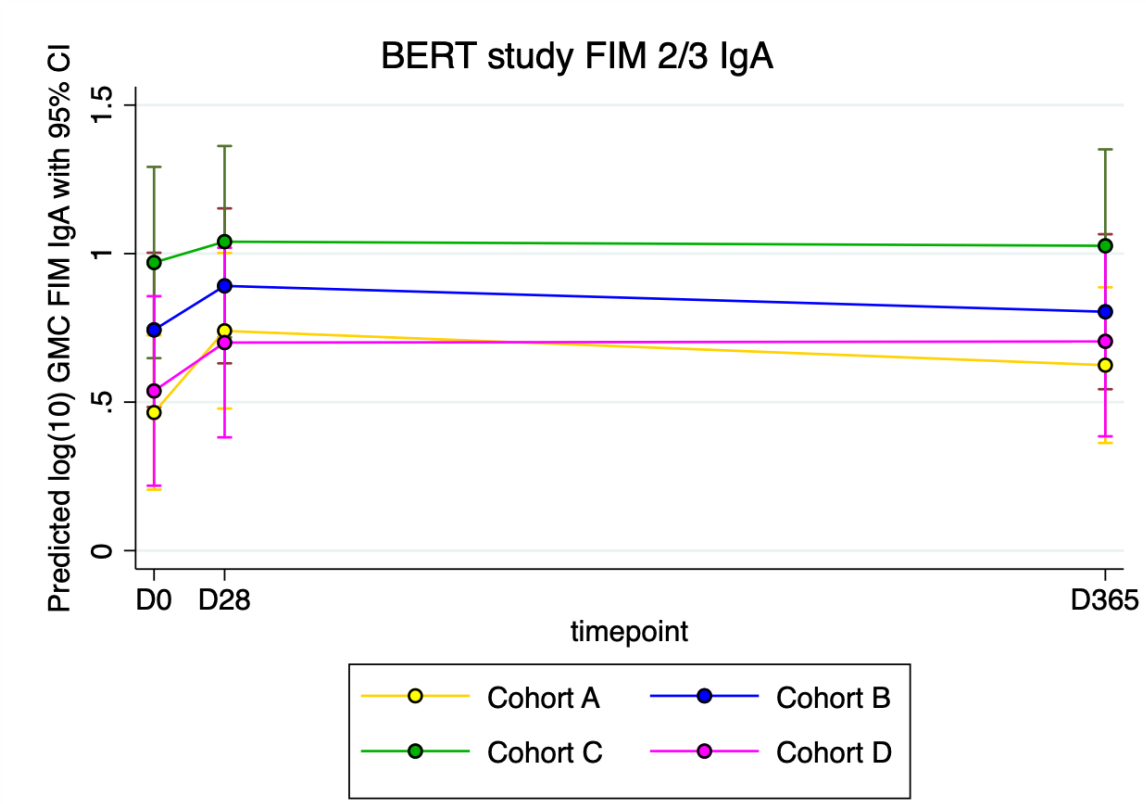


Figure 3.11 Fimbriae (FIM) 2/3 IgA predicted log(10) GMC and 95% CI at baseline, D28 and D365 after booster immunisation

3.4 EVALUATION OF ANTIBODY FUNCTIONALITY IN THE BERT STUDY POPULATION

The functional antibody assays performed in the BERT study population were SBA, OPA and CDA. It was programmed to perform the assays in all participants, but due to constrains of time related with the COVID-19 pandemic not all samples were processed.

The results presented in this DPhil are from the samples with serum available for analysis and with results considered valid. Work on the remaining samples is currently ongoing and will be used for further publications.

3.4.1 Serum bactericidal antibody (SBA)

This novel functional antibody assay, designed to determine vaccine induced antibodies killing of *B. pertussis* in conjunction with complement, was performed in a subset of samples from the BERT study, selected by PHE collaborators.

A total of 76 samples at baseline and 56 samples D28 after booster immunisation were analysed. Although all samples were paired, due to technical issues and impossibility of repeating the assay in a group of samples in time for this DPhil thesis not all results could be used in this thesis.

The SBA GMT at baseline were lower when compared with GMT after immunisation. The cohorts with higher SBA GMT at baseline were adolescents (B) followed by adults (C). The cohort with lower GMT was the children (A) (Table 3.11).

Table 3.11 Serum bactericidal assay (SBA) geometric mean titre (GMT) and 95% confidence interval (95% CI) in the BERT study population at baseline (D0) and D28

SBA	Timepoint		Cohort A	Cohort B	Cohort C	Cohort D
GMT	D0	N	16	16	21	23
		GMT	25.5	40.9	40.5	31.1
		95%CI	8.6, 75.4	21.9, 76.5	17.4, 94.5	14.3, 68.5
	D28	N	13	9	13	21
		GMT	134.1	198.3	232.8	65.4
		95% CI	54.9, 327.3	94.3, 417.1	132.1, 410.2	24.1, 177.3

Four weeks after pertussis immunisation, the elderly (D) showed the least increase in the titres when compared with other cohorts (FC median [IQR]:1.5 [1.0,5.1]). The cohort with the highest FC was cohort B (median [IQR]:8.4 [4.0,10.7]), that also corresponded to the cohort with the second highest GMT (Figure 3.12).

In the analysis of the correlation between pertussis specific antibody titres and SBA titres, anti-Prn IgG antibodies showed the best correlation from the four tested IgG antibodies (PT, FHA, Prn and FIM 2/3). This correlation was found both between baseline SBA and baseline antibody titres in almost all cohorts (Spearman correlation; all population:<0.0001; A [n=16]:0.002; B [n=16]:0.08; C [n=21]:0.01; D [n=23]: <0.0001) and between the titres at D28 (Spearman correlation; all population:<0.0001; A [n=13]:0.0008; B [n=9] <0.0001; C [n=13]:0.0002; D [n=21]: <0.006). At baseline PT IgG did not show any significant correlation with SBA titres in the individual cohorts. Figure 3.12 shows the correlations between IgG pertussis antibody levels and SBA titres after booster immunisation.

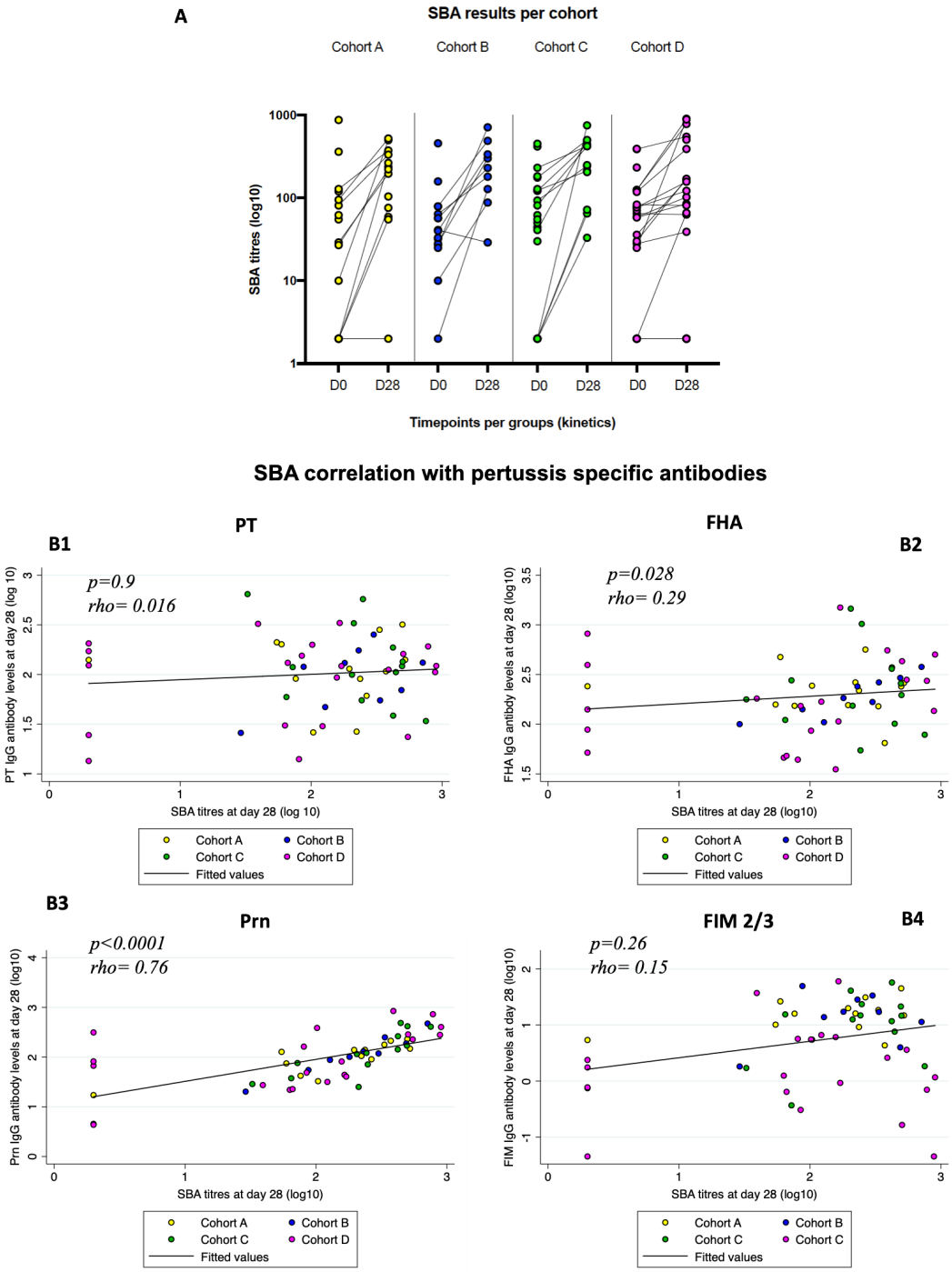


Figure 3.12 Serum bactericidal assay (SBA) results in the BERT population per cohort

A: Kinetics of SBA titers (log10) at baseline and D28 after pertussis booster immunisation per cohort **B:** Spearman correlation between SBA titres at D28 and pertussis specific antibody levels, PT (B1), FHA (B2), Prn (B3) and FIM 2/3 (B4).

No correlations were found between pertussis specific IgA antibodies and SBA titres.

3.4.2 Opsonophagocytosis assay (OPA)

This assay allows the measurement of antibody-complement mediated killing of *B. pertussis*, via the opsophagocytosis process. The higher the fluorescence, the higher the binding of the antibodies to the bacteria.

The OPA was performed in a total of 105 individuals at baseline and 93 individuals at D28. Table 3.12 shows the median and IQR at the two timepoints that the assay was performed.

A statistically significant increase in the OPA mean fluorescence was seen in all cohorts from baseline to D28 (Table 3.12 and Figure 3.13).

Table 3.12 Opsonophagocytosis assay (OPA) median and interquartile range (IQR) in the BERT study population at baseline (D0) and D28

OPA	Timepoint		Cohort A	Cohort B	Cohort C	Cohort D
MFI-C'	D0	N	28	30	24	23
		Median	1,357,942	1,443,134	920,826.1	1,202,649
		IQR	677,434.9-2,003,461	940,664.2-2,787,223	356,424.8-1,286,128	395,288.8-1,629,166
	D28	N	21	24	23	25
		Median	2,802,982	2,603,773	1,630,493	2,326,268
		IQR	2,088,698-3,145,066	1,458,285 – 3,333,320	1,011,286-2,584,870	1,540,806-2,827,134
		p value	0.0007	0.0007	0.0001	0.0001

Wilcoxon signed rank test

The median FC ranged between 1.3 (B) and 2.0 (D). At baseline, the MFI-C' of the OPA assay was statistically significant between cohorts (Kruskal-Wallis rank test *p=0.02*). Adults (C) was the age group with lower MFI-C' at baseline. Twenty-eight days after the booster dose, this difference was lost, and no differences of the MFI-C' were found between groups (Kruskal-Wallis rank test *p=0.2*). When evaluating the correlation of pertussis specific antibodies with the OPA assay, Prn IgG (*p<0.0001*; rho=0.4) showed significant correlation at baseline. After the booster dose, at day 28, Prn (Figure 3.13), IgG had a significant correlation with OPA performed in the samples collected at same timepoint, although the rho was low, perhaps suggesting the role of other antibody specificities in being more important for OPA than SBA.

Considering the total population in the BERT study, no significant correlations were identified at baseline between IgA pertussis specific antibodies (PT, FHA, Prn and FIM 2/3). After the booster dose a significant correlation between FIM IgA and OPA MFI-C' (*p=0.0009*; rho=-0.3) was identified, but not for the other pertussis specific antibodies.

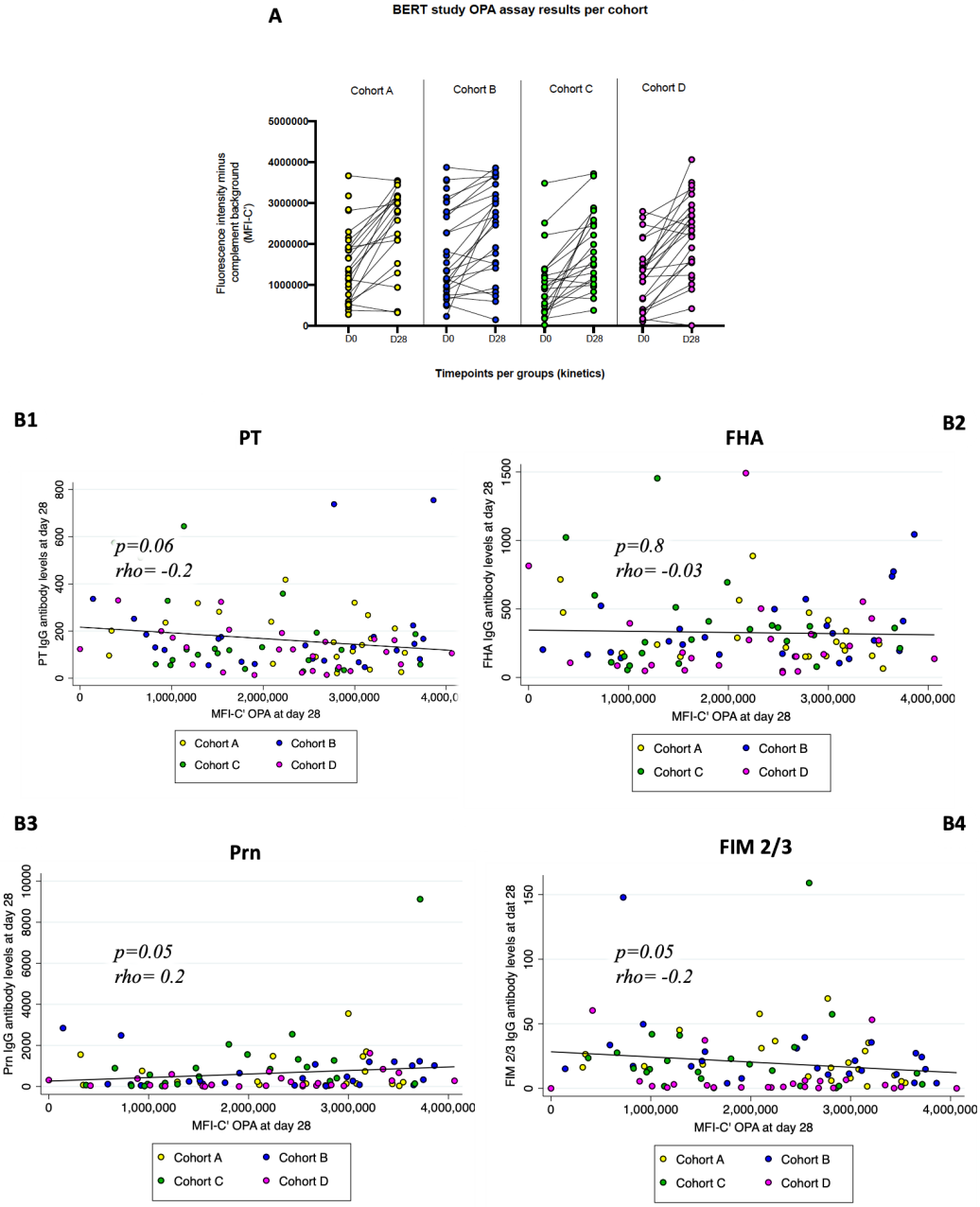


Figure 3.13 Correlation of the opsonophagocytosis assay (OPA) results with pertussis antibody levels in the BERT population (spearman correlation coefficient)

A: Kinetics of the MFI-C' of OPA at baseline and D28 after booster immunisation per cohort

B: Spearman correlation between OPA MFI-C' at D28 and pertussis specific antibody levels, PT (B1), FHA (B2), Prn (B3) and FIM 2/3 (B4).

3.4.3 Complement deposition assay (CDA)

This assay measures the complement adherence to *B. pertussis,* antibody mediated, acting as a surrogate to OPA, when evaluating the deposition of C3 and to SBA when evaluating the deposition of C5. A correlation between these two assays is presented in Supplementary figure 9.

The complement deposition mean fluorescence intensity minus complement background was used as a measure to define complement deposition (MFI-C') in the tested samples and the higher the MFI, the higher the deposition in the bacteria.

A total of 84 samples at baseline and 83 samples at D28 of individuals from the BERT study were studied. At baseline the cohort with the highest median was cohort A for both C3b/iC3 and C5b-C9 (Table 3.13 and Table 3.14).

A significant increase from baseline to D28 after booster immunisation was found for both C3b/iC3b and C5b-C9 in all cohorts (Figure 3.14 and Figure 3.15). A higher mean fluorescence was seen for C3b/iC3 when compared with C5b-C9 at both timepoints. After booster dose the cohort with the highest median was the adult cohort (C) and the cohort with the lowest median was the elderly individuals (D) (Table 3.13 and Table 3.14), that corresponded to the individuals with the highest and lowest median FC respectively (median FC [IQR] C3b/iC3 and C5b/C9 **C:** 22.4 [8.2-77.4] and 14.1 [6.5-55.6] ; **D:** 4.1 [2.1-15.3] and 2.7 [1.7-12.3]).

Table 3.13 Complement deposition assay (CDA) C3b/iC3 MFI-C' median and interquartile range (IQR) in the BERT study population at baseline (D0) and D28

C3b/iC3	Timepoint		Cohort A	Cohort B	Cohort C	Cohort D
MFI-C'	D0	N	19	16	25	24
		Median	148.5	115.3	119.3	92.3
		IQR	102.4-334.3	73.2-182.7	63.8-311.9	66.0 -182.5
	D28	N	19	16	24	24
		Median	1021.1	6705.0	8423.9	329.3
		IQR	470.9-9797.5	870.0 – 12858.8	1281.6-12973.1	146.4-4863.5
		p value	<0.0001	<0.0001	<0.0001	<0.0001

Wilcoxon signed rank test

Table 3.14 Complement deposition assay (CDA) C5b-C9 MFI-C' median and interquartile range (IQR) in the BERT study population at baseline (D0) and D28

C5b-C9	Timepoint		Cohort A	Cohort B	Cohort C	Cohort D
MFI-C'	D0	N	19	16	25	24
		Median	81.7	65.2	57.4	38.4
		IQR	46.6-167.0	39.1-101.8	36.0-110.5	20.7-65.7
	D28	N	19	16	24	24
		Median	355.2	2542.8	3267.9	67.6
		IQR	144.3-3425.4	255.8-4796.6	310.0-3648.6	51.8-1249.8
		p value	0.0007	0.0007	0.0001	0.0001

Wilcoxon signed rank test

Although the differences between cohorts they did not reflect significance at baseline (Kruskal-Wallis C3b/iC3 *p=0.5*; C5b-C9 *p=0.02*), but a significant difference

between cohorts was found at D28 (Kruskal-Wallis C3b/iC3 *p=0.001*; C5b-C9 *p=0.003*).

In the evaluation of the correlation between complement deposition and pertussis, similar trends were found for both C3b/iC3 and C5b-C9, with PT showing no correlation and Prn showing the strongest correlation at both baseline (C3b/iC3 PT *p=0.2*, FHA *p=0.001*, Prn *p<0.00001*, FIM 2/3 *p=0.2*; C5b-C9 PT *p=0.2*, FHA *p=0.002*, Prn *p<0.00001*, FIM 2/3 p=0.07) and D28 (Figure 3.14 and Figure 3.15).

No correlation was found between IgA PT, FHA or FIM 2/3 antibody levels and C3b/iC3 and C5b-C9 complement deposition at D28. This correlation was detected for Prn IgA antibody levels at the same timepoint for all cohorts (C3b/iC3 Cohort A *p=0.01*, Cohort B *p=0.0009*, Cohort C *p=0.004*, Cohort D *p=0.0008*; C5b-C9 Cohort A *p=0.02*, Cohort B *p=0.0008*, Cohort C *p=0.03*, Cohort D *p=0.03*).

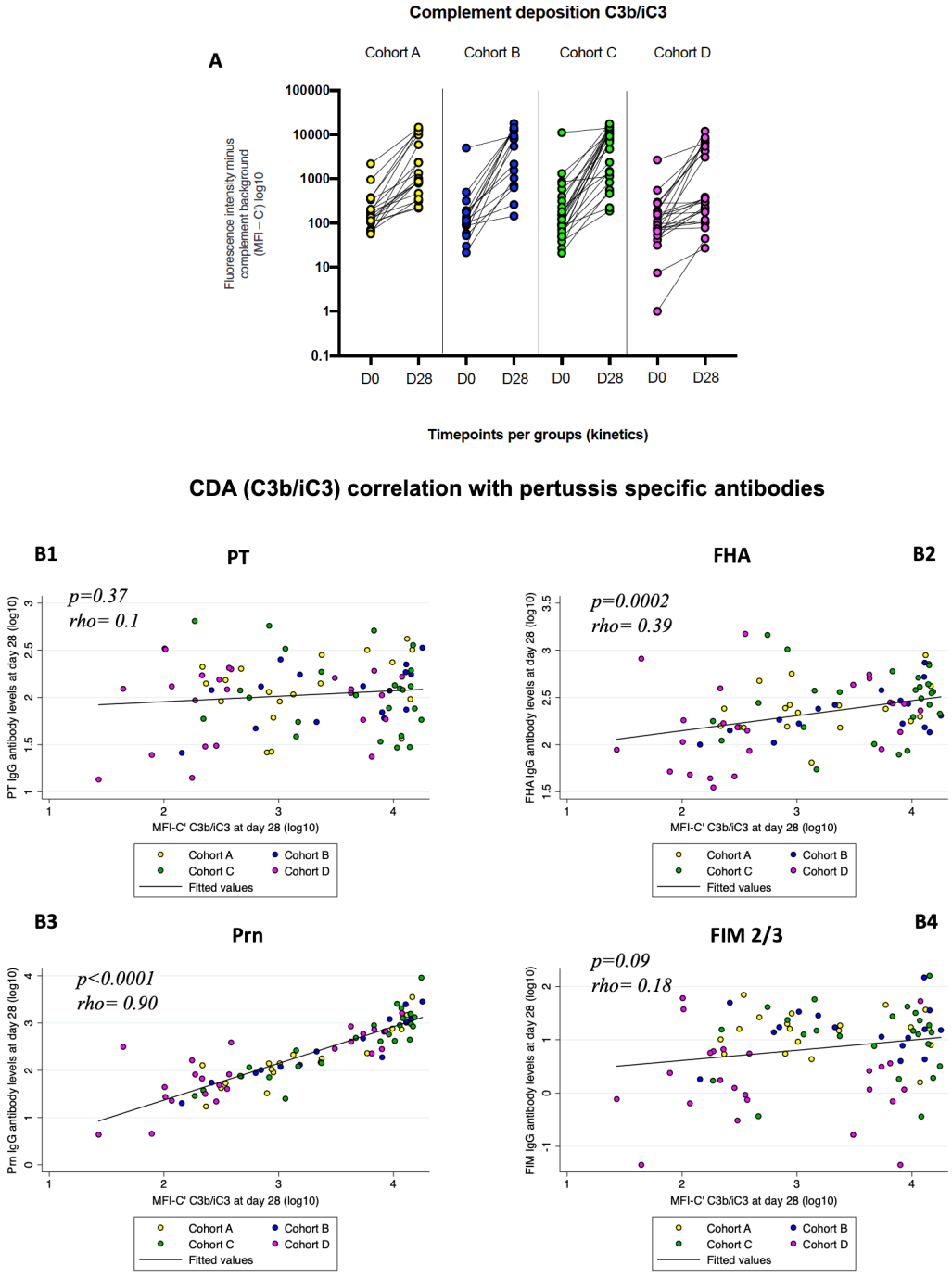


Figure 3.14 Complement deposition assay (CDA) results for C3b/iC3 in the cohorts of the BERT population

A: CDA MFI-C' C3b/iC3 at baseline and D28 after pertussis booster immunisation per cohort (Wilcoxon signed-rank test) **B:** Spearman correlation between CDA MFI-C' C3b/iC3 at D28 and pertussis specific antibody levels, PT (B1), FHA (B2), Prn (B3) and FIM 2/3 (B4).

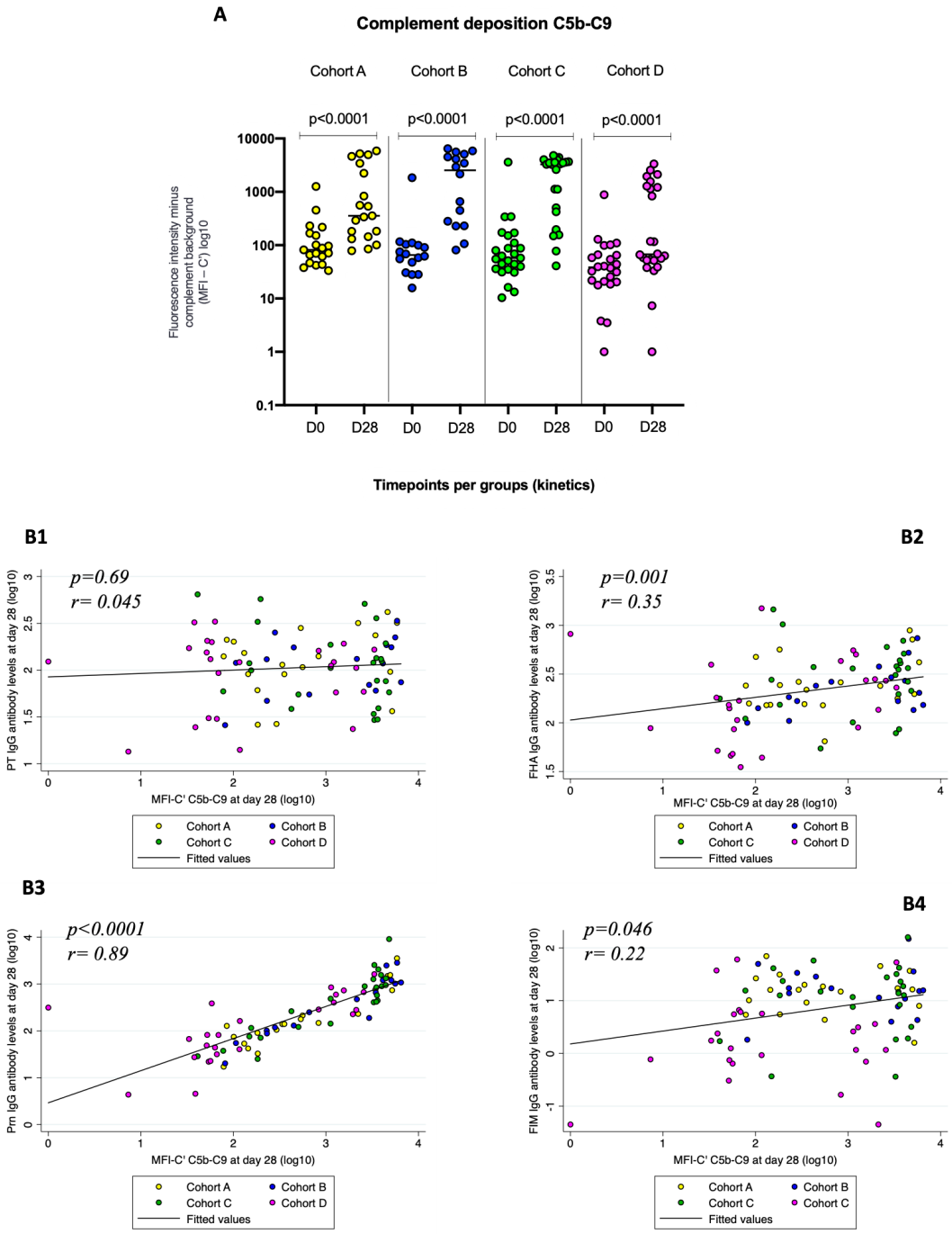


Figure 3.15 Complement deposition assay (CDA) results for C5b-C9 in the cohorts of the BERT population

A: CDA MFI-C' C5b-C9 at baseline and D28 after pertussis booster immunisation per cohort (Wilcoxon signed-rank test) **B:** Spearman correlation between CDA MFI-C' C5b-C9 at D28 and pertussis specific antibody levels, PT (B1), FHA (B2), Prn (B3) and FIM 2/3 (B4).

3.5 COMPARISON OF BERT ACELLULAR PRIMED YOUNG COHORTS (A AND B) BASELINE IMMUNOGENICITY DATA WITH A WHOLE CELL PRIMED BIOBANK UK POPULATION WITH SIMILAR AGE

The biobank UK wP primed population (Children_UK_wP and Adolescent_UK_wP) was used as a comparison for the analysis of baseline pertussis specific antibody levels of the individuals recruited as part of the BERT study that were aP primed (cohort A and B).

3.5.1 Whole cell primed UK biobank populations

From the Children_UK_wP, 43 samples of individuals with ages between 9 and 12 years were available and analysed (median age [IQR]: 11.2 years [10.6, 11.8]). Besides the evaluation of the results in the overall population, the Children_UK_wP population was divided by age within the BERT acellular primed cohorts (A and B). The younger group, matching BERT cohort A had a median age (IQR) of 10.4 (9.9-10.7) years and the adolescent groups, matching BERT cohort B had a median age (IQR) of 11.6 years (11.3-12.2) (Table 3.15).

From the Adolescent_UK_wP a total of 565 samples were available from individuals aged 11 to 20 years (median age [IQR]: 14.8 [13.3, 16.2]). Around 99% were classified as Caucasian. To understand potential differences in the antibodies GMCs in a population with a wide age range, the antibody data was also divided in three different age cohorts: 11 to 13 years, 14 to 16 years and 17 to 20 years of age. A subgroup analysis of individuals matching the adolescent cohort of BERT study (B) was also performed (Table 3.16).

The subgroup had a median age (IQR) of 13.9 (13.0-15.0) years.

Table 3.15 Baseline pertussis specific antibodies levels geometric mean concentrations (GMC) in the Children_UK_wP study population, including results divided by age groups matching BERT study acellular primed cohorts (A and B)

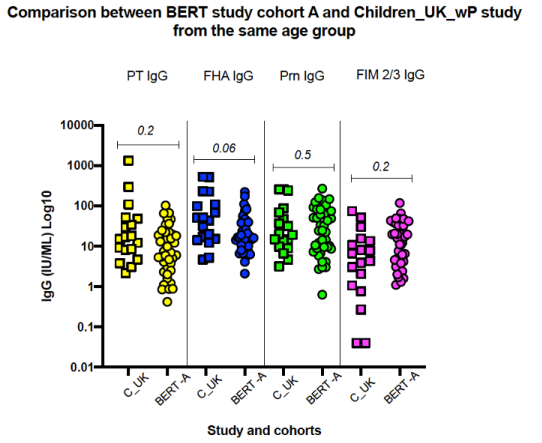
Children_UK _wP		GMC in different age groups		
Antigen	Statistic evaluation	Overall population N=43	**Age group matching BERT cohort A N=18**	**Age group matching BERT cohort B N=25**
PT IgG	GMC	15.5	20.6	12.6
	95%CI	8.8, 27.3	9.1, 46.6	5.6, 28.5
FHA IgG	GMC	40.2	45.7	36.6
	95%CI	26.6, 60.6	22.5, 92.9	21.5, 62.3
Prn IgG	GMC	37.2	26.8	47.0
	95%CI	23.6, 58.6	13.7, 52.5	24.8, 89.2
FIM 2/3	GMC	7.2	3.5	12.1
IgG	95%CI	3.7, 13.7	1.2, 0.9	5.3, 27.5
PT	GMC	15.5	0.32	0.31
IgA	95%CI	8.8, 27.3	0.19, 0.6	0.19, 0.51

Table 3.16 Baseline pertussis specific antibodies levels geometric mean concentrations (GMC) in the Adolescent_UK_wP study population, including results in age group matching BERT study acellular primed adolescent cohorts (B)

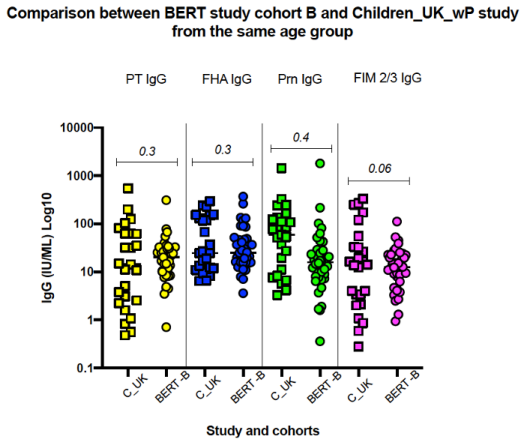
Adolescent_UK _wP		GMC in different age groups				
Antigen	Statistic evaluation	11-13 years N=203	14-16 years N=292	17 to 20 years N=70	Overall population N=565	**11-15 years (Age group matching BERT cohort B) N=402**
PT IgG	GMC	6.1	5.8	7.0	6.1	6.3
	95%CI	4.8, 8.0	4.8, 7.2	4.5, 11.1	5.2, 7.1	5.2, 7.5
FHA IgG	GMC	28.4	21.1	16.7	22.8	24.3
	95%CI	24.4, 33.0	18.4, 24.2	11.8, 23.7	20.7, 20.2	21.8, 27.2
Prn IgG	GMC	18.1	13.8	16.6	15.6	15.5
	95%CI	14.6, 22.5	11.6, 16.5	11.8, 23.3	13.7, 17.7	13.3, 18.0
FIM 2/3	GMC	18.6	14.6	14.6	15.9	15.3
IgG	95%CI	14.6, 23.7	11.8, 17.9	9.5, 22.5	13.7, 18.4	12.8, 18.3
PT	GMC	0.37	0.61	0.87	0.53	0.47
IgA	95%CI	0..31, 0.43	0.54, 0.69	0.66, 1.2	0.48, 0.58	0.42, 0.53

When analysing the different comparisons between the UK wP population and the BERT study at baseline, except for the differences between FIM 2/3 IgG in the comparison between BERT cohort B and Adolescent_UK_wP from the same age groups, no other differences were identified (Figure 3.16).

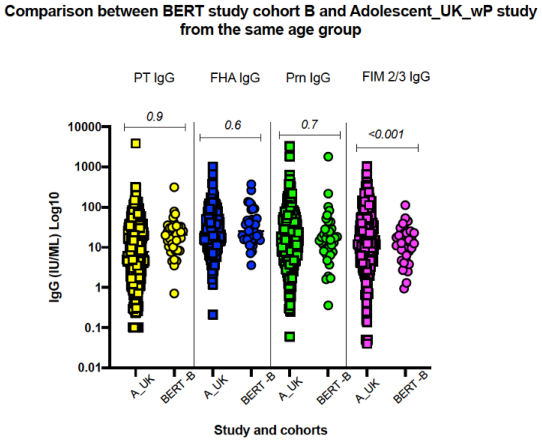
A



B1



B2



C_UK: Children_UK_wP

A_UK: Adolescent_UK_wP

Figure 3.16 Dot plots with comparison between IgG pertussis specific antibodies levels (PT, FHA, Prn and FIM 2/3) in the aP primed BERT study population and biobank samples from the UK studies from the same age ranges, using Welch's T test

A: Comparison between BERT cohort A population (7-10 years of age) and Children_UK_wP population (9-10 years of age); **B1:** Comparison BERT cohort B population (11-15 years of age) and Children_UK_wP population (11-12 years of age); **B2:** Comparison BERT cohort B population (11-15 years of age) and Adolescent_UK_wP population (11-15 years of age).

3.6 DISCUSSION

3.6.1 Summary of the main findings

For the majority of the antibodies no significant differences were found at baseline when comparing individuals from the same age groups with different backgrounds (BERT A and B cohorts - aP primed *versus* Biobank children and adolescent cohorts - wP primed).

In the BERT study a significant increase of IgA and IgG antibody levels after booster immunisation, independently of the immunisation background and age, for all antigens contained in the vaccine (PT, FHA and Prn), was identified.

In general, the IgG antibody with highest levels after booster was Prn, except in the elderly cohort, which was FHA. In response to the vaccine, PT was the antibody with the lowest levels, particularly PT IgA.

For IgG the peak was between D14 and D28, but the antibody levels remained higher one-year post-booster when compared with pre-immunisation levels. The younger cohorts, particularly the children (A), had, in general, the quickest increase of antibody levels after boost, with high antibody levels on D7, showing a quick immune response to the antigen, an important feature of memory.

For IgA, an effect of age was identified, with a tendency for older age groups to have higher IgA antibody levels after booster immunisation when compared with younger individuals.

Although in general the elderly had the poorest responses to immunisation, a tendency to slower waning of antibody levels from D28 to D365 after booster in the older cohort was seen in this population, particularly in Prn.

Higher antibody functionality/quality measured by SBA, OPA and CDA assays had a stronger correlation with Prn IgG antibody levels, when compared with other antibodies like PT and FHA.

3.6.2 Analysis of current results based on the available literature

PT is a secreted toxin that is specific to *B. pertussis* and not found in other *Bordetella* species [287]. Although the presence of other antibodies against other virulence factors like FHA, Prn or FIM can be detected after infection by other *Bordetella* species, it is also expected an increase in these antibody levels after infection with *B. pertussis* [222,284,294-296].

There are few detailed studies of antibody kinetics for any antigen in the first month after immunisation that allow a comparison of the speed of antibody response between groups with different characteristics. For some infections the ability to mount a rapid response to limit bacterial replication and invasion may be important in preventing symptomatic disease once initial infection has occurred. This is a poorly studied aspect of immunological memory which has tended to focus on the final magnitude and functional quality of the secondary antibody response. For all pertussis-specific antigens contained in the vaccine (PT, FHA, Prn) there was a marked and quicker increase in absolute antibody concentration in the younger groups. For PT this was especially marked in the children and adolescent versus adults, worse with the increase of age.

In summary, younger groups started with higher antibodies, but also had a quicker and higher increase in the first seven days. After the 1st week, the older groups that started with lower antibody levels, had steeper increase, with all groups achieving

similar levels at D28, except in the elderly that consistently showed lower levels for IgG antibodies and the highest levels for IgA.

This ability to mount a quicker response could be relevant in terms of disease pathogenesis and could have a role on limiting carriage. Also, this quicker response could be considered an important feature of memory from previous exposure to the antigen. This response was much more marked for IgG pertussis specific antibodies when compared with IgA. The stronger responses in the younger age groups are consistent with previous literature [297] and although it is difficult in this study alone to identify if it is just the effect of age or the combination of immunisation background and time since the last pertussis immunisation, it is well known the lower vaccine responses after a stimulus in later life due to immunosenescence [298]. These overall lower responses do not mean a quicker waning. As described in this study, after the peak IgG responses, the waning of pertussis specific antibodies tends to be faster in the younger groups, with PT IgG antibody showing to be the antibody with the fastest waning. This pattern was previously described after pertussis disease [283], and similar following both aP immunisation and natural infection.

Before the generalised introduction of aP in high income countries it was already a concern from the medical community how to differentiate natural infection from previous immunisation to pertussis. In the early 1980's it was a generalised assumption that IgA could only be detected after pertussis natural infection, but not after immunisation where only IgG and IgM had a significant expression [286].

The low IgA levels and the significant increase of the IgA pertussis specific antibodies with age (highest in the elderly) was seen consistently across all pertussis specific antibodies, although the magnitude of responses was much lower than IgG, similar to what is described in the literature [285,299].

The differences on the behaviour of these two Ig classes after a specific stimulus, like a vaccine, could be related with the route that they encounter the antigens. Pertussis specific IgA antibodies were likely to be stimulated mainly by mucosal antigens due to a previous infection or colonisation [212], with lower stimulation when the main route is via an intramuscular injection, showing that IgA is not a good marker of vaccination. The evaluation of mucosal antibodies would allow us to better understand this role.

The BERT population included a group of people with different ages and consequently different pertussis immunisation backgrounds. In the comparison between the BERT aP primed individuals and the UK wP primed biobank populations, in general, no statistically significant differences were identified, showing an antibody waning and similar baseline antibody levels independently of the background.

Understanding antibody functionality after pertussis immunisation and the differences associated with wP and aP can contribute to a better understanding of pertussis immunology and to define, in the future, a correlate(s) of protection.

Assays have been developed to measure the SBA and OPA of individuals with immunity against *B. pertussis* and both assays have been shown to correlate with antibody-mediated complement deposition. The use of a complement deposition assay can work as an indirect measure of OPA by the C3b/iC3b deposition and SBA by the C5b-9 deposition, with the advantage of being a quicker, less laborious assay, with theoretically similar results. The complement has a relevant role in the elimination of bacteria [172,251]. The basic mechanism is the deposition of C3 in the bacteria surface allowing the phagocytic process to start eliminating the bacteria and also the formation of C5 convertases that contribute to the final composition of MAC lysing the bacteria [172]. Also, the presence of complement in the mucosa has a particular role

considering the pathogenesis of this agent [172]. A significant correlation between CDA and OPA or SBA was identified in this study, with stronger correlations identified between SBA and CDA, particularly post-booster immunisation. No specific or stronger correlation was found between C3b-iC3 and C5b-C9 and OPA or SBA, respectively (Supplementary figure 9).

A significant increase of SBA and complement deposition of both C3b/iC3 and C5b-C9 after the booster immunisation was seen for all cohorts, showing that the vaccine stimulates the production of antibodies with activity against bacteria.

After a booster dose with aP, the adolescents (B) and adults (C) had the highest SBA titres and for the CDA assay the adults (C) had the highest responses. This shows a potential long-term effect of wP priming, although it is not clear if this reflects the antibody quality *per se* or also a wider range of pertussis specific antibodies against additional antigens presented in the wP vaccine but not aP.

Prn IgG showed the best correlation with functional assays. Although not as strong and not always significant, the second antibody with the best correlation was FHA IgG. It can be hypothesised that this correlation is related to these antigens being expressed at the cell surface and not secreted as PT. An exploration of these correlations and the role of antibodies in bacteria adhesion, elimination and role of complement are further explored in other age groups in CHAPTER 5.

Although the unquestionable advantages of the use of functional antibody assays to determine protection against a disease, this approach is more expensive and time consuming when compared with the traditional measure of antibody levels. Furthermore, it is not completely clarified if the results of functional antibody assays 28 days after booster immunisation are associated with an increase in functionality or just a consequence of antibody increase, considering that other vaccine studies

showed that the peak of functionality was achieved later in time when compared with the time needed to achieve the peak of antibody levels [300]. The one-year post-immunisation data would have allowed a better understanding of the kinetics of functionality in association with antibody levels and the correlations between these two variables. A full understanding of these assays could be possibly achieved in the human challenge models, performed as part of the Periscope Consortium where all interventions are controlled.

3.6.3 Limitations

The data presented from the BERT study is only regarding the UK and not the full population enrolled as part of the multicentre study, which could limit the interpretation of the data, since the power calculation was performed based on the original design. In the full multicentre study, power calculation was performed based on the primary endpoint “a sample size of 108 per child cohort, across all countries was estimated to give 80% power to detect a standardised difference in log-PT-IgG at one month post booster of 0.42 IU/ml between age cohorts, allowing a 15% loss” [301]. Considering the UK 36 individuals per paediatric cohort (A and B), we would be able to identify a standardised difference of 0.66 IU/ml, with no loss of follow or 0.74 IU/ml, when considered a potential 15% loss of follow-up.

It is reassuring that the results regarding the serology data already analysed [301] showed similar results to UK study population.

The immunisation backgrounds were not always available for all the study populations and for the individuals with no records it was based on the DOB and NIP implemented in the UK. Although it is likely that the correct immunisation background

was used, we need to acknowledge that potentially mistakes may have happened in a residual number of individuals. Another limitation of this study is the difficulty to distinguish between the effect of age, immunisation background and effect of time since the last pertussis vaccine. Furthermore, recent exposure to disease cannot be accounted for.

Although some differences in responses can be identified in extremes of age, like in the elderly cohort as previously described, it was not possible in this study to identify if the differences found for IgG pertussis specific antibody responses were age related or dependent of previous immunisation background, since individuals from the same age groups had the same primary immunisation. To try to overcome this issue in BERT multicentre study, The Netherlands and Finland were able to include in cohort B (adolescents) individuals from both immunisation backgrounds. This was not possible in the UK, considering the year the aP vaccine was introduced and current immunisation schedules. The low number of individuals in each subgroup in the multicentre study analysis did not allow to draw any definitive conclusions, but no differences were found in the antigens contained in the booster vaccine (PT, FHA and Prn) at baseline and 28 days post-immunisation when comparing wP and aP primed adolescents [301].

Another limitation was the lack of completion of the functional antibody assays, due to the COVID-19 pandemic. This fact led to a lower number of assays performed, lack of one-year post-immunisation results, limiting comparisons between groups and a better understanding of the kinetics of the antibody functionality.

For the comparison with biobank population, one limitation of the interpretation were the differences is the number of contacts with the bacteria through life, with all

individuals from the younger cohorts of the BERT study being exposed potentially to higher rates of disease during the 2011/2012 outbreak, when compared with biobank populations from the 1990's or beginning of the 2000's.

3.6.4 Conclusions

A significant increase in antibody levels for all age groups after a booster immunisation was seen. This shows that booster immunisation with a pertussis vaccine is relevant for protection and should be considered for younger groups, but also other fractions of the population. The question about prevention of colonisation, that could impact disease transmission for vulnerable unvaccinated groups, is still a subject of discussion. The faster increase of antibody levels in younger aP primed age groups can work as an early marker of memory, that is kept years after the last booster immunisation. Despite this fast increase, these cohorts also have a quicker waning of the antibody levels when compared with wP cohort, a potential consequence of the different backgrounds.

Functional antibody assays show that the booster is associated with an increase not only on the quantity but also on the quality of the antibodies, with Prn levels showing to better correlate with this functionality. This antibody is not *B. pertussis* specific but represents an adhesin, that contributes for the first steps of bacterial colonisation and stimulation of innate immunity, contributing to bacteria elimination and potentially for prevention of colonisation. How long this immunity lasts is still not fully understood and functionality could be lost maybe quicker in individuals with no recent immunisations and aP primed.

Understanding the role of cellular immunity, including the kinetics of MBCs after stimulation, T cell patterns and correlation with antibody functionality is explored in the next chapter.

CHAPTER 4

CELULLAR IMMUNE RESPONSES TO PERTUSSIS IN DIFFERENT AGE GROUPS AND DIFFERENT IMMUNISATION BACKGROUNDS

4.1 BACKGROUND

4.1.1 General considerations

After the first contact with a pathogen the immune system has the ability to produce a variety of cells that contribute to the development of memory [219,224]. Antibody response is generated by the underlying B-cells (MBCs and plasma cells) and their interaction with T-cells. Assessing such cellular populations may give more insight into the ability of vaccines to generate long-lived antibody and more pronounced secondary antibody response.

The antibodies are first produced by short lived plasma cells. The antibody levels can be kept by long-lived plasma cells that are present in the bone marrow, designated constitutive humoral memory (CHM), contributing to the neutralisation of the agent. If the antibodies levels are insufficient, the “reactive humoral memory” is induced and pathogen specific MBCs are activated, differentiated into plasmablasts and start producing specific antibodies, with higher affinity when compared with primary infection [219,302].

Different mechanisms are involved in the development of MBCs, the most common process is T cell dependent, where B cells interact with CD4+ T cells in the border of the B cell follicle of the secondary lymphoid organ. The B cells can then

migrate to the germinal centre (germinal centre-dependent MBCs) and undergo clonal expansion and diversification of B cell receptor (BCR) by somatic hypermutation at the dark zone ($CXCR4^{hi}$ $CD83^{low}$ $CD86^{low}$) and affinity selection at the light zone ($CXCR4^{low}$ $CD83^{hi}$ $CD86^{hi}$) [219], leading to the development of high affinity MBCs [303]. These high affinity MBCs are able to bind strongly with specific antigens, with increase B-T_{FH} cell interaction.

A subset of MBCs can be developed by a germinal centre-independent process. They are generated from a CD38/ GL7 precursor after CD40 L signalling from Th cells, particularly if the conjugate formation between T_{FH} and B cells are for a short period, allowing only class switching, but failing somatic hypermutation, giving a wider range of MBCs but with less affinity [219].

Not all MBCs undergo a T cell dependent process. The T cell independent MBCs have been described, although is still not fully known if the type of response is similar to high affinity and quicker response described for T cell dependent MBCs.

Although B cells are associated with antibody protection, the B cells role does not end with antibody production. MBCs can also be detected even in the absence of detectable specific antibody levels in peripheral blood, showing a possible role in long-term protection depending on the presence of the right stimulus [304]. Although this fact, previous studies have shown that immunity wanes rapidly after pertussis immunisation, particularly after the use of aP, suggesting that both vaccines might elicit different immune responses [305].

4.1.2 MBCs responses after immunisation with pertussis vaccines

The circulating B cells just represent a fraction of the available B cells.

In a study by *Stenger et al* using a mouse model, animals primed with either DTP vaccine or a 5-component DTaP, showed that IgG specific pertussis responses peaked at early time points, and waned when evaluated more than one year after primary immunisation [196]. Although this fast decline the long-lived bone marrow plasmablasts increased when compared with the early time-points, although the spleen specific MBCs were undetectable more than a year after immunisation for PT, Prn and FHA. The decline of spleen MBCs until the last time point (> 1 year after immunisation) was only significant in the mice immunised with DTaP [196].

Following the exposure to pertussis it is still not completely defined if the maintenance of long-term memory is dependent on ongoing contact with pertussis antigens that would allow activation of MBCs to ASC or if that occurs without any type of re-exposure but dependent on other factors related to the primary exposure, like type and timing of vaccination or primary infection [196,225]. It is likely that both factors need to be taken into consideration.

If the correlation between antibody levels and MBCs seems not to be consistent for pertussis, exploring antibody functionality as a measure of prediction of long-term memory should be considered. Antibody avidity as a measure of antibody functionality, seems to also influence the maintenance of IgG MBCs and could contribute to the lack of long-term memory [211]. *Van der Lee et al* described a difference in avidity between PT and Prn after a booster dose with aP in adults primarily vaccinated with wP. This study showed that PT- IgG antibodies increased in avidity after the booster dose, change that is not identified with Prn, an antibody that showed at all timepoints (pre-

booster, two weeks and four weeks after) consistently lower mean avidity percentage than PT [222].

When comparing two vaccines with different compositions like wP and aP, we also need to consider that potentially they can stimulate different subsets of B cells, that can contribute differently for long-term immunity. In a novel live intra-nasal pertussis vaccine (BPZE1), the phenotype MBCs were analysed in depth by flow cytometry, showing that the individuals with positive colonisation had an increase of activated MBCs and tissue like MBCs [306]. In a primary vaccinated population with wP and boosted with aP, an increase in several B cell subsets, including plasma, naïve and MBCs were identified, two weeks after booster immunisation [307].

Another concept yet to explore in pertussis, that can contribute to explain some of the differences between vaccines, is the presence or not of atypical MBCs. These cells, with characteristics not fully described to date and identified in chronic diseases like malaria and HIV, seem to decrease the survival of plasma cells, contributing to failure in long-term immunity [218]. Although we can argue that diseases like malaria or HIV have completely distinct characteristics when compared with pertussis, the lack of a durable antibody response or chronic/frequent antigen exposure are some points in common [218].

Data regarding pertussis MBCs responses are heterogenic, by the use of different pertussis vaccines, different timepoints and different stimulation conditions, limiting the conclusions.

4.1.3 Other players of the cellular immune response – T cells

The role of T cells in the immune response to pertussis disease/immunisation was first described in the animal model and can partially explain the differences in the responses to aP and wP.

In CHAPTER 1, the role of T cells in the animal model is described.

The number of T cells studies associated with disease or immunisation against *B. pertussis* infection in humans increased from the 1990's. First, focusing on natural infection, it was described in recent cases of pertussis disease an increased production of IFN- γ, a cytokine produced after Th1 induction [308,309]. In another study by *Ausiello et al* comparing aP primed children with children with previous pertussis disease, both induced IFN- γ responses, but previously aP vaccinated individuals showed mixed Th1/Th2 responses [310]. These findings were described in naïve young infants, where Th1 type responses were induced by natural infection in children with no previous immunisation [311]. In the following years, several studies have showed that pertussis immunisation was associated with mixed Th1/Th2 responses, with wP primary immunised infants showing a skewed Th1 response when compared with aP primed [312-314].

One of the most relevant studies in humans, by *Sette's* group, had the aim to compare the T cell responses in populations with two different pertussis immunisations backgrounds, aP and wP, after a booster immunisation with aP [259]. This study, that used cultured PBMC, showed that wP primed individuals had a higher proliferation of pertussis specific $CD4^+$ T cells when compared with aP [259]. Also, the wP individuals had a skewed response type Th1/Th17, translated by the polarization of IFN- γ/IL-17 and an increased IgG1, showing differences in the type of immune response

considering that aP primed individuals had an IL-4/IL-9 polarization, showing skewed Th2 response [259].

As part of the PERISCOPE consortium studies, using BERT study samples from 73 Dutch participants, an evaluation of *B. pertussis* specific $CD4^+$ T cell responses was performed in a whole blood (WB) assay [260]. The levels of cytokines were considered generally low (300pg/ml), a finding that was attributed to the short incubation period. A significant increase in IFN-γ (Th1) in response to PT, followed by an increase in IL-17 (Th17) was reported [260]. The responses to FHA showed a higher increase in IL-17 levels, but in general the cytokine levels were considerably lower when compared with PT. Although the IL-5 and IL-13 levels (Th2 responses) remained stable from baseline and after pertussis booster immunisation, in individuals with aP primary immunisation background, IL-5 levels were significantly higher when compared with wP primed individuals [260].

This assay had two read-outs performed immediately after harvesting the supernatant and the cells after 24 hours of WB culture (using a multiplex cytokine measurement platform for cytokine determination, and by flow cytometry for determination of cell population frequency), without any intermediate freezing step. It was proposed by the consortium members the development of a simpler variant of the original assay, with the following basic principles: 1) to omit the secretion blocking step; 2) to freeze the harvested supernatant after WB culture at -80°C. This freezing step would allow to perform the cytokine read out at a convenient time for the trial, centralized for all research centres and maximizing the number of samples at the multiplex. To achieve this aim we performed a pilot T cell assay study in the BERT UK population.

The methods are described in CHAPTER 2 and the results of the assay are presented in this chapter.

4.2 BERT STUDY: UK STUDY POPULATION

4.2.1 BERT study population used to evaluate B cell responses

The BERT study (CHAPTER 2 section 2.2.1) was used to compare the MBCs responses at baseline and after pertussis booster immunisation. MBCs kinetics will be described considering the following timepoints: baseline, D28 and D365 after booster immunisation with Boostrix®. We pre-selected a representative subgroup of participants from the paediatric cohorts (A1, B1 and B2) in order to match the number of individuals available from adult cohorts. For the evaluation of plasma cells, we pre-selected individuals from cohort A and B with visits schedule at D7 (A1 and B2). MBC and plasma cell ELISpot were programmed to be performed in all individuals from cohorts C and D. For MBC the assay was only performed in individuals with PBMC's available at baseline.

MBCs ELISpot assays were done in a total of 85 patients. The mean age from cohort A population was 9.26 years and from cohort B 12.72 years. The mean age of the individuals in cohort C was 26.23 years and of cohort D was 65.33 years old.

Also, to complete the cellular immunity analysis, a pilot study with whole blood samples to study T cell responses by the analysis of cytokine patterns at baseline and after booster immunisation was performed. Samples from a total of 18 participants were processed, 10 from aP primed population and 8 from older individuals (cohort C and D).

As previously described, individuals from cohort A and B were primarily immunised with aP, individuals from cohort C were primarily immunised with wP and the majority of individuals from cohort D were not immunised with any pertussis vaccine in the first years of life.

4.3 PERTUSSIS SPECIFIC MBCS RESPONSES IN THE BERT POPULATION

4.3.1 MBCs frequencies at baseline and after booster immunisation

Methods:

A full description of MBCs ELISpot method is available in CHAPTER 2, section 2.3.4.

Measurement of MBCs IgG responses to specific pertussis antigens (PT, FHA, Prn, FIM 2/3) was performed using a cultured B-cell ELISpot assay from frozen PBMC previously isolated from fresh whole blood as part of the BERT study.

A mixed-effects model was used to look at differences between groups and time-points on log(10) transformed data. For MBCs the frequency in the wells with no response, was arbitrarily assigned a value of 0.01 in order to allow log(10) transformation when needed for analysis or plotting. Although no negative 95% CI were identified when using the raw data, the log10 transformation lead in some specific antigens (particularly when the overall frequencies were considered low) to negative lower limit of 95% CI that could be assumed as 0, since no negative counts are of physiologic nature.

Results:

In general, antigen-specific MBCs could be detected for all antigens and there was evidence of vaccine induced boosting for the antigens contained in the vaccine across all ages with a peak response at D28. FHA IgG MBCs had the highest overall GM frequencies, with PT showing the lowest responses.

The cohort with the lowest MBCs responses was the elderly (D).

a. PT IgG specific MBCs

PT IgG MBCs had the lowest baseline GM from all tested antigens, a pattern that was maintained after the peak of booster response (D28) (Table 4.1 and Figure 4.1). No differences between cohorts for this antigen were identified. The adolescents (B) had the highest MBCs GM frequencies at D0, similar to the antibody responses at the same timepoint. These high frequencies in cohort B were considered significantly higher in comparison with adults (C: *p=0.008)* and elderly cohorts (D: *p=0.001*). By D28 there was a significant increase in MBC GM frequencies in all cohorts, and the differences detected at baseline between cohorts were lost. Of note, by D365 although there was a decrease in MBCs GM frequencies, they remained above baseline levels for all cohorts, showing the same pattern seen in D28, with no significant differences identified between cohorts (Figure 4.1).

Table 4.1 PT IgG memory B cells (MBCs) geometric mean (GM) frequencies and 95% CI per cohort at baseline (D0), D28 and D365 in the BERT study population

Antigen	Timepoint		Cohort A	Cohort B	Cohort C	Cohort D	All cohorts
PT (IgG) MBC Spots per 10^5 PBMC	D0	N	17	23	24	21	85
		GM	0.6	1.1	0.4	0.3	0.5
		95% CI	0.3, 0.6	0.6, 2.1	0.2, 0.7	0.2, 0.5	0.4, 0.7
	D28	N	17	23	24	21	85
		GM	2.4	3.8	2.7	2.1	2.7
		95% CI	1.6, 3.6	2.3, 6.2	1.5, 4.7	1.1, 4.0	2.1, 3.5
	D365	N	16	22	21	20	79
		GM	1.3	1.9	1.0	1.0	1.3
		95% CI	0.8, 2.3	1.2, 3.0	0.6, 1.9	0.5, 1.9	1.0, 1.7

Legend: 95% CI: 95% confidence intervals; PBMC: Peripheral blood mononuclear cells

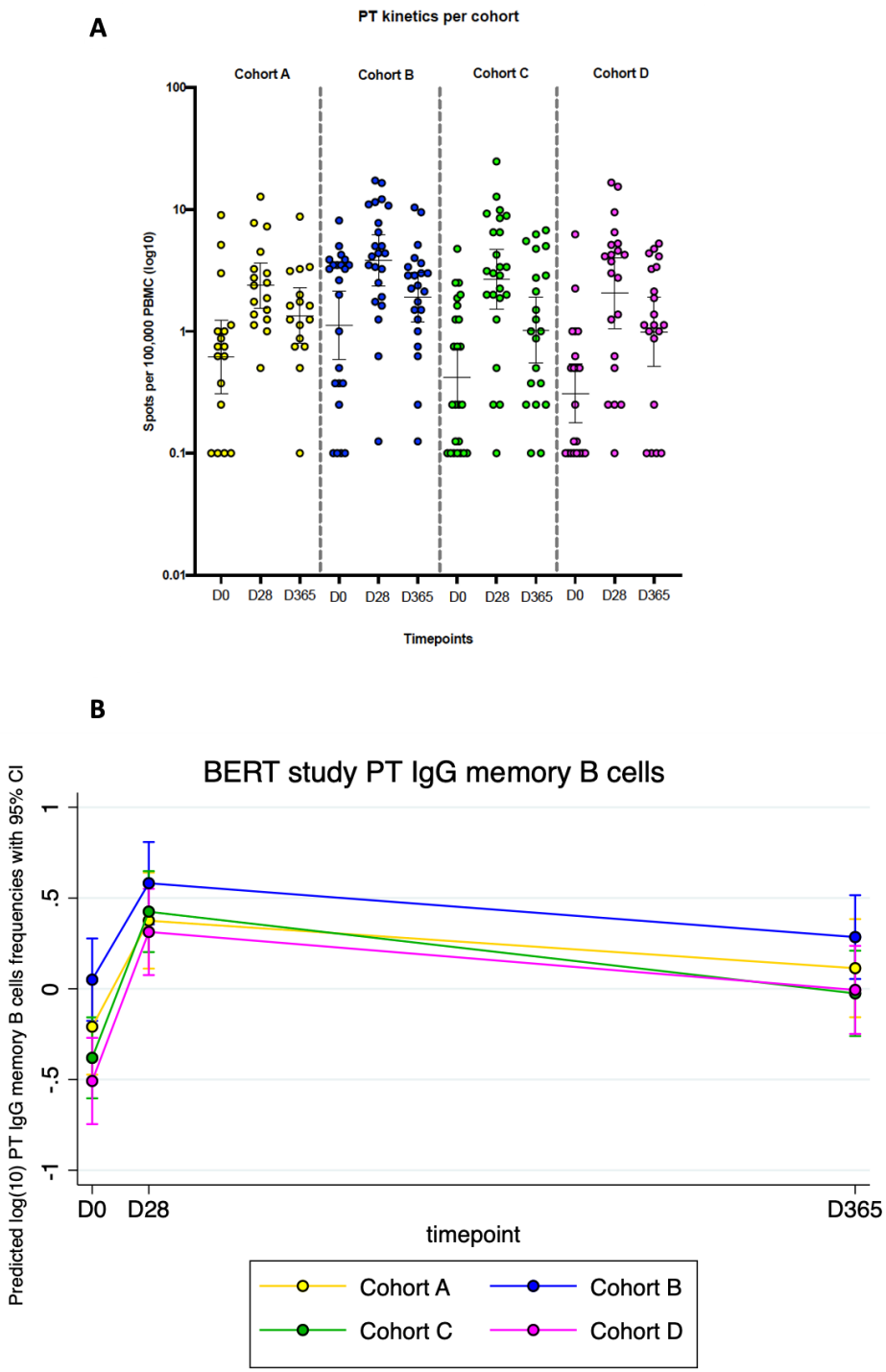


Figure 4.1 Pertussis toxin (PT) IgG memory B cell responses (MBCs)

A: Geometric mean (GM) frequencies and 95% confidence intervals (spots per 100,000 PBMC) per cohort at baseline, D28 and D365 after booster immunisation; **B:** Predicted log(10) PT IgG MBCs frequencies and 95% CI, per cohort

b. FHA IgG specific MBCs

FHA IgG MBCs GM frequencies were higher in the younger cohorts (A and B) when compared with the older individuals (C and D).

Overall, for most of the participants, FHA IgG MBCs was the MBCs with highest baseline GM frequencies when compared with other pertussis specific MBCs (Table 4.2).

At D0 the younger cohorts (A and B) had significantly higher MBCs than the older cohorts (C and D). Following the booster dose, at D28, a significant increase in all cohorts was identified, with the adolescents (B) continuing to have the highest MBC GM frequencies, but with significant differences only identified between elderly (D) and the adolescents (B) (*p=0.02).* From D28 to D365 a waning of MBC was also significant in all cohorts. At D365 these remained above baseline in all cohorts, but significant differences were identified between the elderly (D), that showed the lowest MBCs GM frequencies and the other cohorts (A, B and C) (Figure 4.2).

Table 4.2 Filamentous haemagglutinin (FHA) IgG memory B cells (MBCs) geometric mean (GM) frequencies and 95% CI per cohort at baseline (D0), D28 and D365 after booster immunisation in the BERT study population

Antigen	Timepoint		Cohort A	Cohort B	Cohort C	Cohort D	All cohorts
FHA (IgG) MBC Spots per 10^5 PBMC	D0	N	17	23	24	21	85
		GM	2.1	3.6	0.7	0.8	1.4
		95% CI	1.2, 3.6	2.3, 5.5	0.4, 1.3	0.5, 1.2	1.1, 1.9
	D28	N	17	23	24	21	85
		GM	12.7	15.1	10.7	7.3	11.1
		95% CI	9.9, 16.3	12.2, 18.7	8.2, 14.0	5.0, 10.7	9.6, 12.8
	D365	N	16	22	21	20	79
		GM	4.6	4.0	3.4	1.3	3.0
		95% CI	3.1, 6.9	2.5, 6.5	2.2, 5.2	0.7, 2.4	2.3, 3.8

Legend: 95% CI: 95% confidence intervals, PBMC: Peripheral blood mononuclear cells

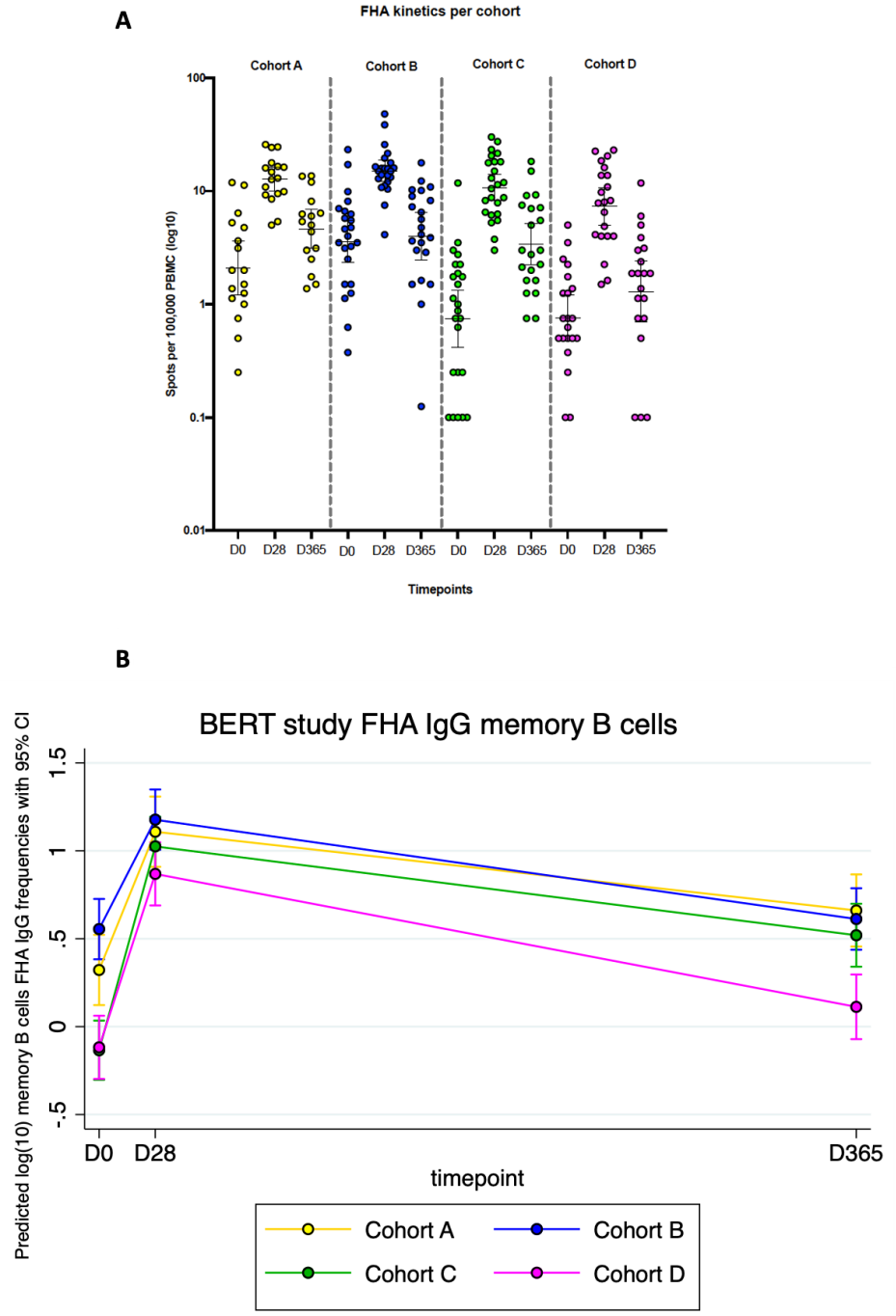


Figure 4.2 Filamentous haemagglutinin (FHA) IgG memory B cell (MBCs) responses

A: Geometric mean (GM) frequencies and 95% confidence intervals (spots per 100,000 PBMC) per cohort at baseline, D28 and D365 after booster immunisation; **B:** Predicted log(10) FHA IgG MBCs frequencies and 95% CI per cohort

c. Prn IgG specific MBCs

Overall, Prn IgG specific MBCs had the second highest GM frequencies of all tested pertussis specific antigens and similar to other MBCs peaked 28 days after booster, with a significant increase in all cohorts. Although younger cohorts showed the highest GM frequencies, at D0 no significant differences were identified between cohorts. At D28, a significant increase was identified in all cohorts, with adults (C) showing the highest GM frequencies (Table 4.3). At this timepoint significant differences were only identified between adults (C) and elderly (D) ($p=0.005$), but not between other cohorts. A significant waning from D28 to D365 was identified (Table 4.3 and Figure 4.3), but MBC GM frequencies remained above baseline for all pertussis specific antigens by D365 with no significant differences between cohorts (Figure 4.3).

Table 4.3 Pertactin (Prn) IgG memory B cells (MBCs) geometric mean (GM) frequencies and 95% CI per cohort at baseline (D0), D28 and D365 after booster immunisation in the BERT study population

Antigen	Timepoint		Cohort A	Cohort B	Cohort C	Cohort D	All cohorts
Prn (IgG) MBC Spots per 10^5 PBMC	D0	N	17	23	24	21	85
		GM	1.0	1.0	0.6	0.7	0.8
		95% CI	0.6, 1.5	0.6, 1.6	0.3, 1.1	0.4, 1.2	0.6, 1.0
	D28	N	17	23	24	20	84
		GM	6.3	6.7	11.8	4.2	7.0
		95% CI	3.2, 12.4	3.6, 12.6	7.8, 17.9	2.3, 7.6	5.3, 9.2
	D365	N	16	22	21	20	79
		GM	2.1	2.2	2.5	1.2	1.9
		95% CI	1.2, 3.8	1.4, 3.6	1.4, 4.2	0.6, 2.5	1.5, 2.6

Legend: 95% CI: 95% confidence intervals, PBMC: Peripheral blood mononuclear cells

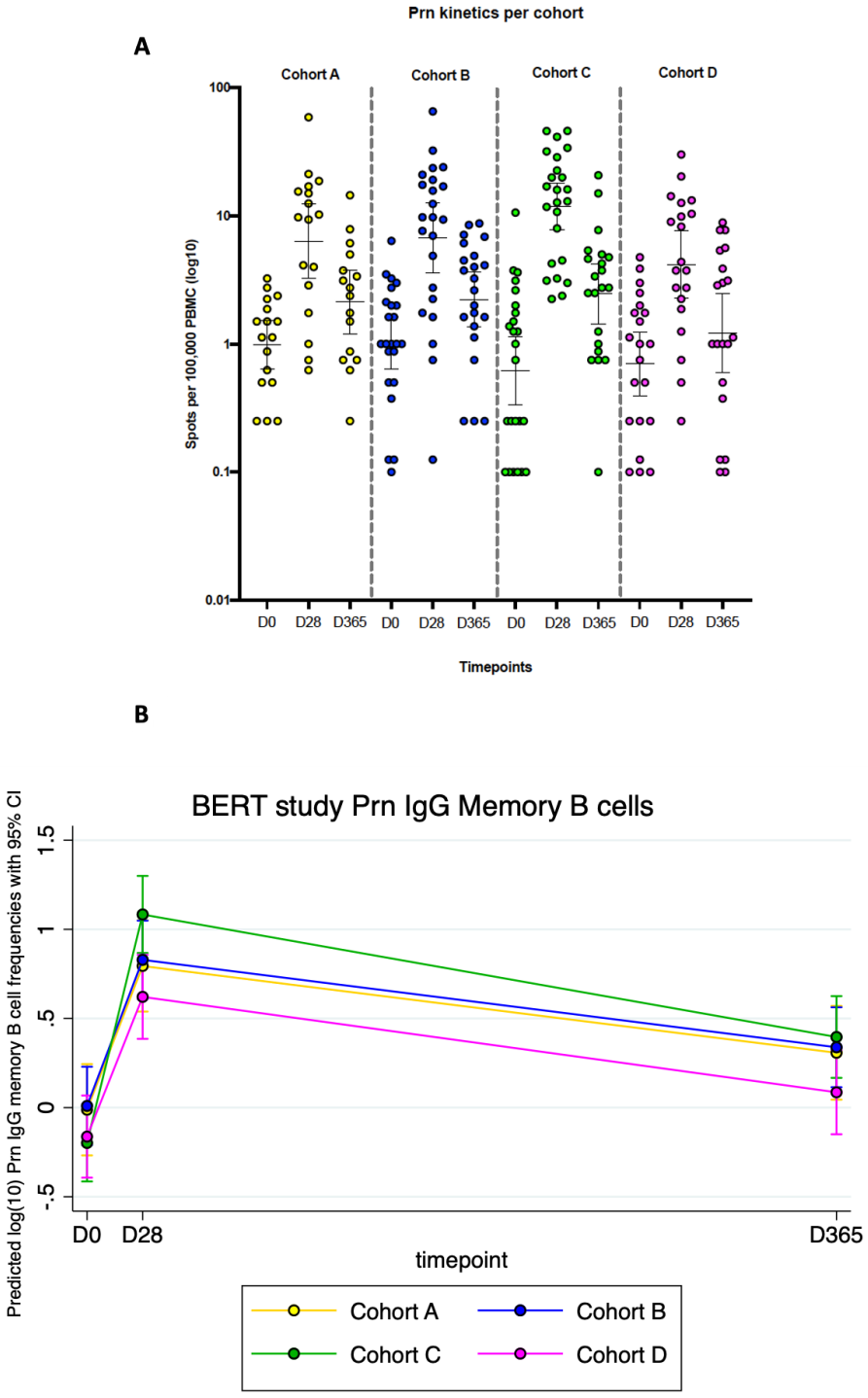


Figure 4.3 Pertactin (Prn) IgG memory B cell (MBCs) responses

A: Geometric mean (GM) frequencies and 95% confidence intervals (spots per 100,000 PBMC) per cohort at baseline, day 28 and day 365 after booster immunisation; **B:** Predicted log(10) Prn IgG MBCs frequencies and 95% CI per cohort

d. FIM 2/3 specific MBCs

For this antigen not contained in the vaccine, no differences between timepoints or between cohorts were identified. The MBCs frequencies were consistently low (Table 4.4 and Figure 4.4).

Table 4.4 Fimbriae (FIM) 2/3 IgG memory B cells (MBCs) geometric mean concentrations (GMC) and 95% CI per cohort at baseline (D0), D28 and D365 after booster immunisation in the BERT study population

Antigen	Timepoint		Cohort A	Cohort B	Cohort C	Cohort D	All cohorts
FIM 2/3 (IgG) MBC Spots per 10^5 PBMC	D0	N	17	23	24	21	85
		GM	0.4	0.6	0.5	0.5	0.5
		95% CI	0.2, 0.9	0.3, 0.9	0.3, 0.7	0.3, 0.8	0.4, 0.6
	D28	N	17	23	24	19	83
		GM	0.5	0.8	0.5	0.5	0.6
		95% CI	0.3, 0.9	0.5, 1.3	0.3, 0.9	0.2, 1.1	0.4, 0.7
	D365	N	16	22	21	20	79
		GM	0.7	0.5	0.5	0.5	0.5
		95% CI	0.3, 1.3	0.3, 0.8	0.3, 0.8	0.3, 0.9	0.4, 0.7

Legend: 95% CI: 95% confidence intervals, PBMC: Peripheral blood mononuclear cells

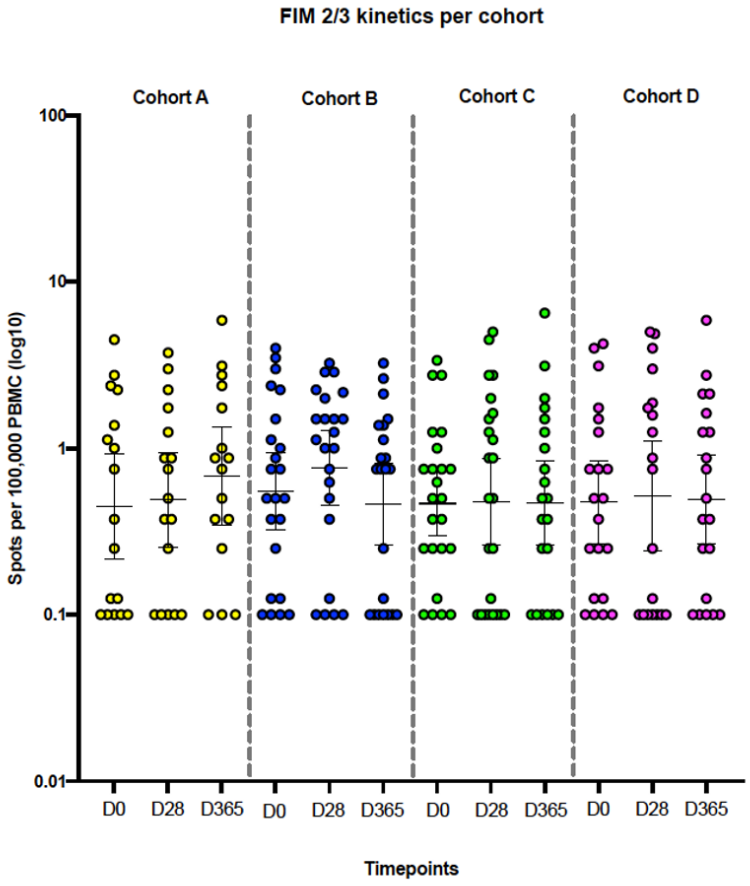


Figure 4.4 Fimbriae (FIM 2/3) IgG memory B cell (MBCs) geometric mean (GM) frequencies (spots per 100,000 PBMC) and 95% confidence intervals per cohort at baseline, D28 and D365 after booster immunisation

4.3.2 Correlation between MBCs frequencies and pertussis specific antibody levels

Background:

The MBCs populations are responsible for the maintenance of persistent antibodies through renewal of long-lived plasma cells and for the pronounced secondary response on encounter with antigen. The relationship with antibody was

explored through investigating the correlation between antigen-specific MBCs, baseline antibody and the response to boosting.

Results

Although no consistent correlation pattern was seen between MBCs at baseline and antibody levels at the same timepoint, the adolescents (B) showed the most significant correlations (Table 4.5).

Table 4.5 Correlation between memory B cells (MBCs) frequencies at baseline and pertussis specific antibodies at baseline in the BERT study population per cohort

Correlation between baseline MBCs and antibody levels at baseline (spearman correlation)						
Antigen	Statistic evaluation	Cohort A N=17	Cohort B N=23	Cohort C N=24	Cohort D N=21	Overall population N=85
PT IgG	rho	0.12	0.44	0.42	0.18	0.39
	p value	0.6	0.03	0.04	0.4	0.0002
FHA IgG	rho	0.53	0.75	-0.17	-0.08	0.31
	p value	0.03	<0.0001	0.4	0.7	0.004
Prn IgG	rho	-0.05	0.41	0.27	-0.13	0.17
	p value	0.9	0.05	0.2	0.6	0.1
FIM 2/3	rho	-0.02	0.62	0.20	0.42	0.29
IgG	p value	0.9	0.002	0.3	0.06	0.007

For the study population MBCs detected at baseline did not correlate with any of the pertussis specific antibody levels present in the vaccine present in the study (PT, FHA and Prn) measured at D28.

In this study population a correlation between MBCs frequencies at D28 after booster immunisation and antibody at D365 was identified for all pertussis specific antigens (Figure 4.5).

Per individual cohort, Prn was the only antigen that all cohorts showed to have a significant correlation (A rho: 0.66 $p=0.004$; B rho:0.73 $p=0.0001$; C rho: 0.51 $p=0.02$; D rho:0.85 $p<0.0001$) with baseline MBCs.

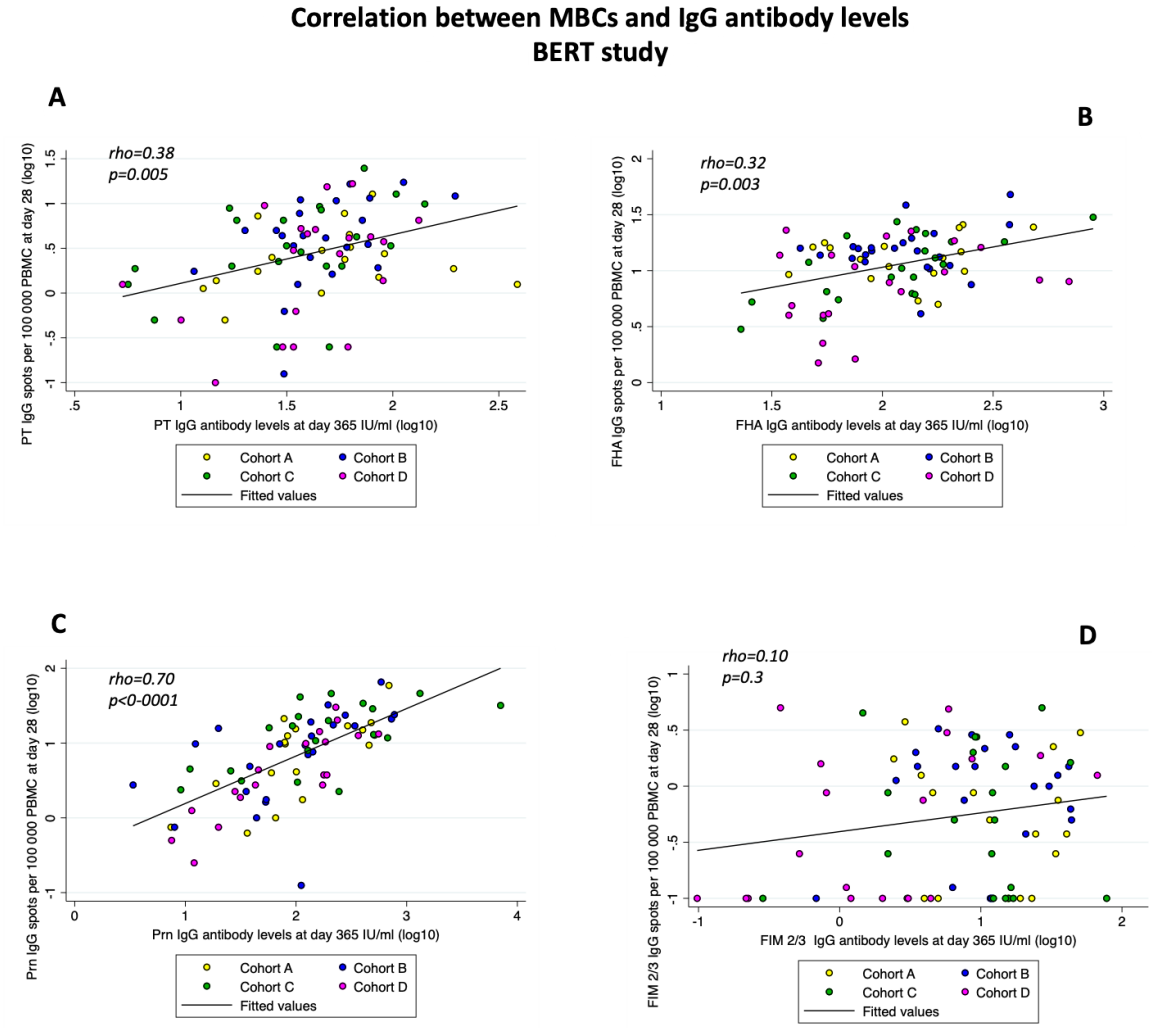


Figure 4.5 Correlations between memory B cells (MBCs) frequencies detected at D28 after booster immunisation and antibody levels at D365 after booster immunisation

Legend: A: Studied antigen pertussis toxin (PT); **B:** Studied antigen filamentous haemagglutinin (FHA), **C:** Studied antigen pertactin (Prn); **D:** Studied antigen fimbriae (FIM 2/3)

4.4 PLASMA CELL RESPONSES AFTER BOOSTER PERTUSSIS IMMUNISATION IN THE BERT POPULATION

4.4.1 Plasma cell frequencies

Methods

Plasma cells were measured using an ELISpot assay from unstimulated PBMC's after isolation from fresh blood, seven days after booster immunisation with Boostrix®. Antigen-specific plasma cell frequencies in the resting state are invariably very low or undetectable for most antigens and it was therefore decided not to measure them at baseline. A full description of the methods is available at CHAPTER 2, section 2.3.4.

Results

The assay was performed in a total of 68 participants. For PT and FHA, a statistically significant difference between cohorts was identified (Figure 4.6).

Adolescents (B) had the highest plasma cells median frequencies for all specific antibodies (PT, FHA, Prn and FIM) and the elderly (D) the lowest, when compared with other cohorts. The specific plasma cells with highest frequencies were FHA IgG (Table 4.6).

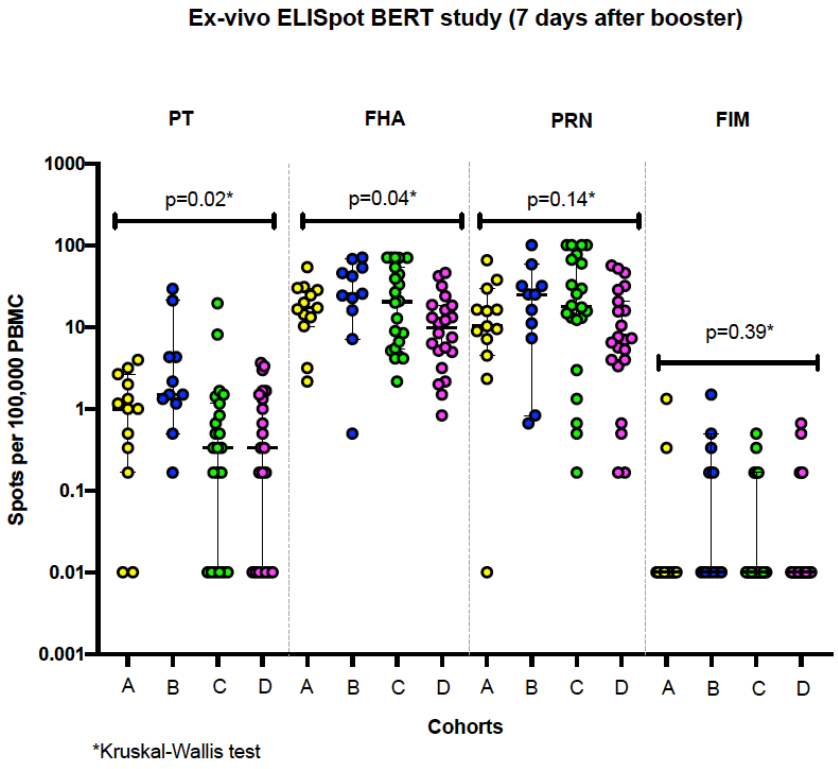


Figure 4.6 Plasma cell frequencies seven days after booster immunisation for all pertussis antigens and per cohort

Table 4.6 Plasma cell frequencies after booster immunisation per cohort and for all participants in the BERT study

Plasma cells		Median in different age groups Spots per 10^5 PBMC				
Antigen	Statistic evaluation	Cohort A N=13	Cohort B N=11	Cohort C N=22	Cohort D N=22	Overall population N=68
PT IgG	Median	10	15	3.3	3.3	5.8
	IQR	3.3-20	11.7-43.3	0.1-11.7	0.1-15	1.7-15.8
FHA IgG	Median	173.3	258.3	205	99.2	165
	IQR	133.3-286.7	161.7-540	55-541.7	50-185	60-327.5
Prn IgG	Median	103.3	251.7	180	71.7	152.5
	IQR	71.7-165	73.3-321.7	123.3-675	40-206.7	49.2-321.7
FIM 2/3	Median	0.1	0.1	0.1	0.1	0.1
IgG	IQR	0.1-0.1	0.1-3.3	0.1-1.7	0.1-0.1	0.1-1.7

Legend: IQR: Interquartile range, PBMC: Peripheral blood mononuclear cells

4.4.2 Correlation with pertussis antibody levels

For the antigen that was not present at the booster vaccination with Boostrix®, FIM 2/3, no correlations were identified between plasma cell frequencies and antibody levels detected at the three defined timepoints – baseline, D28 and D365.

Significant correlations were identified for all pertussis antigens present in the study vaccine (PT, FHA and Prn) between plasma cells and IgG antibody levels at D28 and D365 (Figure 4.7).

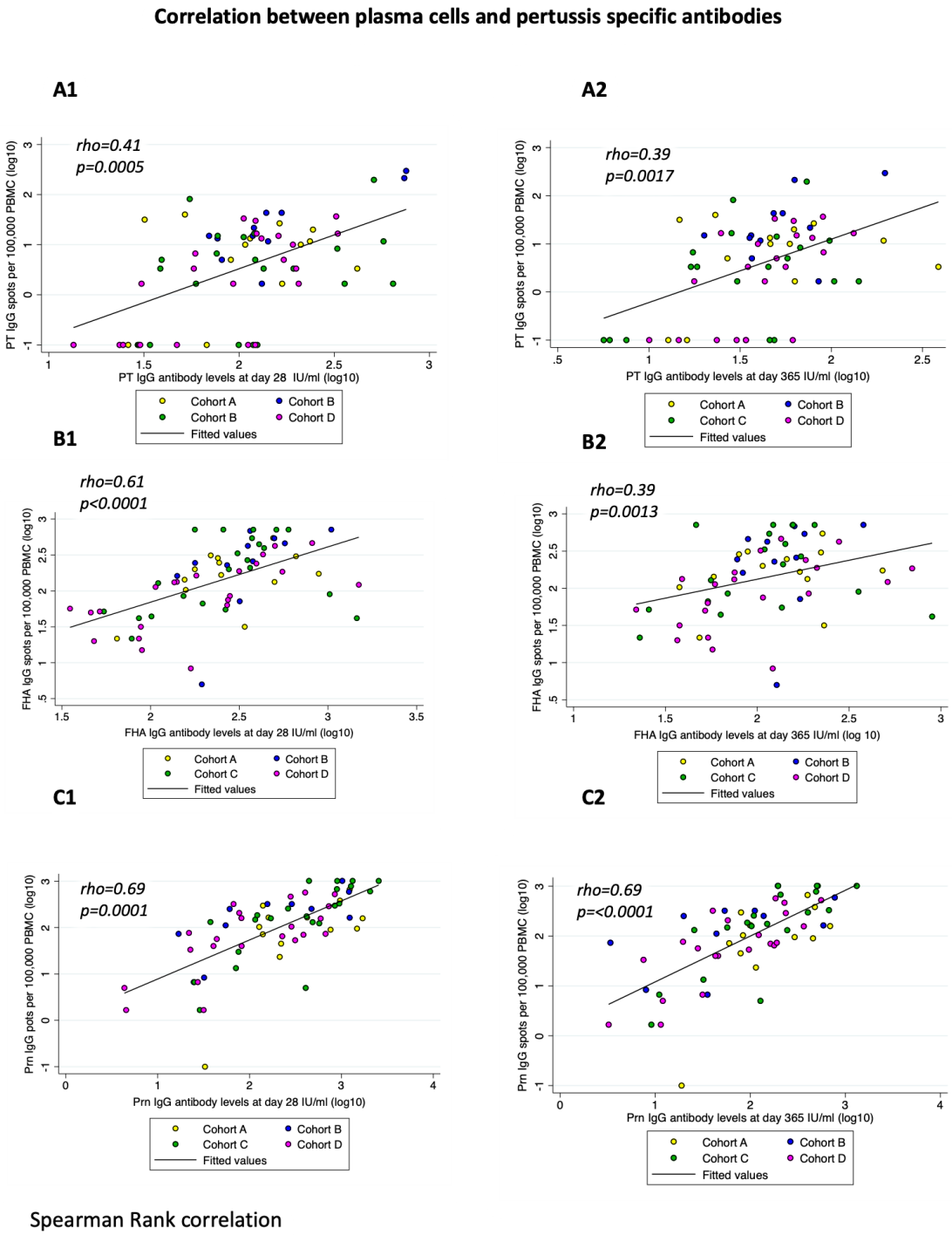


Figure 4.7 Correlation between plasma cells frequencies detected 7 days after booster immunisation and pertussis specific antibody levels at D28 (1) and D365 (2) after booster immunisation.

A1 (N=68**) and A2** (N=64): PT IgG; **B1**(N=68**) and B2** (N=64): FHA IgG; **C1**(N=68**) and C2** (N=64): Prn IgG.

Considering these data per cohort some significant correlations were lost. Prn specific plasma cells was the antigen that showed the highest correlation profile in terms of cohort, with a significant correlation with both IgG Prn antibody levels at D28 (**A:** rho=0.41, *p=0.2*; **B**: rho=0.74, *p=0.009*; **C:** rho=0.78 *p<0.0001*; **D:** rho=0.67, *p=0.0006*) and D365 (**A:** rho=0.48, *p=0.1*; **B:** rho=0.48, *p=0.013*; **C**: rho=0.78, *p:0.0001*; **D:** rho=0.76, *p=0.0001*).

4.5 WHOLE BLOOD T CELL PILOT STUDY RESULTS

The whole blood T cell assay was performed in a total of 18 samples (Cohort A:8; Cohort B:2; Cohort C:3 and Cohort D:5) as a pilot for other studies, like AWARE. Paediatric samples were collected at D0, D7 and D28 and adult samples at D0, D14 and D28.

The Th1 response to the aP booster immunisation was evaluated by IFN-γ and IL-2, Th2 responses by IL-5, IL-10 and IL-13 and Th17 responses to IL-17A and IL-17F.

After testing all conditions, a final protocol was decided. The results and steps to define the final conditions are presented in Supplementary data from Chapter 4. Other conditions defined below resulted from data of the original whole blood T cell assay [260].

In summary and per stimulation condition, 200µl of WB in prediluted form should be used, reaching a final dilution factor of 4: 1) CM plus co-stimulatory antibodies (mock/negative control); 2) pertussis toxin heat inactivated (HI) plus co-stimulatory antibodies; 3) FHA plus co-stimulatory antibodies; 4) additional antigen of choice (Bp lysate or Bp peptide pool) plus costimulatory antibodies and 5) SEB without

costimulatory antibodies (positive control), the last two decided based on previous studies. After an incubation period of 48 hours three aliquots of 150µl supernatants should be harvested and frozen at -80°C and T cell specific cytokines (IL-17A, IL-17F, IFN-γ, IL-13, IL-5, IL-10, and IL-2) determined using a multiplex bead format at a time determined by the research team.

The cytokine indicator lineage for CD4+ Th1 is IFNγ, for CD4+ Th2 is IL-5 and IL-13 and for CD4+ TH17 is IL-17A and IL-17F. IL-10 will be used as an indicator cytokine for regulatory Th cells, and IL-2 is a cytokine associated with T-cell fitness and proliferative capacity.

The results after 48 hours stimulation with the use of co-stimulants (CD28/CD49d) from the BERT UK population used in this pilot study for PT and FHA are presented below. Due to the low number of samples, cohort A and B were analysed together as cohort C and D. No formal statistical analysis was undertaken given the small sample size and a descriptive analysis follows.

In the PT stimulated wells, in the aP primed cohorts, the baseline cytokine levels for IL-13, IL-17A and IL-17F were higher than the levels found D28 after booster immunisation, although the cytokine levels increased from baseline to day seven and waned afterwards, for the Th17 cytokines. The IL-17F had a higher increase in the older cohorts when compared with the paediatric cohorts (Table 4.7 and Table 4.8).

Table 4.7 Median and interquartile range of cytokine levels after pertussis toxin (PT) stimulation and use of co-stimulants, with 48 hours incubation in paediatric cohorts of the BERT study

PT stimulation		Paediatrics (Cohort A and B) Median (Interquartile range - IQR)		
	Antigen	D0 N=10	D7 N=9	D28 N=8
Th1	IL-2 (pg/ml)	253.8 (135.9-343.5)	169.4 (53.5-370.3)	193.9 (146.0-345.8)
	IFN-γ (pg/ml)	970.8 (289.7-1283.2)	1616.8 (557.8-2497.2)	1174.4 (481.2-2360.4)
Th2	IL-5 (pg/ml)	138.9 (92.9-213.2)	62.4 (46.7-115.6)	16.9 (0.01-63.4)
	IL-10 (pg/ml)	2351.6 (1358.7-4279.5)	3008.6 (2213.5-4369.6)	2583.8 (2099.7-3601.7)
	IL-13 (pg/ml)	1838.4 (1146.6-3906.6)	1218.5 (1100.4-2049.6)	910.9 (24.6-1413.5)
Th17	IL-17A (pg/ml)	84.3 (0.01-306.4)	116.8 (77.2-255.4)	15.1 (0.01-87.3)
	IL-17F (pg/ml)	2762.8 (870.8-4794.8)	4202.8 (3243.5-4337.5)	2426.9 (1210.9-4295.8)

Legend: Th: T helper cells

Table 4.8 Median and interquartile range of cytokine levels after pertussis toxin (PT) stimulation and use of co-stimulants, with 48 hours incubation in adult cohorts of the BERT study

PT stimulation		Adults (Cohort C and D) Median (Interquartile range – IQR)		
	Antigen	D0 N=8	D14 N=7	D28 N=8
Th1	IL-2 (pg/ml)	64.6 (12.0-593.3)	322.4 (119.9-390.5)	173.2 (129.1-333.5)
	IFN-γ (pg/ml)	3486.4 (122.0-28066.6)	1114.3 (929.8-3512.7)	386.9 (71.5-1578.8)
Th2	IL-5 (pg/ml)	36.8 (0.01-173.7)	58.1 (0.01-141.4)	15.1 (0.01-74.0)
	IL-10 (pg/ml)	1694.9 (1101.4-3665.9)	1367.3 (891.3-5926.0)	961.5 (197.8-2026.1)
	IL-13 (pg/ml)	568.9 (333.9-1649.3)	879.0 (206.2-1942.5)	780.8 (573.1-1262.7)
Th17	IL-17A (pg/ml)	127.7 (0.01-720.3)	237.7 (105.0-344.5)	85.5 (43.9-120.0)
	IL-17F (pg/ml)	2737.3 (777.7-29365.3)	7857.5 (4289.8-17462.5)	3620.5 (3254.0-3898.9)

Legend: Th: T helper cells

IFN-γ had higher baseline levels in the older cohorts, but an increase from baseline to intermediate timepoints (D7 in paediatric and D14 in adults) was found in paediatric cohorts only. In the older cohorts, IFN-γ levels at D28 were lower than the levels at baseline (Table 4.7 and Table 4.8).

Th17 responses were the strongest in both age groups, particularly at the intermediate timepoints and higher in the older individuals (D7 for paediatric cohorts and D14 for adult cohorts). The paediatric cohorts, aP primed, also showed a stronger Th2 response, when compared with the adult cohorts (Figure 4.8).

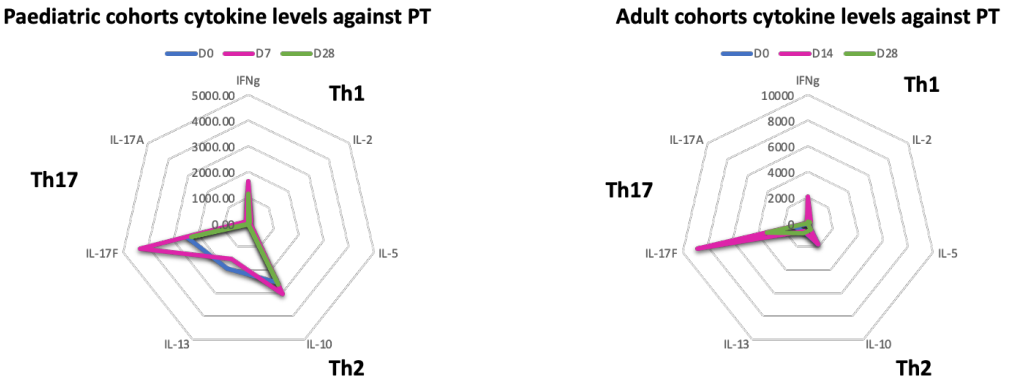


Figure 4.8 Differences between cytokine levels in the pertussis toxin (PT) stimulated wells from baseline and after booster immunisation with aP in the BERT study per paediatric and adult cohorts

The FHA levels for all cytokines were lower when compared with the responses to PT, except for IL-2. In the paediatric cohorts, a decrease from baseline to D7 with a subsequent increase at D28 was found for IL-2 (Th1) and IL-5 (Th2) (Table 4.9 and Table 4.10).

Table 4.9 Median and interquartile range of cytokine levels after filamentous haemagglutinin (FHA) stimulation and use of co-stimulants, with 48 hours incubation in paediatric cohorts of the BERT study

FHA stimulation		Paediatrics (Cohort A and B) Median (Interquartile range – IQR)		
	Antigen	D0 N=5	D7 N=4	D28 N=8
Th1	IL-2 (pg/ml)	1028 (657.9-2431.1)	614.1 (353.3-1100.3)	1622.0 (935.9-4988.8)
	IFN-γ (pg/ml)	28.0 (21.3-94.8)	45.6 (19.9-94.7)	23.0 (7.2-106.8)
Th2	IL-5 (pg/ml)	36.7 (5.0-95.4)	24.9 (0.01-56.7)	55.4 (37.0-506.5)
	IL-10 (pg/ml)	0	0	0
	IL-13 (pg/ml)	375.1 (323.3-1284.8)	449.2 (80.35-831.7)	942.7 (400.3-3731.0)
Th17	IL-17A (pg/ml)	26.1 (0.01-66.7)	32.6 (5.0-57.5)	11.8 (2.7-38.1)
	IL-17F (pg/ml)	114.62 (0.01-239.4)	0	0.01 (0.01-0.01)

Legend: Th: T helper cells

Table 4.10 Median and interquartile range of cytokine levels after filamentous haemagglutinin (FHA) stimulation and use of co-stimulants, with 48 hours incubation in adult cohorts of the BERT study

FHA stimulation		Adults (Cohort C and D) Median (Interquartile range – IQR)		
	Antigen	D0 N=5	D14 N=4	D28 N=8
Th1	IL-2 (pg/ml)	17.6 (0.01-187.4)	550.2 (295.3-1638.6)	767.4 (550.8-868.5)
	IFN-γ (pg/ml)	0.01 (0.01-0.01)	37.9 (0.01-110.2)	0.01 (0.01-0.01)
Th2	IL-5 (pg/ml)	0.01 (0.01-2.2)	0.01 (0.01-19.5)	0.01 (0.01-2.2)
	IL-10 (pg/ml)	0	0.01 (0.01-3.8)	0
	IL-13 (pg/ml)	0	0.01 (0.01-660.4)	0.01 (0.01-301.7)
Th17	IL-17A (pg/ml)	0.01 (0.01-0.01)	0.01 (0.01-15.7)	0.01 (0.01-4.2)
	IL-17F (pg/ml)	0	0.01 (0.01-0.01)	0

Legend: Th: T helper cells

4.6 DISCUSSION

4.6.1 Summary of the main findings

The MBCs showed the lowest frequencies at baseline, with a maximum increase at D28, statistically significant in all cohorts. Although the waning of MBCs

frequencies from D28 to D365, the frequency levels were never below the frequencies measured at baseline.

In the BERT population, FHA specific MBCs had the highest frequencies at baseline and after booster immunisation, although it was Prn specific MBCs that showed the highest fold change.

At baseline the MBCs in the adolescent cohort correlated with all pertussis specific antibodies at the same timepoint. This cohort also had the highest PT IgG frequencies at baseline, significantly different from other cohorts.

From all pertussis specific antigens present in the booster vaccine, immune response against Prn showed the best correlations in terms of long-term protection, particularly the correlation between Prn specific MBCs at D28 and Prn antibody levels measured at D365 and correlation between Prn specific plasma cells and Prn levels at D28 and D365.

In the T cell pilot study, the highest responses were seen in the youngest cohorts, with stronger Th17 responses in both cohorts. The aP primed cohorts (A and B) showed higher Th2 responses when compared with older cohorts.

4.6.2 Analysis of the current results based on the available literature

MBCs are developed as part of a response to a specific stimulus, allowing that in a second encounter a quicker production of antibodies with higher affinity [219,224,315].

Differences in recovery rates in adult and paediatric cohorts can make comparisons between long-term immunity outcomes in those populations difficult [225]. We used the most common described methods for recovery of these cells by

isolating PBMCs and doing polyclonal stimulation in order to induce the proliferation and differentiation of small number of resting MBCs in ASC [225,316,317].

MBCs were detectable one-year post-booster for all antigens and in higher frequencies when compared with baseline, independently of the primary immunisation. This increase in MBCs after stimulation with waning after a longer period has been described in pertussis immunisation studies [313,318,319].

At baseline, MBCs frequencies were higher for FHA when compared with other pertussis antigens, particularly for the younger cohorts (A and B). The frequencies for FHA and Prn in the older cohorts were similar. These findings are consistent with previous studies in adults, with FHA and Prn showing the highest frequencies and PT showing the lowest baseline levels [222,225]. Both FHA and Prn are antigens that are not *B. pertussis* specific and could be present in other *Bordetella* species [4]. This could partially explain why MBCs and associated antibodies could be present in higher levels, since a higher number of stimuli could contribute for the production of MBCs against that specific antigen. After stimulation, in the first month after immunisation, this increase was higher, in general, to IgG Prn. If we analyse per cohort, the adults had a higher increase after the booster dose, particularly for Prn. If we compare the frequencies between cohorts, statistically significant differences were detected for PT and FHA, due to the higher frequencies in adolescents. These differences were not maintained after the booster dose for PT but detected for Prn, not found at baseline.

Without a specific stimulation the resting MBCs in the peripheral blood are naturally low. The higher baseline MBCs frequencies identified in the younger cohorts could be related with the time since the last pertussis vaccine, that was less for those groups. These higher frequencies of MBCs in aP primed cohorts have been previously described [313,318].

We could extrapolate that the group that was primed with wP had the strongest response after the booster dose, independently of not having the highest baseline frequencies. In a study from *Schure et al*, aP primed individuals had a stronger response shortly after booster immunisation (4 weeks) for PT, FHA and Prn when compared with wP primed individuals, but Prn showed higher frequencies in wP primed individuals two years after booster [313].

If we compared the two adult cohorts, elderly showed a weaker response, with small fold increase when compared with younger adults. As previously described the younger adults were all wP primed but the older individuals (cohort D) were likely not to have received any primary immunisation against pertussis, considering the year of birth. This fact, associated with some degree of immunosenescence, typical of this age, could have contributed for these findings [320]. This could be relevant in terms of a higher risk for these individuals to contract the disease, due to a poor T and B cell response, and although not as severe as seen in young vulnerable infants, could have some impact in quality of life and contribute to an increased risk of disease transmission [117]. This knowledge could also contribute to design vaccines tailored to this age group, with specific adjuvants [320].

In the BERT study, correlation between MBCs frequencies and antibody levels at baseline was identified for PT, FHA IgG but not Prn. This pattern changed after booster, with strongest correlations in general seen between plasma cells/MBCs and Prn IgG MBCs.

In general, human studies in this area showed a lack of consistent correlation between the IgG pertussis specific antibody levels and antigen specific MBCs, making the assumption that MBCs could contribute independently for long-term immunity and on that basis likely to need to be considered when defining a CoP

[222,223,225,281,318,321]. In a study by *Van der Lee S et al* [222], evaluating adults after booster immunisation with three pertussis component (PT, FHA and Prn) Tdap, a significant correlation was found between IgG PT and Prn MBCs two weeks after immunisation and antibody levels one year after immunisation, but not for FHA. This correlation was not described by *Carollo et al*, that studied children boosted also with a 3-component pertussis vaccine (PT, FHA and Prn), where no correlation was found between PT and FHA [223]. In another Swedish study, in adolescents primed with an aP, two types of a second booster pertussis vaccine were given to two distinct groups: group 1 received a 1-component pertussis vaccine and group 2 received a 5-component vaccine [281]. In this case, around the first month post-immunisation, a correlation was only found in group 1 for PT IgG, but no correlations were found in group 2 for PT, FHA or Prn [281].

As part of the cellular immune response, $CD4^+$ T helper responses to pertussis immunisation have been explored in a more detailed way when compared with B cell responses, particularly in the animal model. A skewed Th1/Th17 response has been associated with wP priming, contributing not only to protection against disease but also colonisation [197,202,259].

The whole blood T cell assay was a pilot study, with a small number of participants and of descriptive nature. So, it is important to highlight that the most relevant finding was the reassurance that this method can be applied in a larger scale, feasible in infant populations, like the AWARE study and easy to be performed in a clinical trial setting.

In this study we saw an increase in IL-2 levels from baseline to D14 in the adult population with decline from D14 to D28, but with levels higher than baseline. In the paediatric cohort, for IL-2, the baseline levels were the highest when compared with

other timepoints. For INF-γ, in the paediatric aP primed population from baseline to D7 an increase was also identified, and although a decline from D7 to D28 was found, it was higher than baseline. A similar pattern after aP booster immunisation was described [322]. These results can be due to downregulation, but in some cases where the cytokine levels were not that elevated at baseline and an increase was seen in the first two weeks post-booster, with waning by D28, might show an early peak, with a quick waning after stimulation.

IL-2 is considered an inflammatory cytokine produced as part of the Th1 response, as well as IFN-γ [198]. Also, IL-2 is one of the first cytokines produced by naïve Th cells as part of an initial response to a stimulus, contributes to cell proliferation as a signaling molecule, but seems to have a role not only in the activation of Th1, but also Th2 responses [323]. After that initial increase in IL-2 as this initial response, after induction of Th1 responses, IL-2 levels can start declining, with increase of the IFN-γ levels [323]. Our results can be explained by the role of IL-2 in cell proliferation, but also due to the high baseline levels of IFN-γ.

Th2 cytokines levels were relatively stable at all timepoints for both populations, a finding already described by *Lambert et al* when studying the Dutch BERT samples [260]. Higher levels were found in the aP primed population. This finding was also described by *Lambert et al* in the Dutch BERT population, particularly in the adolescent cohorts [260].

Cytokine levels in healthy individuals vary and a group of cytokines used in these studies is described to have a high variability in the baseline levels between individuals, including IFN-γ and IL-2 [324]. A study from *Karanfilov et al* showed that baseline cytokine levels associated with both Th1 and Th2 responses tend to be significantly higher in young individuals, when compared with elderly individuals [325].

Immunosenescence can explain the decline in $CD4^+$ T cell responses, but the behavior of cytokines produced as part of Th1 and Th2 stimulation is not consensual between studies [326].

4.6.3 Limitations

The number of *ex-vivo* assays performed was smaller when compared with the individuals that were tested for specific antibody levels against pertussis. The correlations identified in this analysis are based in lower number of individuals, that could mean that for specific antigens, some correlations might not have been identified.

The individuals with data available for plasma cells results are not the same that we have serology available regarding D14 after immunisation, so it was not possible to study the behaviour of the paediatric cohorts. This is also valid for the participants that MBCs ELISpots assays were performed, so this information is not available for paediatric cohorts.

As previously explained (CHAPTER 3), in the BERT study it was not possible to define if MBCs responses are only influenced by age, primary immunisation background or both. In the analysis of data from the multicentre study, similar to the serological studies, the adolescent cohort, had individuals with different backgrounds, but the final analysis is ongoing and no conclusions can be taken.

Regarding the T cell optimization assay it is relevant to point that the low number of samples per cohort and the high confidence intervals show a high variance of the cytokine levels and limits the interpretation of the data.

4.6.4 Conclusions

These data showed that after pertussis booster immunisation an increase in MBCs is seen in individuals of different ages and independently of their immunisation background. The presence of higher frequencies of MBCs after one year, when compared with baseline levels, showed that although immunity and memory wanes, the vaccine effect lasts at least after one year, showing the relevance of booster doses.

The best correlation between MBCs/plasma cells after booster was with Prn IgG antibody levels.

The T cell optimisation assay allowed the development of an assay with an applicability in large clinical trials and with small blood volumes.

CHAPTER 5

WHOLE CELL PERTUSSIS VACCINE: ASSESSMENT OF FUNCTIONAL ANTIBODY ASSAYS AND THE EFFECT OF A PREVIOUSLY DESCRIBED HLA VARIANT THAT IS ASSOCIATED WITH PROTECTION AND DISCRIMINATES WP RESPONSE FROM AP

5.1 BACKGROUND

5.1.1 HLA definition and role in immune response

The MHC is located in the short arm of chromosome 6 (6p21.31) [327]. This region contains less than 4MB and although limited in size is considered a region with one of the greatest levels of variability within the human genome [226,327]. The genes contained in this region encode different molecules and are classified into three different classes: the classical HLA class I and class II and located between them class III [327]. Both classes I and II are recognised by the role of their molecules in antigen presenting processes, to $CD8^+$ T cells in the case of HLA class I (*HLA-A, B and C*) and $CD4^+$ T cells in the case of HLA class II genes [328].

HLA-DP, HLA-DQ and HLA-DR loci are part of class II HLA and in general it is assumed that the expression of molecules from these loci are restricted to antigen presenting cells (APC), like dendritic cells, B cells and macrophages although non-APC can induce HLA II gene expression [328-330]. The $\alpha\beta$ T cell receptor (TCR) of $CD4^+$ T recognises the combination of specific peptide sequences derived from the vaccine protein (HLA II peptides) that are expressed in complex with MHC class II

molecules on the surface of antigen presenting cells [328,330,331]. Briefly, after a pathogen is internalised by a phagocytic cell, the pathogen is broken down in antigenic peptides (processed) and placed into a phagosome that fuses with a lysosome inside the APC cell [328,330]. The two chains of MHC class II, α and β, inside the endoplasmic reticulum together with the invariant chain form a complex that migrates to the MHC class II compartment. The phagolysosome fuses with the MHC class II compartment and the antigenic peptides are degraded. The peptide fragments bind to the MHC class II complex after an exchange with the modified invariant and migrate to the cell surface to be presented to the TCR, resulting in T cell activation and production of different cytokines [328]. The activated T cell CD40 ligand can then bind to CD40 of the B cell, and activate the B cells, leading to proliferation and to differentiation to antibody secreting plasma cells, with the ability to produce specific antibodies against the pathogen [332].

The T cell receptors have high diversity due to three gene segments (V- variability J- joining D- diversity), that by undergoing somatic recombination contributes for heterogenicity and composition of TCR [329]. This diversity in TCR combined with polymorphisms in HLA loci are responsible for differences in individual immune responses, that can go from a weak immune response, with low antibody levels and poor function to an autoimmune response against the self [226,329]. Although the diversity of TCR can be grouped together when sharing conserved motifs and a CDR3 sequence, the complexity is to match a disease susceptibility to a specific polymorphism [329]. The challenge is to identity a significant association between these genes and specific immune response or diseases, considering the presence of high number of polymorphisms and a strong linkage disequilibrium, characteristic of this complex [329]. One of the first associations between HLA variation and disease

was with Hodgkin lymphoma around half a century ago [329]. The mechanisms associated with disease and HLA variants are not completely understood and the same HLA variant could be associated with increased susceptibility to multiple diseases, with polymorphisms being caused by different molecular mechanisms. Conversely, different polymorphisms (each capable of association with separate diseases) may be associated with a single disease.

5.1.2 HLA role associate with infectious diseases

The fundamental key players in infectious diseases are the pathogen, environment and host, including the host genetic factors, that can determine susceptibility to disease [226].

Chronic viral infections progress and outcomes have been associated with different HLA variants and polymorphisms. A study from *Ranasinghe et al* showed an association between HLA-DRB1 and virus replication, with HIV viremia levels being able to be stratified by high and low, depending on different HLA polymorphisms [333]. Higher risk of progression from HIV infection to AIDS was found in individuals from West Africa with HLA-B*08:01 and HLA-B*18:01 alleles when compared with individuals without this variant [334,335]. On the other hand, lower susceptibility was found in an European population with HLA-B*57:01 [336]. Implications in disease progression and viral load were also found related to Hepatitis B and C and malaria [337,338]. Class II HLA were also associated with different susceptibilities and severity of high prevalent diseases, like tuberculosis and malaria [338,339]. The HLA-DRB1*1302 was associated with higher protection against severe malaria in homozygotes in an African population [340] and rs5570011, located between HLA-

DQA1 and HLA-DRB1, seemed to be associated with higher risk of development of tuberculosis [341].

In pertussis the initial presentation of the agent occurs in the respiratory epithelial cells of the upper airways, stimulating the innate immune response. It is well known that with the role of MHC class II in antigen presentation, susceptibility to pertussis disease could also be determined by different alleles. A work by *McMahon et al* showed an association between self-reported whooping cough from the ALSPAC and 1958 Birth Cohorts of two British studies and variants in HLA class II [342]. Among those, the alleles HLA-DQB1*0602, HLA-DQB1*0501 and HLA-DRB1*0301 and the SNP rs9271768 were associated with higher susceptibility to the disease [342].

5.1.3 HLA role in responses to immunisation

Following vaccination some peptides within the vaccine can be presented to $CD4^+$ T cells in the context of MHC/HLA class II molecules. As expected, different HLA polymorphisms can influence antigen presentation and consequently individual vaccine responses, even with epitopes that share higher and more sustainable protection [226].

Several studies have been focusing in identifying HLA alleles and SNP's that can be associated with stronger or absence responses to immunisation. After immunisation with a trivalent influenza vaccine in an UK population, a higher percentage of individuals with HLA-DRB1*07 allele were not able to develop hemagglutination inhibition (HAI) titres, showing a lack of response to immunisation [227]. In the same study, individuals with HLA-DQB1*0603-9/14 were more likely to have protective titres after vaccination [227]. Another study in the UK, investigating

also a trivalent influenza vaccine, showed that individuals with HLA-DRB1*04:01 or HLA-DPB1*04:01 were more likely to develop protective antibody titres [228].

Class II alleles were also associated with antibody functionality, with an increase in neutralizing antibodies after the administration of inactivated Japanese encephalitis vaccine in individuals with DQB1*02:01 allele [343].

In a meta-analysis, the increase in hepatitis B antibody titres was associated with specific alleles, including DRB1*01, DRB1*1301 and DQB1*0602, although the last two showed high specificity with low sensibility [229].

Different alleles could be associated with protection in the same vaccine, but it is very likely to differ with different vaccines, even if the type and the target are similar.

A GWAS study in African populations from Burkina Faso (VAC050) identified a significant association between groups of class II HLA alleles (HLA-DRB1-233Thr, pooled p value 1.16e-20, minor allele frequency (MAF) 0.44) and antibody responses to PT in infants primed with wP. This association with higher PT antibody levels in a wP primed population was not found for aP primed infants, from South Africa (*Mentzer et al*, DPhil thesis) [230].

The observed patterns of HLA II in association with PT levels in the wP primed populations were identified in an independent GWAS of self-reported whooping cough in UK, the same cohorts of the *McMahon et al* study [342]. The HLA-DRB1-233Thr was highly correlated with the lack of self-reported whooping cough in this population. These findings allowed the authors to hypothesise that specific HLA alleles could work as a determinant of wP related protection against *B. pertussis,* and with that we can also question if HLA-II could work as route to establish functional antibody correlation of wP related protection against this agent.

The general acceptance that aP primed individuals normally mount higher PT IgG antibody responses after immunisations in comparison to wP leads to the idea that the associations between HLA and PT levels could be related to differential presentation of PT- derived peptides by dendritic or B cells. This differential presentation has downstream effect on T cell induction, Th1, Th2 and Th17, in the antibody quality/function and generation of B cell memory.

Several questions could be posed based on these findings: are these correlations independent of the PT antibody levels and more a measure of antibody functionality, and can these patterns be relevant across different populations and not only African populations as first described? To answer these questions, individuals from different studies and of different ages, that also contribute to disease transmission, like adolescents and adults, were selected with the aim to identify if the same association between antibody levels, particularly PT IgG as described by *Mentzer et al,* was replicable in other populations and not specific of Africans.

Also, antibody function was measured to identify how these populations antibodies perform in terms of antibody quality.

5.2 STUDY SUMMARY AND STUDY POPULATIONS

The studies that were used to explore antibody functionality and to identify if HLA-DRB1-233Thr variant associations are present are described in Table 5.1. The full details of the studies design are described in CHAPTER 2, section 2.2.2.

A description of the immunisation schedules of the biobank populations are represented in Figure 5.1.

Table 5.1 Description of the studies used to study antibody functionality and evaluation of association between HLA-DRB1-233Thr and pertussis immunological responses

	Pertussis immunisation background					
	Whole cell pertussis vaccine/ Natural infection			Acellular pertussis vaccine		
	Study (year of study)	Assay	Samples with genotyping available (N)	Study	Assay	Samples with genotyping available (N)
Infants	Infants_African_wP	Ab, BAI, CDA	353	Infants_African_aP	Ab, BAI	755
	Infants_Asian_wP	Ab	284			
Children	Children_UK_wP	Ab, BAI	24	BERT – cohort A	Ab, SBA, OPA, CDA	27
Adolescents	Adolescent_UK_wP	Ab, avidity, BAI	450	BERT – cohort B	Ab, SBA, OPA, CDA	34
Adults	BERT – cohort C	Ab, SBA, OPA, CDA	23			
Elderly	BERT – cohort D	Ab, SBA, OPA, CDA	25			

Legend: Ab: antibody levels, BAI, bacterial adherence assay, CDA: complement deposition assay, OPA: Opsonophagocytosis; SBA: serum bactericidal antibody assay.

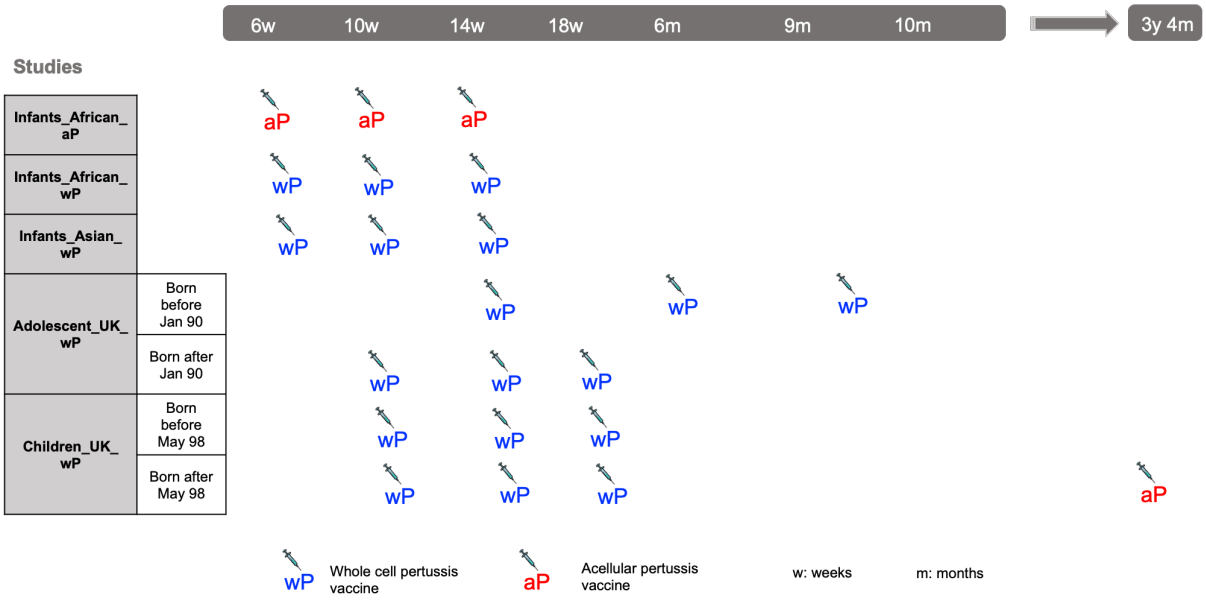


Figure 5.1 Representation of the immunisation schedules of the pertussis vaccine in the populations of different biobank studies

5.3 METHODS

A full description of the laboratory assays for evaluation of pertussis serology (MIA assay) and antibody functionality (PT IgG avidity, BAI, CDA, OPA, SBA) can be found in CHAPTER 2, section 2.3.

5.3.1 DNA extraction and genome wide genotyping

For the biobank studies (Children_UK_wP, Adolescent_UK_wP and Infants_Asian_wP) and African infant populations, the genomic data has been previously used for other research projects and already available for analysis; therefore, I was not involved in the DNA extraction process or genotyping. I was responsible for the DNA extraction in the BERT study samples and a full description of the methods are presented in 5.3.1.1 section.

For the UK biobank samples, the methods of DNA extraction and genotyping were described by Daniel O'Connor [232,344]. For Infants_Asian_wP study genotyping (*Kandasamy R et al),* DNA was extracted from blood clots using QIAsymphony. In summary, the blood clots were transferred to a 1.5 mls micro-centrifuge tube and ATL (QIAGEN, Hielden, Germany) buffer and protein kinase K (QIAGEN, Hielden, Germany) added. The mixture was incubated overnight at 56°C. After incubation AL buffer was added and vortexed. After the lysis process a 400 μl sample was run in QIAsymphony using QIAsymphony DSP Mini kit (QIAGEN, Hielden, Germany), with an elution volume of 100μl. Samples were quantified using Qubit (Invitrogen, Carlsbad, CA, USA) or Nanodrop (Thermofisher, Waltham MA, USA). The DNA was genotyped using Illumina infinitum Global screening arrays (version 2).

5.3.1.1 BERT study

DNA extraction from the BERT samples was performed using frozen PBMC's. The PBMC's were defrosted using the same method and reagents described in section 2.3.4. In summary, 10 mls aliquots of pre-warmed (37°C) TM were prepared in 15 mls falcon tubes. PBMC were defrosted in warm water by swirling the cryovials and when almost defrosted, 0.5 mls (for 0.5 mls vials) or 1 ml (to 1 ml vials) of warm TM was transfered to the vial and gently mixed. The full content of the cryovial was transferred to the falcon tube. The falcon tube was centrifuged at 400g for 10 minutes and the supernatant poured after checking for the presence of the cell pellet. After resuspension of the cell pellet, a second centrifuging cycle was performed with the same conditions. After the second cycle, the supernatant was poured, and the cell pellet was resuspended. For this specific procedure, the first 10 samples were counted using the Muse™ (method described in section 2.3.4) and compared with the pre-

freezing concentration. Considering that the cell viability was higher than 80% for the samples counted, for the remaining samples, the cell counts registered after cell isolation and before freezing were used to guide the volume of lysis buffer reagents used in the final step. For cell concentrations estimated to be higher than $4x10^6$/ml, after the second wash the cells were resuspended in 2 mls of CM (composition and regents as described in section 2.3.4) and the volume corresponding to $4x10^6$/ml taken out of the cell mixture. The sample was centrifuged at 18,000g for 5 minutes and the cells resuspended as described below to concentrations between $2x10^6$/ml - $4x10^6$/ml.

For cell counts estimated to be between $2x10^6$/ml - $4x10^6$/ml, 150μl of PBS (Gibco, Life Technology Corporation, Grand Island, USA) was added first to resuspend the cell pellet and the cells vortexed followed by 100μl of the lysis buffer ATL (QIAGEN, Hielden, Germany). For samples with cell counts estimated to be $\leq 2x10^6$/ml, cell pellet was resuspended in 100μl of the lysis buffer ATL. After adding ATL the cell suspension was vortexed and 20μl of Proteinase K (QIAGEN, Hielden, Germany) was added. The samples were heated at 56°C in a heat plate, for 2 hours. After this period 130μl of AL lysis buffer (QIAGEN, Hielden, Germany) was added to each sample. After preparing the samples, the final volume of each sample was transferred to 2mls pre-labelled tubes and added to the QIA symphony racks.

QIAsymphony (QIAGEN, Hielden, Germany) was set up following the protocol Tissue HC 200DSP, using a QIAsymphony DSP Mini kit (QIAGEN, Hielden, Germany), that by the use of a magnetic particle's technology allows an automated isolation of DNA [345]. In general, a magnetic rod enters the tube/well that contained the sample to test and attract magnetic particles previously added that were able to bond to the DNA on the sample. The rod cover that was on the magnetic rod, after

that step, releases the magnetic particles with the DNA into another well. In this assay, the DNA is separated from the magnetic beads and 200μl were eluted to a plate [345].

After the DNA was extracted, it was quantified using Agilent 2200 TapeStation system, including reagents from the same company (Agilent, Santa Clara, CA, USA). After launching the TapeStation Controller Software, the Genomic DNA ScreenTape device and the tips were loaded to the machine. A ladder was prepared by mixing 10μL Genomic DNA Sample Buffer with 1μL Genomic DNA Ladder. After this step, the samples were prepared by mixing 10μL Genomic DNA Sample Buffer with 1μL Genomic DNA sample. The plate with the samples was centrifuged and vortexed at 2000 rpm for 1 minute. The samples were loaded into the TapeStation machine and DNA quantified. After this process, the plates were sealed and storaged at -30°C before genotyping. The plate was transferred in dry ice to Wellcome Centre for Human Genetics and the following steps performed under the orientation of Dr Mentzer.

Before genotyping, all samples were treated to have a final DNA 200ng on the plate after rehydration. In summary, the volume of DNA to transfer was determined by DNA wanted on the final plate (200ng) divided by the concentration of DNA estimated by TapeStation procedure. The calculated volume was transferred to a 96 well plate, the plate was sealed and stored at -20°C. Immediately before the samples were sent to be genotyped, the plate was defrosted, vortexed and centrifuged. After these steps the plates were placed in a fume-hood with extraction on and left overnight, allowing the samples to evaporate. On the following day the samples were rehydrated to 4μL with Tris-EDTA (TE buffer). After sealing the plate, it was vortexed, centrifuged and placed in a plate shaker for 2 hours before sending to genotyping. At the Oxford Genomics Centre, a Genome Wide Genotyping Screening on 120 Human genomic

DNA samples using Infinium® HTS assay on Human Global Screening array-24 Beadchips v3.0 per 24 samples was done.

5.3.2 Genotype imputation and association analysis

For the BERT study, Children_UK_wP, Adolescent_UK_wP, HLA alleles were imputed using as reference panel the Type 1 Diabetes Genetic Consortium (T1DGC) panel. This panel was constructed with 5225 unrelated individuals in a total of 10450 haplotypes, containing 5,868 SNPs and 4-digit classical HLA types for the classical class I molecules (HLA-A, B and C) and classic class II molecules (HLA-DPA1, DPB1, DQA1, DQB1 and DRB1) [346].

The reference panel used for Infants_Asian_wP study was the Pan-Asian reference panel [347,348]. This panel is composed by data from 441 individuals, with 4603 SNPs in Chromosome 6 MHC region, including the following HLA genes: HLA-A, B, C, DRB1, DPA1, DPB1, DQA1 and DQB1[347,348].

For the African infant populations, as previously described, the imputation and an association analysis had been previously performed and presented by Dr Mentzer and the reference panel used described in *Mentzer, A.* DPhil Thesis [236]. For the purpose of this thesis, I used the available files and phenotypic data, and I added the results of functional antibody assays not yet analysed (Table 5.1). I performed the association analysis for the new variables (Infants_African_wP: CDA and BAI; and Infants_African_aP: BAI), using the same method described below.

The method used for the imputation analysis was SNP2HLA, using PLINK toolset. Before the imputation between reference panel and genotyped participants data of the SNPs of HLA region of interest was done, I performed a LiftOver step. This

first step allows the genetic analysis to the same reference build – build 36 for SNP2HLA.

Antibody (serology and functionality) as well as MBCs data were normalised using log10 transformation and inverse normal transformation before doing the association analysis using a linear regression model, adjusted for age and sex. This analysis allowed us to identify regions on the MHC region and HLA variants associated with the variation of antibody responses to vaccination. A *p value* ≤0.05 was considered statistically significant. The data will be mainly presented in forest plots. As previously described in the BERT study, individuals from cohort A and B were primed with aP and individuals from cohort C primed with wP. Considering the DOB and timing of the introduction of wP in the UK, individuals from cohort D were unlikely to be vaccinated. Despite this fact, the high rates of disease during the first years of life of this group, it was considered that was highly likely that cohort D have had contact with bacteria and developed immunity associated with natural infection in the first year of life. For the purpose of maximizing group sizes for downstream analysis, cohorts C and D were evaluated together.

Based on *Mentzer* data, we assumed that the top PT-associated variant in HLAII (HLA-DRB1-233Thr) that we aim to replicate has a frequency of nearly 50% in African and European populations [236]. The aim of this chapter is to replicate the described variant and not to identify novel associations, so for this case we considered a lower threshold of significance (*p<0.05*) when compared with usual GWAS. Using the genetic power calculator (http://zzz.bwh.harvard.edu/gpc/) it was determined that it was needed a population of 75 individuals to achieve 80% power (p<0.05). As shown in Table 5.1, when analysing the BERT population, even when combining paediatric

(A+B) and adults' cohorts (C+D) the sample size was underpowered for both age groups (paediatric: 61% power and adults:72% power).

5.4 RESULTS

5.4.1 Antibody functionality in African infant populations previously tested for HLA-DRB1-233thr

As previously described in the Infants_African_wP population the variant HLA_DRB1_233thr was associated with higher PT IgG antibody levels by *Mentzer et al* [230], but not in the Infants_African_aP population. The individuals of both studies were from African origin and maternal ethnicity was collected in both studies. In the Infants_African_aP a higher percentage of mothers were from Zulu ethnicity (36.8%) and from the Infants_African_wP population where of Gouin ethnicity (47.3%) [236].

An evaluation of the antibody functionality in these populations and association with this HLA variant was performed.

5.4.1.1 Bacterial adherence assay

This assay is based on the principle that the presence of mucosal antibodies is expected to prevent attachment of *B. pertussis* to the respiratory epithelium and with that the initiation of colonisation. Higher dilutions represented a better antibody functionality, determined by the half maximum effective concentration, EC50.

In the Infants_African_wP, the results of 69 samples were analysed, in infants with a median (IQR) age of 12 months (11-14), 52.2% female. From the Infants_African_aP, samples from two different timepoints, at 6 months of age (N=21;

57.1% males) and 12 months of age (N=52 57.7% male) were analysed. Twelve of these samples were paired.

The highest median (IQR) was from Infants_African_wP study (N=69; 56.1 [20.3-146.8]), followed by Infants_African_aP population shortly after the last pertussis primary dose at 6 months of age (N=21; 29.7 [9.2-618.3]), although in the 12 months samples the median was similar with no significant differences (N=52; 27.5 [22.3-44.2]) (Figure 5.2).

A significant correlation between BAI and FHA and Prn IgG (FHA IgG rho:0.28, *p=0.03*; Prn IgG: rho:0.26, *p=0.03*) was identified for the Infants_African_wP, but not for PT IgG (rho=0.20; *p=0.1*). No significant correlations were found between BAI and pertussis specific antibody levels in the Infants_African_aP at any of the timepoints (6M: PT: rho: 0.03, *p=0.9*; FHA: rho -0.31, *p=0.2*; 12M: PT IgG rho=-0.2, *p=0.3*, FHA IgG rho=-0.07, *p=0.6*), although Prn IgG was not tested since it was not present in the vaccine. No significant associations were found between the BAI results (EC50) and HLA_DRB1_233thr for any of the African infant populations (Infants_African_wP beta coefficient: 0.42, SE:0.2, *p value=0.2;* Infants_African_aP beta coefficient: 0.25, SE:0.2, *p value=0.2)*.

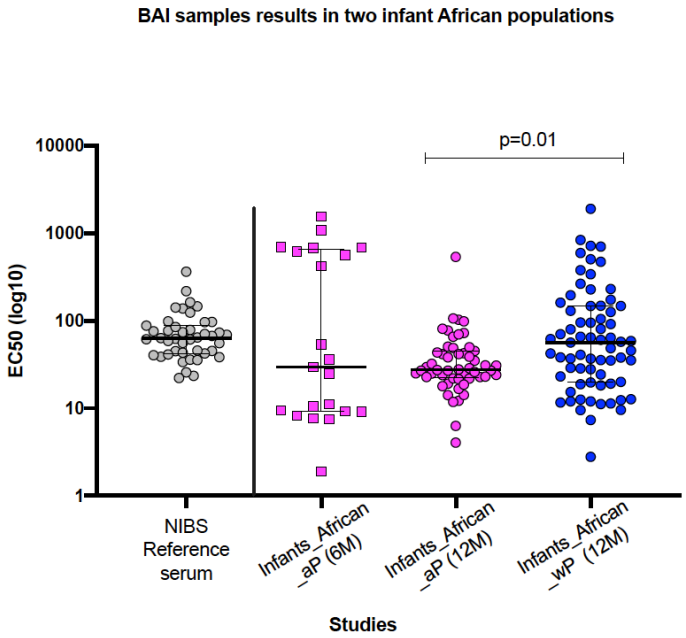


Figure 5.2 Dot plot of BAI results, represented by half maximum effective concentration (EC50) in two infant African populations

Populations: Infants_African_aP at 6 and 12 months of age, primary immunised with aP and Infants_African_wP with median age of 12 months primary immunised with wP.

5.4.1.2 Complement deposition assay

CDA was limited to the Infants_African_wP, not allowing comparisons between populations.

This assay was performed in 124 samples, with a median (IQR) age of 13 months (11-14), 50.8% female. C3b/iC3 had the highest median (C3b/iC3 median [IQR] = 23,020.5 [12,216.3-43107.9]; C5b-C9 median [IQR]=6123 [1723.7-22568.7]) (Figure 5.3).

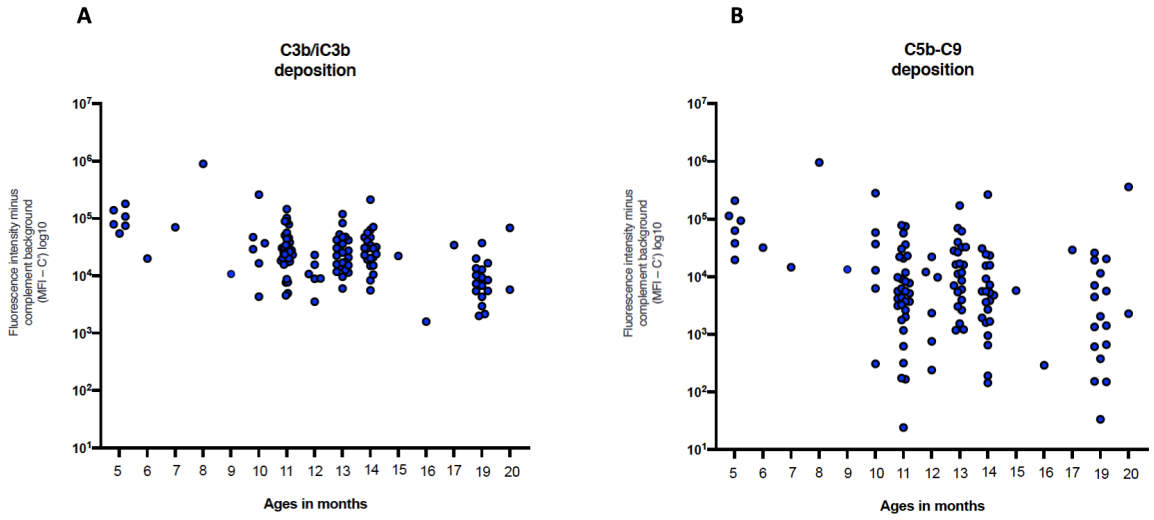


Figure 5.3 Complement deposition assay results (C3b/iC3 and C5b-C9) measured by mean fluorescence intensity minus complement (MFI'-C) in Infants_African_wP population

As previously described, a contamination was detected in a set of samples and although it is likely that the gating excluded potential interference and the correlation data presented based in the overall samples, contaminated samples were marked in a different colour (yellow) in Figure 5.4 to better represent the real results of this assay (Supplementary data from Chapter 2).

A correlation between antibody levels and complement deposition of C3b/iC3b and C5b-9 was performed, and Prn IgG showed in general the strongest correlation (Figure 5.4).

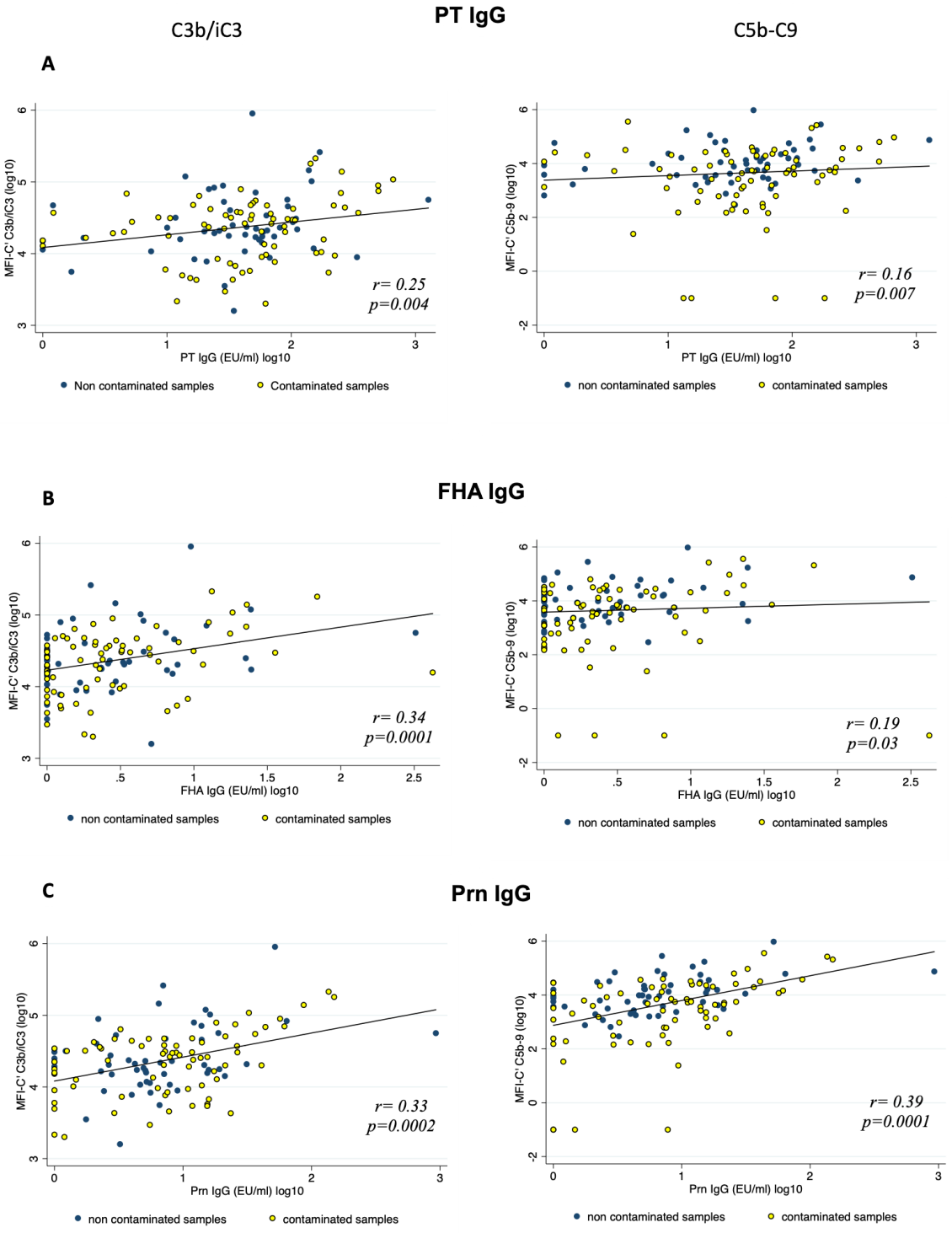


Figure 5.4 Correlation between pertussis specific antibody levels and CDA results for both C3b/iC3 and C5b-C9 in the Infants_African_wP study tested population

No significant associations were found between the CDA results and HLA_DRB1_233thr in this population (C3b/iC3 beta coefficient: 0.42, SE:0.4, *p value=0.5;* C5b-C9 beta coefficient: 0.42, SE:0.5, *p value=0.8).*

5.4.2 Whole cell primed infant Asian population (Infants_Asian_wP): Replication of the association of HLA-DRB1-233Thr variant with PT IgG antibody levels

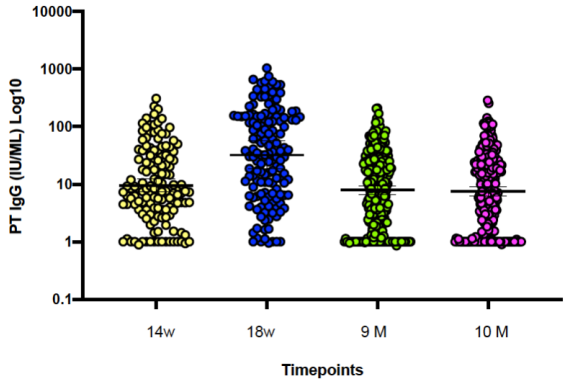
To evaluate if the HLA variant described in the infant African wP primed population was not specific of this population and could be replicated, data from the Infants_Asian_wP was analysed.

As described by *Kandasamy et al* a total of 304 individuals received a wP (DTwP-HBV-Hib) at 6, 10 and 14 weeks of age. The mean age of the full study population at time of randomisation was 6.6 weeks (0.4) and 48% were female [241].

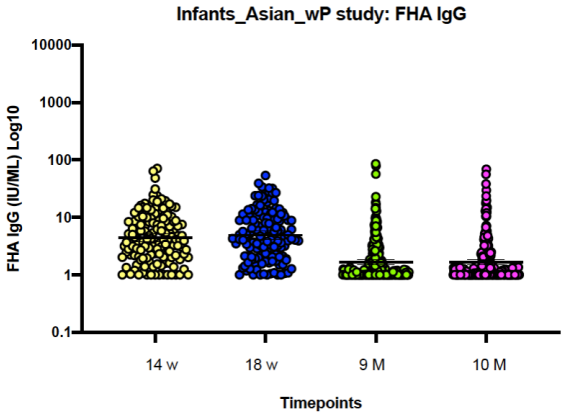
5.4.2.1 Pertussis specific antibody levels after primary immunisation at different timepoints

In total, 287 individuals had a pertussis serology available from at least one timepoint, with PT showing the highest GMC at all timepoints (Figure 5.5). From the early timepoints (14 and 18 weeks) immediately after primary immunisation to the nine months sample, a waning of the antibody levels was identified for all pertussis specific antibodies. Between 14 weeks of age, four weeks after the second wP dose (group 1) and the nine months blood collection the antibodies waned 1.3, 2.4 and 2.3 times for PT IgG, FHA IgG and Prn IgG, respectively. In group 2, at 18 weeks of age, four weeks after the third wP, to the nine months timepoint, the waning of antibodies was higher (PT IgG:4.7 times, FHA IgG: 3.1 times and Prn IgG:7.3 times).

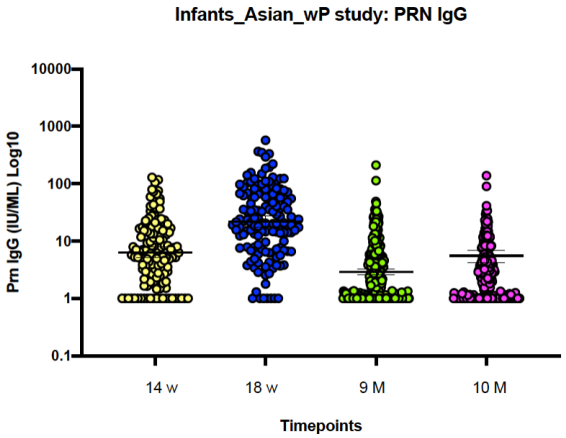
A



B



C



D

Infants_Asian_wP	N	PT IgG GMC (95% CI)	FHA IgG GMC (95% CI)	Prn IgG GMC (95% CI)
14 weeks	137	8.99 (7.01-11.54)	4.34 (3.69-5.09)	6.69 (5.33-8.40)
18 weeks	142	32.82 (24.22-44.48)	4.96 (4.20-5.85)	20.70 (16.34-26.22)
9 months	280	7.91 (6.63-9.43)	1.67 (1.52-1.84)	2.86 (2.51-3.25)
10 months	274	7.70 (6.45-9.02)	1.66 (1.51-1.83)	2.66 (2.34-3.02)

Figure 5.5 Dot plot of pertussis specific IgG antibody levels

A: Pertussis Toxin (PT) IgG; **B**: Filamentous haemagglutinin (FHA) IgG; **C**: Pertactin (Prn) IgG after primary immunisation with wP in the Infants_Asian_wP population. **D:** Table of geometric mean concentrations (GMC) and 95% confidence interval (CI) per timepoint and pertussis specific antibody

5.4.2.2 Association analysis between HLA-DRB1-233Thr and pertussis specific antibody levels in the Infants_Asian_wP

In this population the HLA-DRB1-233Thr variant showed a significant association with IgG PT antibody levels for all timepoints (Figure 5.6). The minor allele frequency (MAF) of this variant (with Threonine residue) in this population was 64%.

This association had a larger effect size and significance in the early timepoints, four weeks after second primary wP immunisation in group 1 *(*beta coefficient: 0.5*)* or the third wP in group 2 (*(*beta coefficient: 0.41*)*. This effect continued at the latest timepoints, although less significant.

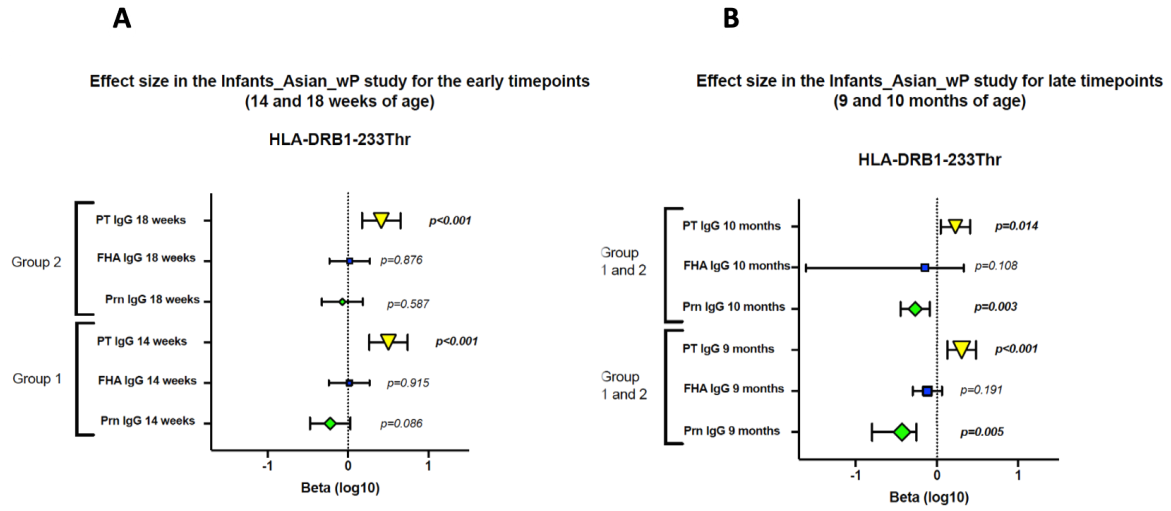


Figure 5.6 HLA-DRB1-233Thr effect size in the Infants_Asian_wP population in association with different pertussis specific antibodies at 14 weeks, 18 weeks, 9 and 10 months of age

A: Effect size in the early timepoints, at 14 weeks of age (Group 1: four weeks after second wP primary dose) and 18 weeks of age (Group 2: four weeks after third wP primary dose); **B:** Effect size for the later timepoints, at nine and 10 months of age, for individuals from group 1 and 2

FHA IgG did not show any significant association with this variant at any timepoint and Prn IgG at the earliest timepoints (14 and 18 weeks). Considering the latest timepoints Prn IgG showed a significant association, in the opposite direction of the variant, showing a significant association with a variant HLA-DRB1-233Arg (9 months age: beta coefficient: 0.25; 10 months of age: beta coefficient: 0.25) (Figure 5.6).

5.4.3 Whole cell primed paediatric UK population (Children_UK_wP and Adolescent_UK_wP): antibody functionality and association of HLA-DRB1-233Thr variant with serology

Original analysis from *Mentzer et al [230]* showed that self-reported whooping cough protection in cohorts from the 1950's had negative association with HLA-DRB1-233Thr. In order to investigate if this variant had an association with PT IgG antibody levels in a UK population, where the association with protection was found, an analysis of samples from two wP primed paediatric populations was performed (Children_UK_wP and Adolescent_UK_wP). The serology results of this population were presented in CHAPTER 3, section 3.5.

5.4.3.1 Serologic responses and antibody functionality

In this population, FHA IgG had the highest GMC's, followed by Prn IgG. In the Adolescent_UK_wP, the PT IgG was the antibody with the lowest GMC.

To evaluate the antibody functionality in this population, with no recent pertussis booster, the following functional assays were performed:

a. Avidity assay:

The avidity assay was performed in 245 individuals from the Adolescent_UK_wP study with a median age (IQR) of 14.8 years (13.3-16.3), 52.24% females. The sample selection was based in the following criteria: PT IgG antibody levels > 5 IU/ml, genotype data and available volume to perform the assay.

The avidity was measured using NH4SCN as a dissociating agent and avidity index (AI) was calculated based on pertussis specific antibody levels measured before adding the dissociating agent. The median (IQR) of the IgG PT (IU/ml) after incubation with NH4SCN was 17.0 97.3-37.4.

The median (IQR) of AI in this population was 75.2% (66.7-81.8%).

b. Bacterial adherence assay

In the Adolescent_UK_wP, the BAI was performed in 63 individuals, with a median age (IQR) of 12.9 years (12.5-13.2). All individuals had an age between 11 and 13 years, inclusive (within the age range of individuals enrolled in cohort B of the BERT study). In the Children_UK_wP study a total of 29 samples were analysed, from individuals with a median age (IQR) of 11.3 years (10.6-11.8). The median (IQR) EC50 in the Children_UK_wP was 29.3 (15.2-64.4) and in the Adolescent_UK_wP was 26.3 (11.8-51.6), with no statistically significant differences between groups (Figure 5.7). In the Children_UK_wP the strongest correlation was found between Prn IgG and for the Adolescent_UK_wP with PT IgG. In the latest population significant correlations were also identified for FHA IgG and Prn IgG (Figure 5.8).

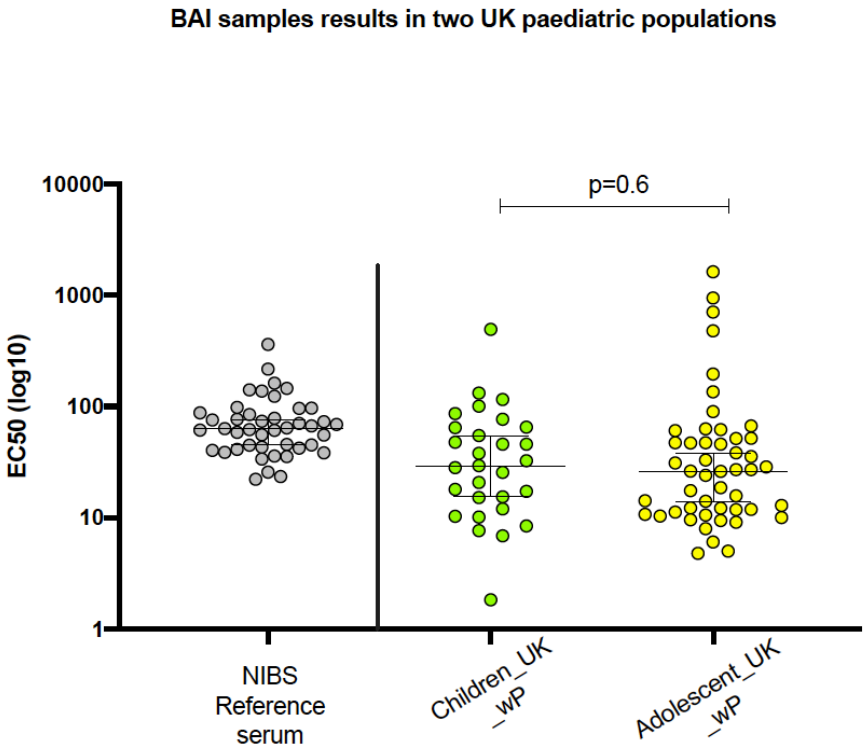


Figure 5.7 Dot plot of BAI results, represented by half maximum effective concentration (EC50) in UK paediatric populations: Children_UK_wP and Adolescent_UK_wP

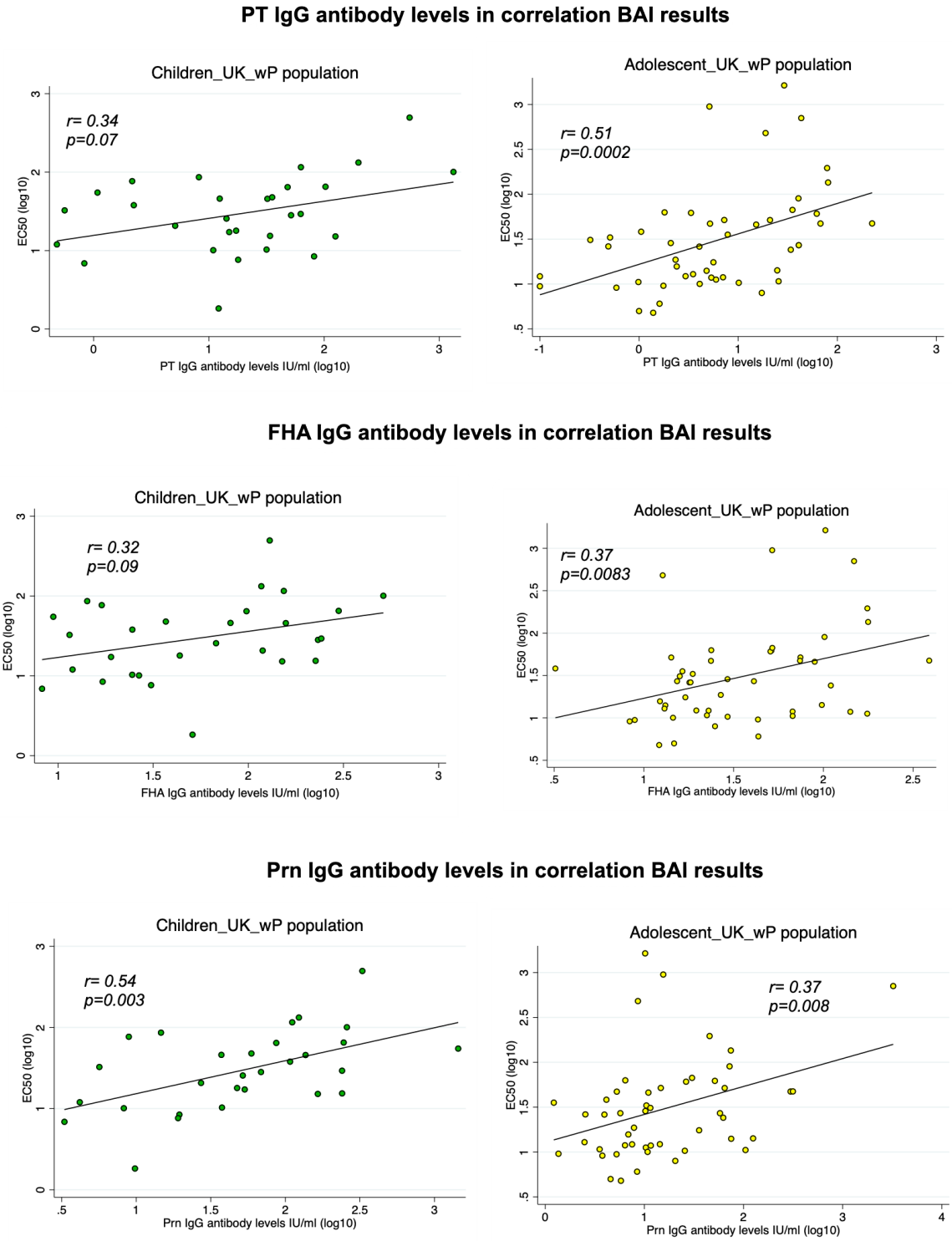


Figure 5.8 Correlation between pertussis specific antibodies levels (IU/ml) and bacterial adherence results (BAI) represented by half maximum effective concentration (EC50) in the Children_UK_wP and Adolescent_UK_wP population

5.4.3.2 Association analysis between HLA_DRB1_233Thr and serological responses in wP primed UK paediatric populations

For the association analysis the Children_UK_wP and the Adolescent_UK_wP populations were analysed together. In these populations the MAF of this variant was 62.5%. The HLA-DRB1-233Thr variant was significantly associated with IgG PT antibody levels (beta coefficient:0.14, *p=0.03*). No association was found with other pertussis specific antibody levels (Figure 5.9).

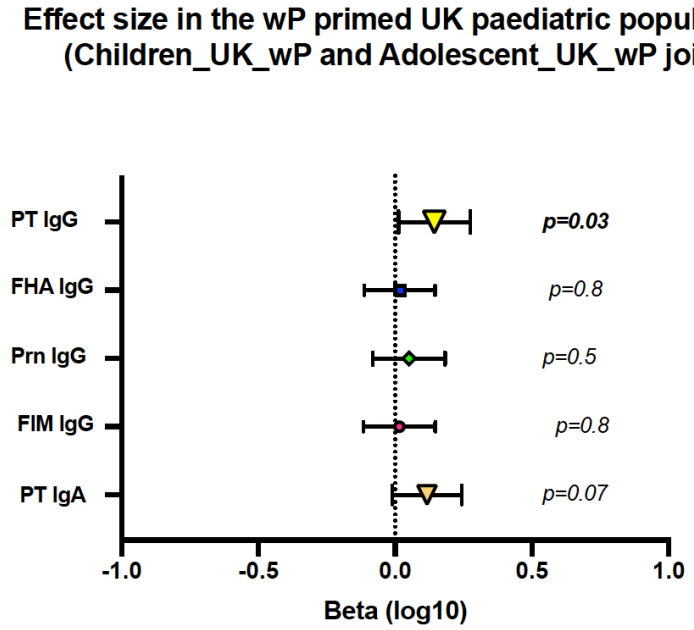


Figure 5.9 HLA-DRB1-233Thr effect size in the UK wP primed population from the Children_UK_wP and the Adolescent_UK_wP populations together, in association with different pertussis specific antibodies

Legend: PT: pertussis toxin; FHA: Filamentous haemagglutinin, Prn Pertactin, FIM: Fimbriae 2/3

When evaluating this association with antibody functionality, an association between avidity of PT IgG antibodies (beta coefficient:1.7, *p=0.001*) and HLA-DRB1-233Thr was found. The same association was not found with BAI (Figure 5.10).

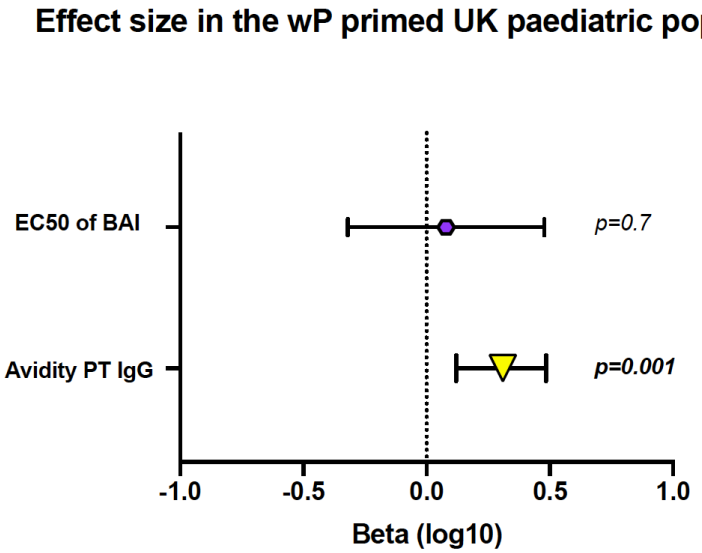


Figure 5.10 HLA-DRB1-233Thr effect size in the UK wP primed population from the Children_UK_wP and the Adolescent_UK_wP populations together, in association antibody functionality (Bacterial adherence assay (BAI) and avidity to pertussis Toxin (PT) IgG)

5.4.4 BERT study: association of HLA-DRB1-233Thr variant with serology in a UK population from different ages and different immunisation backgrounds

In the population from the BERT study the children and teenagers were aP primed. In the BERT study, the MAF frequency of the HLA-DRB1-233Thr variant was 64.7%. For this population, no association with variant HLA-DRB1-233Thr was found for any of pertussis specific antibodies at baseline and after booster immunisation with aP (Figure 5.11).

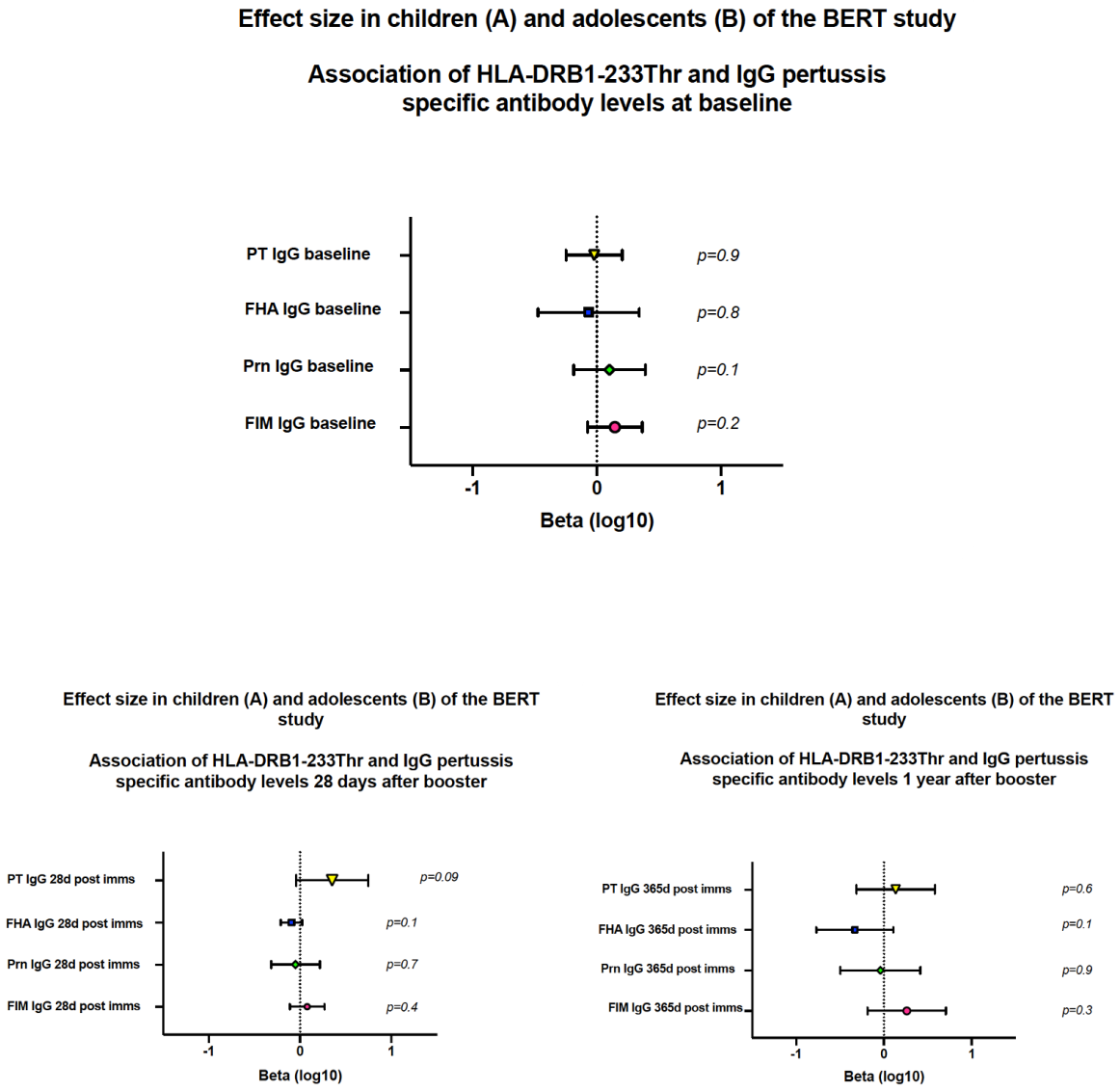


Figure 5.11 HLA-DRB1-233Thr effect size in the aP primed BERT population (serology IgG)

Cohort A and B in association with different IgG pertussis specific antibodies (PT IgG, FHA IgG, Prn IgG, FIM 2/3 IgG) at baseline (N=62), D28 (N=59) and D365 (N=59) after booster immunisation.

For the older populations in the BERT study, individuals that were wP primed (cohort C) or had no previous primary immunisation in the first year of life (cohort D),

showed a lack of association between the HLA-DRB1-233Thr and pertussis specific antibodies (Figure 5.12).

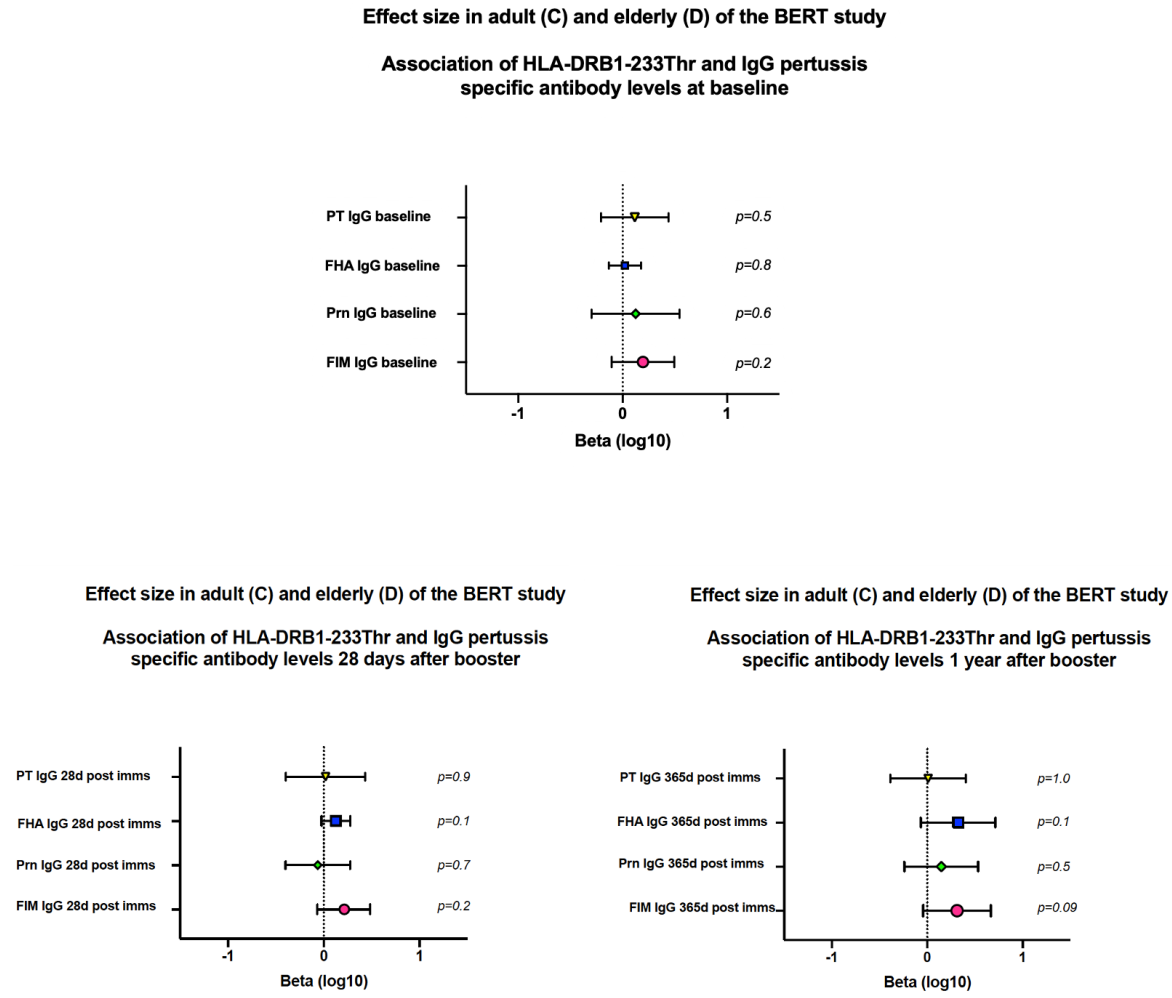


Figure 5.12 HLA-DRB1-233Thr effect size in the adult BERT population (serology IgG)

Cohort C and D in association with different IgG pertussis specific antibodies (PT IgG, FHA IgG, Prn IgG, FIM 2/3 IgG) at baseline (N=49), D28 (N=49) and D365 (N=45) after booster immunisation

If we analyse only cohort C, to include only individuals wP primed, no significant associations were found between HLA-DRB1-233Thr and PT IgG antibody levels (*D0 p=0.91; D28 p=0.66; D365 p=0.94*).

No association between IgA pertussis specific antibody levels and HLA-DRB1-233Thr was identified (Figure 5.13 and Figure 5.14).

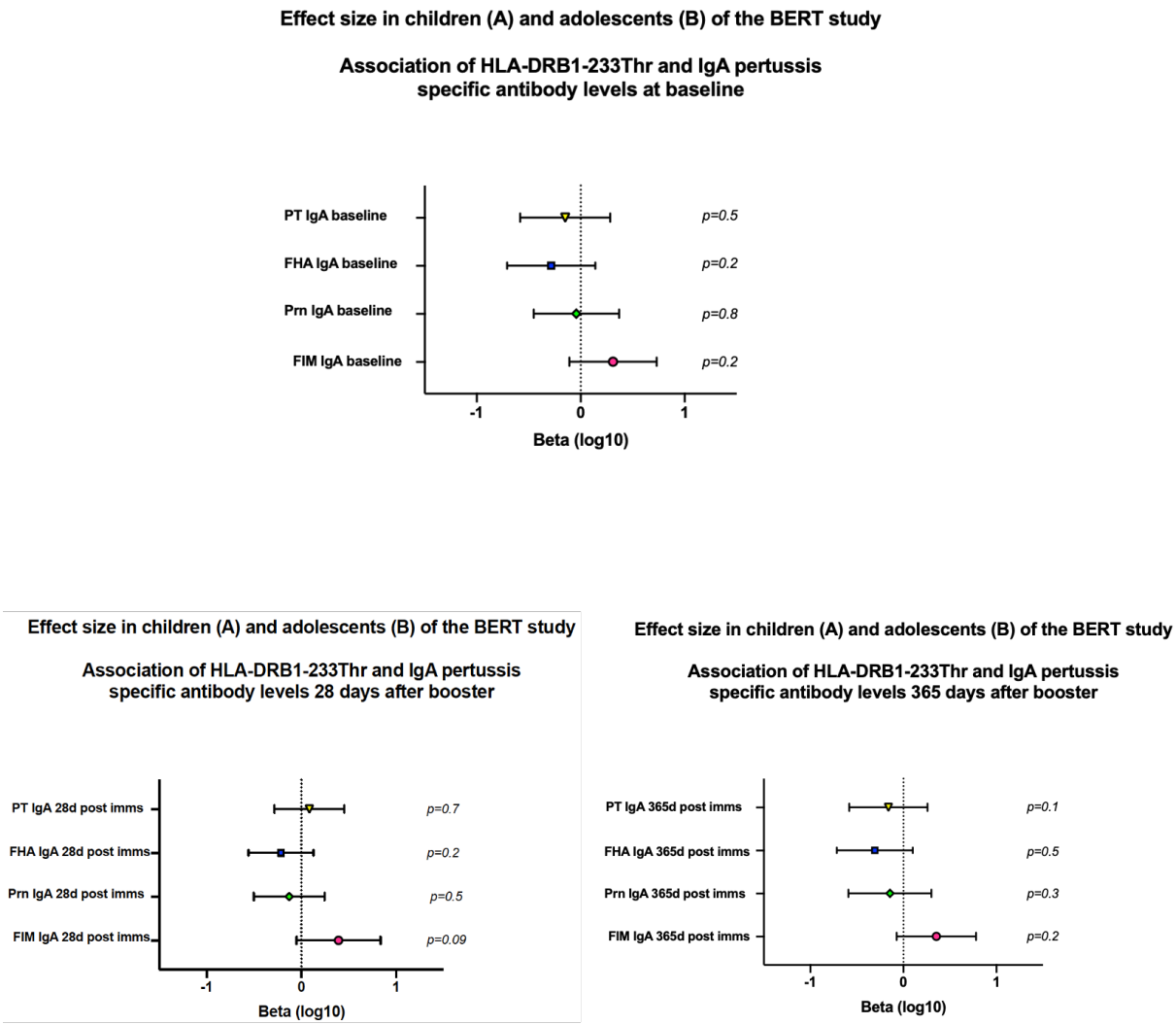


Figure 5.13 HLA-DRB1-233Thr effect size in the aP primed BERT population (serology IgA)

Cohort A and B in association with different IgA pertussis specific antibodies (PT IgA, FHA IgA, Prn IgA, FIM 2/3 IgA) at baseline (N=62), D28 (N=59) and D365 (N=59) after booster immunisation

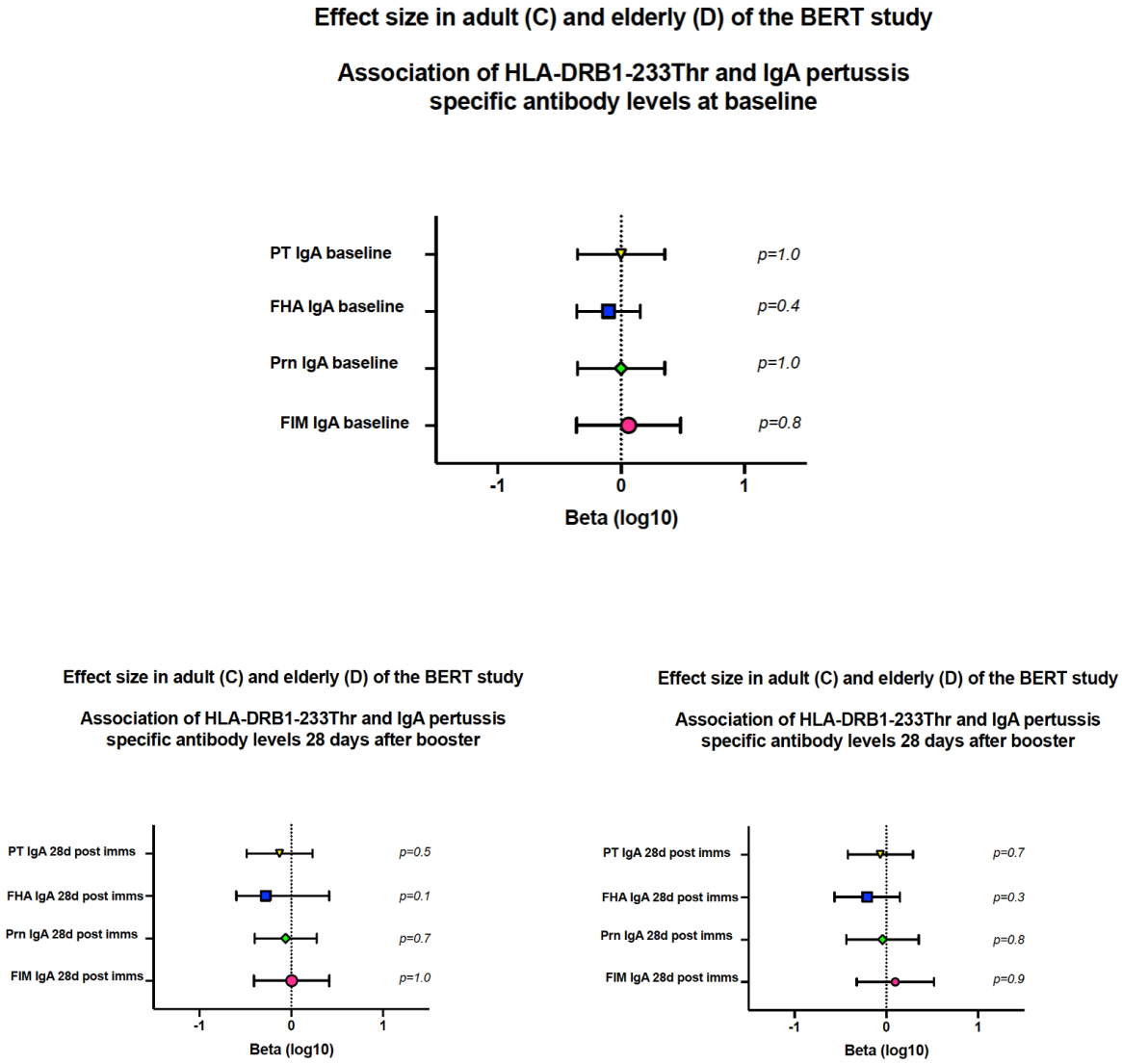


Figure 5.14 HLA-DRB1-233Thr effect size in the adult BERT population (serology IgA)

Cohort C and D in association with different IgA pertussis specific antibodies (PT IgA, FHA IgA, Prn IgA, FIM 2/3 IgA) at baseline (N=49), D28 (N=49) and D365 (N=45) after booster immunisation

No association was found between SBA and CDA for any of the BERT cohorts at any of the timepoints (Figure 5.15 and Figure 5.16).

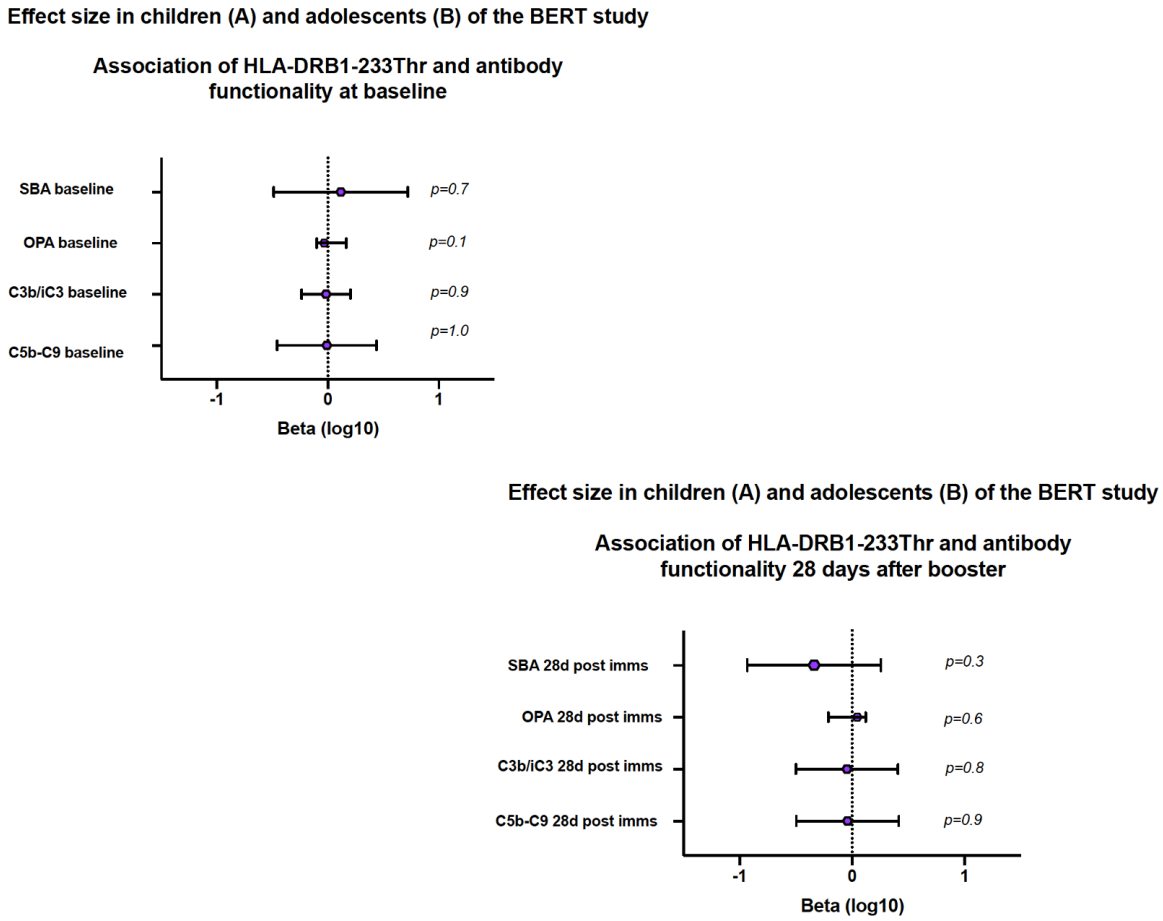


Figure 5.15 HLA-DRB1-233Thr effect size in the aP primed BERT population (functional assays)

Cohort A and B in association antibody functionality, measured by serum bactericidal assay (SBA), opsonophagocytosis (OPA) and complement deposition assay (C3b/iC3 and C5b-C9) at baseline (SBA N=32, OPA= 53, CDA N=62) and D28 (SBA N=31, OPA= 41, CDA N=59)

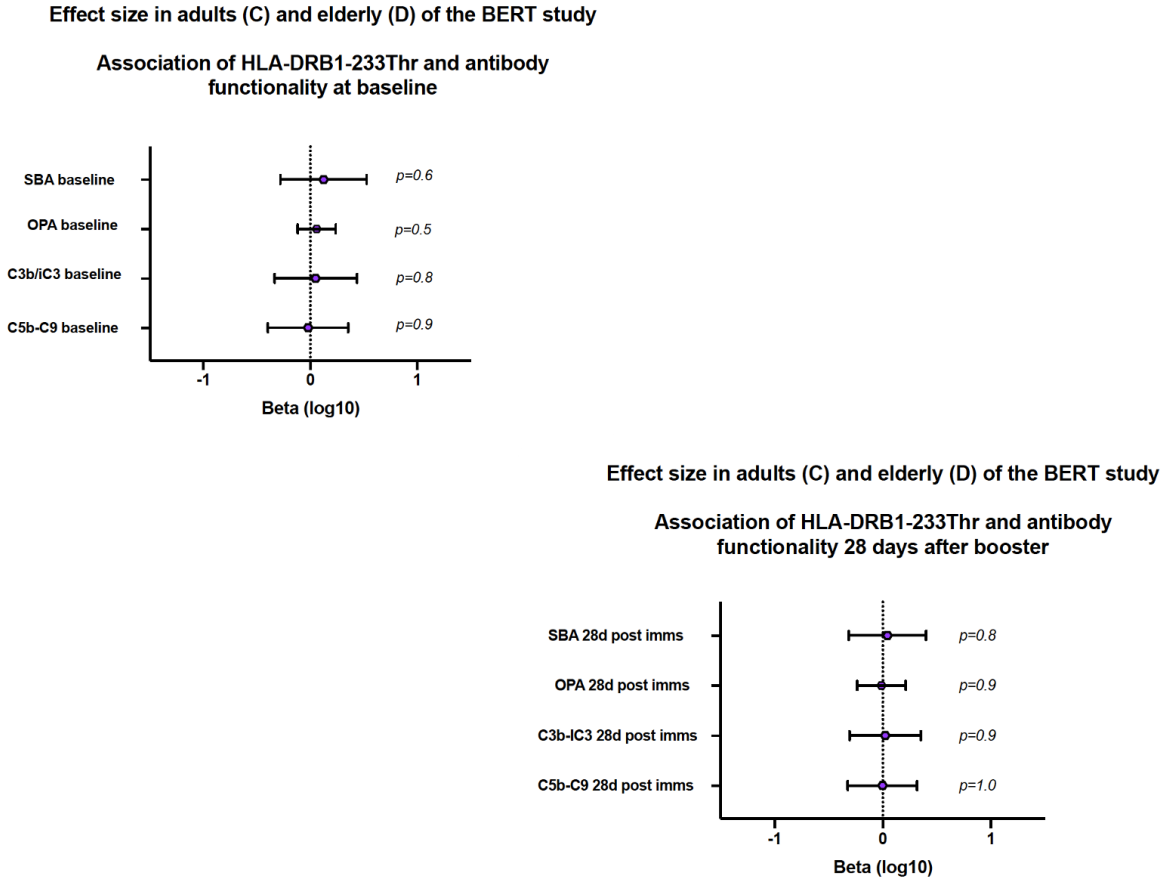


Figure 5.16 HLA-DRB1-233Thr effect size in the adult BERT population (functional assays)

Cohort C and D in association antibody functionality, measured by serum bactericidal assay (SBA), opsonophagocytosis (OPA) and complement deposition assay (C3b/iC3 and C5b-C9) at baseline (SBA N=47, OPA= 45, CDA N=49) and D28 (SBA N=48, OPA= 46, CDA N=49)

When evaluating association between HLA-DRB1-233Thr variant and MBCs pre- and post-booster immunisation with aP, the only association found was with Prn MBCs 28 day after immunisation. (beta coefficient: 0.56, *p=0.04*) (Figure 5.17).

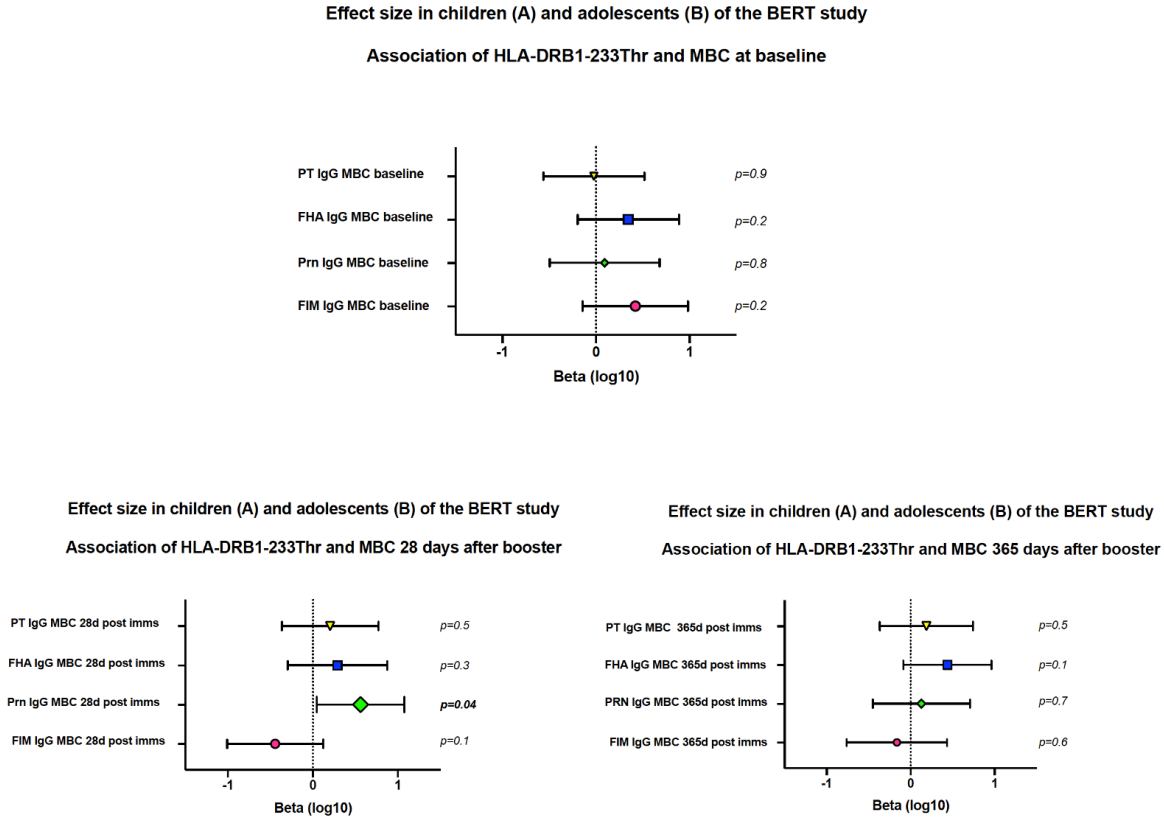


Figure 5.17 HLA-DRB1-233Thr effect size in the aP primed BERT population (MBCs)

Cohort A and B in association with pertussis specific memory B cells (MBCs) (PT, FHA, Prn, FIM 2/3 IgG) at baseline (N=35), 28 days (N=35) and 365 days (N=22) after booster immunisation

When considering older cohorts at baseline, FIM IgG MBCs showed a significant association with HLA-DRB1-233Thr, that was lost after immunisation, although the booster vaccine did not contain FIM antigen in the composition. This baseline association remained significant when analysing cohort C participants only (beta coefficient: 0.60, *p=0.006).*

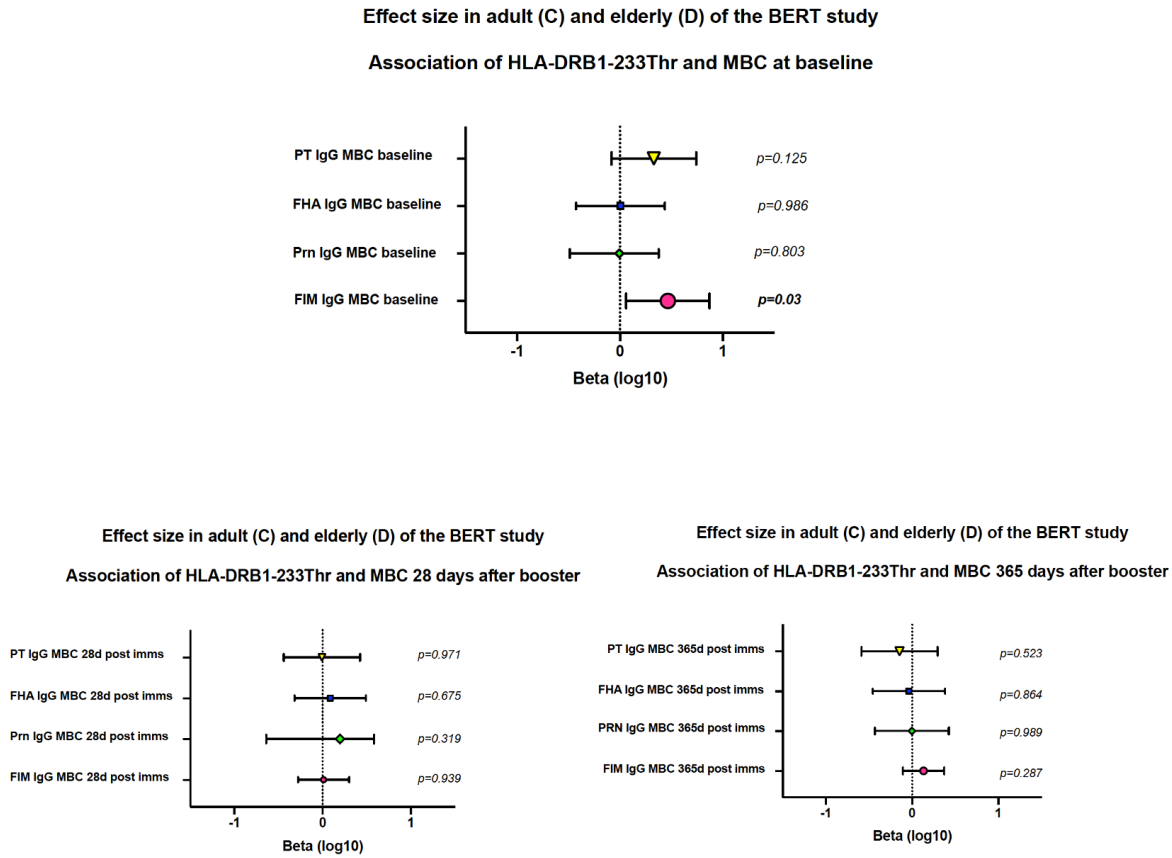


Figure 5.18 HLA-DRB1-233Thr effect size in the adult BERT population (MBCs)

Cohort C and D in association with pertussis specific memory B cells (MBCs) (PT, FHA, Prn, FIM 2/3 IgG) at baseline (N=44), 28 days (N=44) and 365 days (N=40) after booster immunisation

5.5 DISCUSSION

5.5.1 Summary of the main findings

A significant association between IgG PT and HLA-DRB1-233thr was found in the Infants_Asian_wP population in the first year of life.

The association of this variant with PT IgG antibody levels and no other pertussis specific antibodies was also replicated in the adolescent wP primed population.

When evaluating the performance of the inhibition of *B. pertussis* to the respiratory epithelial cell line (BAI), wP primed infants had higher EC50 levels, meaning lower bacterial adherence, when compared with aP primed infant population. The wP adolescent population had an EC50 similar to the aP primed African infants.

Antibodies anti-Prn IgG showed in general the best correlation with functional antibody assays.

Except for the association between PT IgG avidity and HLA-DRB1-233Thr found in wP primed adolescent biobank population, no other associations with this variant were found between the functional antibody assays performed and any of the study populations, wP or aP primed.

5.5.1 Analysis of the current results based on the available literature

Previous work in wP primed infants by *Mentzer et al* suggested that antibody responses against PT could potentially be used as correlate of protection against *B. pertussis* and the levels of these antibodies seemed to be significantly affected by differential HLA II alleles.

The Infants_Asian_wP study replicated the same findings, with HLA-DRB1-233Thr variant being associated with IgG PT antibody levels at all timepoints.

Considering a total of 568 samples from the Infants_African_wP study with an age range between 5 and 21 months, a similar pattern to the Infants_Asian_wP study, with PT IgG antibody levels considered the highest, were found.

Although it is difficult to compare the antibody levels between Infants_African_wP and Nepalese population (Infants_Asian_wP) due to the high range of ages, the fact that the same pattern is presented is reassuring of the similarities in the humoral response to immunisation with wP.

This HLA allele effect associated with PT antibody levels is likely to be wP specific and maybe more apparent in the short-term following primary immunisation, as seen in the infant studies.

Those findings were not found in aP primed infants in previous studies (*Mentzer et al)*.

Considering that both aP and wP lead to significant increase in antibody levels for different pertussis antigens, [207-209], it was suggested that other features may be the true determinant of protection and antibody function/quality might need to be accounted for.

In the Children_UK_wP and Adolescent_UK_wP, an association with the same HLA variant was also found (*p=0.033*), showing that this signature could be maintained in wP primed individuals for a longer period of time. In the BERT study for adults and elderly, this association was not replicated, although at baseline, before the aP booster immunisation, the direction of the signal was similar. These findings are likely related with the small study population and lack of power to detect the variant.

As shown for the aP primed infants, a lack of association was also found in aP primed individuals from the BERT, children and adolescents from cohort A and B.

The lack of HLA II associations for aP raises the possibility of differences in antigen processing/presentation for wP versus aP individuals with similar HLA background. Another explanation is that the genetic effects are not fully related with peptide biding, but instead with gene expression levels in particular cells, like

monocytes and MBCs (*Mentzer et al*, unpublished data). The mechanisms behind this phenomenon are not clarified, but the activation of monocytes as part of the innate immune response to the aP could have differences when compared with wP and natural infection. The aP more widely used in the current immunisation schedules around the world have aluminum ($Al(OH)_3$) as an adjuvant, a component that *per se* is able to induce strong immunity, including the stimulation of innate immune responses via monocytes, also contributing to antigen presentation [349]. It was shown by *Kooijman et al* that the effect on antigen presentation of $Al(OH)_3$ *per se* remained similar to the $Al(OH)_3$ adjuvanted DTaP vaccine, but in monocytes the signaling is stronger with $Al(OH)_3$ alone and the vaccine formulation seems to partially antagonize those responses [349]. This could in part explain why the HLA-DRB1-233Thr variant was found in wP primed population but not in aP primed individuals.

If we look into the antibody quality the only association with HLA-DRB1-233Thr was found between avidity to IgG PT. No association was found between the ability of the bacteria to be killed when using complement, evaluated by SBA and CDA, or to inhibit adherence to the respiratory epithelium in different populations independently of the background. It needs to be highlighted that Prn IgG and not PT IgG, showed the best correlation with these assays as described here and in CHAPTER 3 and other HLA variants are likely to be implicated in this mechanism.

Avidity for IgG PT was only performed in the adolescent population from the UK, wP primed. Avidity has been described as an indirect measure of long-term protection and could be used to evaluate antibody functionality after immunisation [300,350].

In the BERT study MBCs were evaluated in all populations. A significant association of HLA-DRB1-233Thr with Prn MBCs 28 days after booster immunisation

was found in aP primed individuals. This association was not present at baseline and was lost at D365. The low number of individuals in this study limits the interpretation of the signal in this population, as well of their potential maintenance, although we could hypothesise that booster aP immunisation in an aP primed population can induce long-term memory.

5.5.2 Limitations

The number of individuals in the BERT study representing each one of the immunisations backgrounds was very small, particularly when we consider only the individuals that were effectively wP primed, from only cohort C. That limitation does not allow us to interpret the meaning of the lack of significance between the HLA-DRB1-233Thr variant and PT IgG antibody levels as found in other populations. These assumptions are also valid in the analysis of association with long-term memory and antibody functionality. The aim of this study was to replicate a previously described top associated allele variant and not to identify novel associations, that would implicate larger populations, not explored in this thesis. This approach could be considered a limitation and although the identification of other alleles could have been explored in these datasets, it would always be as part of a secondary analysis.

5.5.3 Conclusions

This project showed that the HLA class II variant DRB1-233Thr association with PT IgG antibody levels was replicated in an infant Asian population and in an European adolescent population, both wP primed. This variant was present in different

populations and was maintained several years after primary immunisation with a wP vaccine. Also, HLA-DRB1-233 seems to be associated with long-term protection, as seen by the significant association with avidity in the same population. The association between PT IgG antibody levels was not found in the BERT study, both in the aP primed children and adolescents' cohorts and the older individuals from cohort C (wP primed) and cohort D (natural infection immunity).

CHAPTER 6

CONTINUOUS TEMPERATURE MONITORING AS A METHOD TO STUDY REACTOGENICITY

6.1 BACKGROUND

6.1.1 Adverse events and the impact in vaccine acceptance

One of the most important aspects when studying a new vaccine or on post-marketing studies is vaccine safety. Recording AEFI allows us to monitor the safety of a vaccine, although cause-effect interpretation can be challenging [351,352]. Safety concerns with a vaccine, particularly due to their preventive nature, can compromise vaccine acceptance and consequently coverage, that ultimately leads to increase in disease rates and loss of heard immunity in some cases [266,268,353]. A report from the European Commission (EU) from 2018, covering the 28 EU members countries at the time, has shown that although in general 90% of the European population believe that immunisations are important for children and 86.5% believe they are effective, only 82.1% agreed that vaccines are safe [267]. Confidence in vaccination is an issue, currently growing in high income countries.

6.1.2 Adverse events after immunisation: fever as an objective representation of vaccine reactogenicity

Fever is considered one of the most commonly reported adverse event and according to the US vaccine adverse events reporting system 1991-2001, accounting

for 26% of all AEFI [354,355]. The frequency of fever events depends on the type and combination of vaccines administered [356]. Fever represents an endogenous increase in the body core temperature [357,358]. In summary, innate immune cells sense the presence of exogenous stimulus by the binding of molecules that act as pathogen-associated molecular patterns, like lipopolysaccharide (LPS) to pathogen recognition receptors [359]. The binding leads to the production of prostaglandins E2 (PG2) from peripheral tissues in very early phases and from brain cells, that after release in the brain parenchyma will act in the preoptic nucleus of the hypothalamus, stimulating neural pathways that would be responsible for the increase in the core body temperature [357,359,360].

Although a wide variation between methods has been shown, it is generally accepted that rectal or tympanic temperatures are normally considered to be higher when compared with axillary or oral temperatures [356,361-363].

One of the most consensual definitions for AEFI is from the Brighton Collaboration: “Fever is defined as the endogenous elevation of at least one measured body temperature of ≥38°C” [277], but no specific method is defined, leading to differences in the frequency of the event for the same vaccine [364,365].

The perception of fever by parents varies, with a study describing that around 18% were concerned about the consequences of fever to the child’s health, 1/3 about risk of dehydration and febrile convulsions and 13% about risk of brain damage [366]. It is important to explain to the general public that high reactogenicity or increased fever rates are not equal to safety concerns of a vaccine.

6.1.3 High reactogenicity vaccines: the old (wP) and the new (4CMenB)

The wP is a good example of how concerns with high reactogenicity were associated with a decrease in vaccine acceptance and vaccine coverage in the 1970's [109]. Associated with the fever episodes, febrile convulsions were estimated to occur in 1/16,000 immunisations, a significant higher number when compared with the currently available aP (1/200,000) [367]. Although it is proven that the febrile convulsions are not associated with long-term neurological illness or sequelae [368], at the time, it was suggested that DTP had causal relationship with neurological complications [109,112,157,369,370].

Another example of a highly reactogenic vaccine is the 4CMenB [365,371]. This high reactogenicity was associated with high fever rates, particularly increased when the vaccines were given alongside with routine immunisations [364,365]. High fever rates were also reported with a vaccine containing only OMV's, designed to control the New Zealand outbreak [372]. This finding was also shown in a mouse model [373]. The full mechanism is yet to be described and other antigens containing in the vaccines with pyrogenic activity, like LOS, might also be involved [2,374].

In 2015 the UK was the first country in the world to introduce the 4CMenB vaccine in the NIP [263,375], but due to concerns of high rates of fever and presumably how they could lead to increase in hospital AE admissions [376,377] and impact in vaccine acceptance, it was recommended the use of three doses of paracetamol after vaccine administration at the two and four months doses [264].

6.1.4 Continuous temperature monitoring methods

Many advantages can be attributed to the use of continuous temperature monitoring methods: allow a better understanding of temperature trends, association with circadian rhythms and if applicable, more reliable fever rates.

Evaluation of temperature patterns have been used in clinical studies to understand the probability of predicting disease outcomes, as an early indicator of disease severity, like sepsis and to potentially guide treatment interventions [378,379].

The same methods can be applicable to understand fever rates post-immunisation and to define strategies when a vaccine is given universally. Also, the correlation of these findings with other relevant aspects like gene expression signatures and immunogenicity [2] allows distinction between fever associated with an actual severe condition or as an adverse event.

Not all available continuous temperature methods are applicable to humans, particularly when using invasive techniques, or can be difficult to use outside medical facilities [270,378,380,381].

The iButton® is a small wireless device that was validated to be used in the human skin to monitor temperature [271,272]. A full description of the device is presented in CHAPTER 2, section 2.6.

The aim of this chapter is to explore the role of a continuous monitoring method in a high reactogenic vaccine and compare with the results of conventional intermittent methods currently used in vaccine clinical trials. This was initially planned to be applied in the AWARE study (comparing wP and aP).

This chapter was written based on the work that I performed in this area on the 4CMenB vaccine, from two studies (EUCLIDS and Sched 3 study) [1-3]. The iButton®

results for the EUCLIDS study were partially explored in two publications [1,2]. The iButton® results from the Sched 3 study were published in full in a paper dedicated to this subject, that I first authored [3].

6.2 METHODS

Methods used for the performance of these studies are explained in CHAPTER 2, including iButton® specifications and the general lines of the statistical analysis. In this section specifications of each study are explored.

6.3 RESULTS

6.3.1 EUCLIDS study

The EUCLIDS trial, that occurred between July 2014 and June 2016, recruited a total of 187 infants, 94 randomised into the test group and 93 in the control group.

The rates of fever detected were higher in the test group at both timepoints when compared with the control group, independently of the temperature evaluation method (Table 6.1). When considering the full study population in the test group and the control group, the same number of individuals had a fever after the two immunisation visits (test group: N=53; 56,4%, control group: N=24; 25.8%). For a subgroup of study participants, it was not possible to collect temperature records due to failure of the equipment or misplacement. Table 6.1 shows the number of individuals with valid temperature readings for each one of the methods and proportion of participants with fever episodes. The differences between the number of fever

episodes detected by each method were considered statistically significant in between groups (Pearson Chi-squared test p <0.001).

Table 6.1 Number of individuals in each group with at least one fever episode detected by each one of the two study methods in the first 24 hours after immunisation

	Test group (Routine imms+4CMenB) N=94		**Control group (Routine imms) N=93**		Comparisons
Age	Axillary temp (1^{st} 24h)	iButton® (24h period after vaccines)	Axillary temp (1^{st} 24h)	iButton® (24h period after vaccines)	p value *test *vs* control using iButton **Axillary *vs* iButton®
4 months	12/94 12.8%	53/89 59.6%	6/93 6.5%	24/88 27.3%	**<0.0001* *** Test: <0.0001* ***Control: 0.0003*
12 months	10/94 10.6%	53/90 58.9%	7/93 7.5%	24/84 28.6%	**<0.0001* *** Test: <0.0001* ***Control: 0.0003*

For the individuals where readings from both methods were available, the iButton® did not detect fever in a group of participants as represented in the venn diagram in Figure 6.1.



Figure 6.1 Venn diagram representation of the participants with temperature measurements by the two study methods and with at least one episode of fever in the first 24 hours after immunisation (at four and 12 months of age)

To better understand the concordance between the methods, a minute-to-minute match between the temperatures recorded by the parents at four- and eight-hours post-immunisation and the temperatures recorded by the iButton® was performed (Figure 6.2). The Bland-Altmann plot shows the comparison between the two methods, the x-axis represents the mean of the axillary and iButton® temperatures and the y axis the temperature difference between axillary and iButton® temperatures. For the 4 months timepoint most of the measurements fell into the agreement limits (+/- 2 standard deviation of the mean of the temperature differences), but the wide limits were in favour of a moderate to weak agreement as shown below.

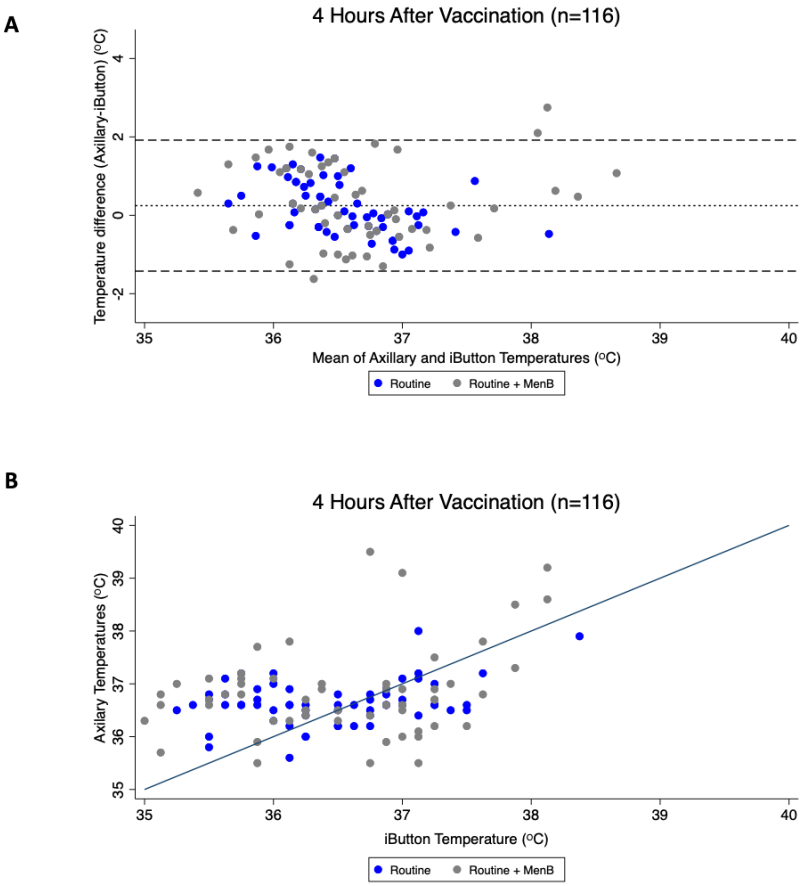


Figure 6.2 Bland-Altman plot and scatterplot representing the concordance between axillary and iButton® temperature records around four hours after immunisation at four months of age

Bland-Altman plot legend (A): Grey short dash dot (.-.-.-.-): Mean of the temperature difference (axillary and iButton® ºC)**;** Grey dash (- - - -) upper and lower dash line represent the +/- 2 standard deviation (SD) of the mean of the temperature differences

Considering the temperatures recorded at four and eight hours after immunisation, although the concordance was considered to be statistically significant at both timepoints and visits, the concordance was considered moderate to weak (Figure 6.2 and Table 6.2).

Table 6.2 Lin's concordance correlation between the iButton® and axillary temperature measures for all study participants at different study visits and timepoints

	Lin's concordance correlation					
	4 hours		8 hours		24 hours	
	N ρ^c (95%CI)	p-value	N ρ^c (95%CI)	p-value	N ρ^c (95%CI)	p-value
4 months	N=116 0.29 (0.13-0.45)	**<0.001**	N=75 0.25 (0.06-0.45)	**0.01**	N=8 0.24 (-0.13-0.61)	0.2
12 months	N=100 0.34 (0.19; 0.48)	**<0.001**	N=98 0.29 (0.13-0.45)	**<0.001**	N=8 -0.08 (0.79-0.63)	0.827

Focusing on the iButton® only data, the time for the first fever was 445 minutes (7.4 hours) [215.5-611.0] in the control group and 431 minutes (7.2 hours) [329.0-633.0] in the test group at the 4 months visit. Figure 6.3 represents the 12 months visit.

12 months immunisations

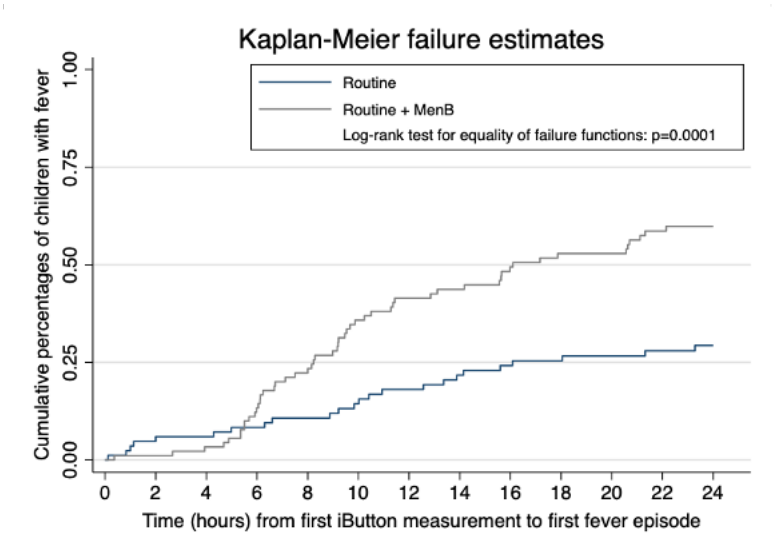


Figure 6.3 Kaplan-Meyer curves with cumulative percentages of individuals with at least one fever episode representing the time for the first fever at 12 months

Although differences were detected between the groups regarding the presence of fever, no differences were detected in severity: number of minutes of fever (4 months median: test group=66.0 minutes; control group=35.5 minutes, Mann-Whitney *p=0.4 /* 12 months median: test group=95.0 minutes; control group=100 minutes, Mann-Whitney *p=0.5)* and median temperature of fever episodes (4 months median: test group=38.2ºC minutes; control group=38.1ºC, Mann-Whitney *p=0.3 /* 12 months median: test group=38.2ºC minutes; control group= 38.3ºC, Mann-Whitney *p=0.1).*

Considering that in this particular study anti-pyretic treatment was only given on demand, since use of paracetamol was not recommended routinely, an analysis of the use of paracetamol in this study and differences in fever episodes detected by the iButton® was performed.

After the four months immunisations 76/94 infants from test group and 71/93 infants from the control group had information available about the use of paracetamol. In the test group paracetamol was administered in 56.4% and in the control group in 32.2% of the infants.

6.3.2 Sched 3 study

The sched 3 study enrolled 213 individuals between September 2015 and June 2016. From the total participants, 106 infants were randomised to the PCV 2+1 group (median age 60 days) and 107 infants to the PCV 1+1 (median age 59 days).

The iButton® detected a significantly higher number of individuals that had at least one episode of fever in the first 24 hours after immunisations. If we considered the number of fever events across all visits, the iButton® detected 4.1 times more episodes when compared with the axillary thermometer (Table 6.3). Considering all participants, at each visit, a statistically significant difference between the number of fever episodes detected by the two methods was found *($p<0.001$).*

Table 6.3 Number of individuals in each group with at least one fever episode detected by each one of the two study methods – axillary temperature and iButton® in the first 24 hours after immunisation, after each visit

		V1	V2	V3	V4
PCV2+1	Axillary temp (7 days diary)	20/106 18.9%	7/105 6.7%	30/102 29.4%	38/100 38%
	iButton® (24 hours period after immunisation)	69/99 69.7%	29/101 28.7%	63/102 61.8%	75/98 76.5%
PCV1+1	Axillary temp (7 days diary)	15/104 14.4%	12/105 11.4%	24/102 23.5%	33/89 37.1%
	iButton® (24 hours period after immunisation)	72/101 71.3%	48/103 46.6%	47/98 48.5%	64/90 71.1%

Adaptation of table published in the paper Valente Pinto, et al Vaccine 2020 [3]

The 12 months immunisation timepoint, when 4CMenB vaccine was administrated but paracetamol was not given, showed in general the higher rates of fever when considering the two study groups and both methods (Table 6.3). This timepoint also represented the 24 hours with the higher mean maximum temperature detected by the iButton® (PCV 2+1: 38.38 ºC and PCV 1+1: 38.37 ºC).

At three months of age, when 4CMenB vaccine was not given to any of the participants, but also no paracetamol was recommended at a fixed regime, the fever rates were the lowest detected by the two methods in both groups. Despite the lower rates of fever, this was the only immunisation timepoint that showed statistically significant differences between the number of individuals with at least on fever episode detected between the two groups (*p=0.008*).

A minute-to-minute match between the axillary temperature and the temperature recorded by the iButton® was performed. The Lin's concordance correlation showed a weak agreement between the two methods particularly at 4- and 24-hours post-immunisation measurements (Figure 6.4 and Table 6.4). This finding is demonstrated in the Bland-Altmann plots (Figure 6.4) of the 4 hours post-immunisation temperature evaluations at all study timepoints (2, 3, 4 and 12 months) by the wider agreement limits (+/- 2SD of the mean of the temperature differences) and the presence of some points not falling in-between those limits (Figure 6.4).

A stronger and significant agreement was seen at the temperatures collected at 8 hours post-immunisation in the two, three and four-months visits (ρ_c 0.337 to 0.390, *p<0.0001*). The agreement was weaker at the 2- and 12-months visits.

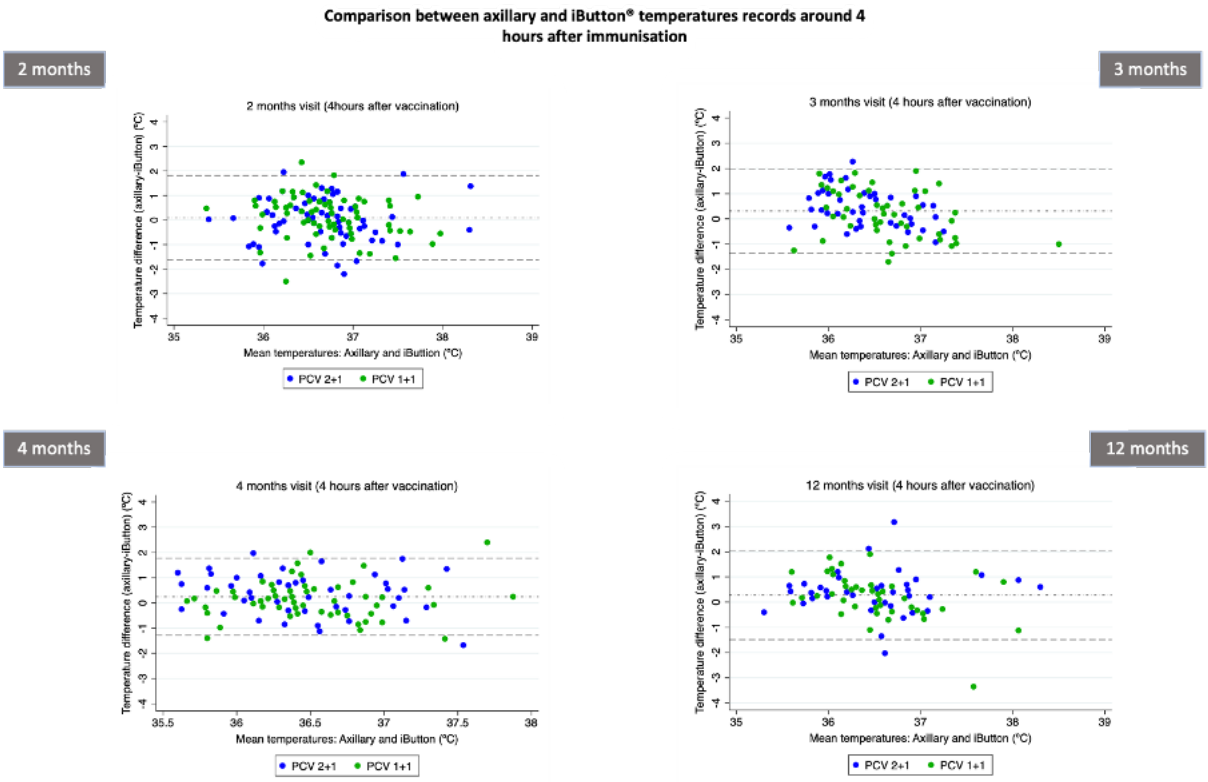


Figure 6.4 Bland-Altman scatterplots representing the concordance between axillary and iButton® temperature records around four hours after immunisation at two, three, four and twelve months after immunisations.

Legend: Grey short dash dot (.-.-.-.-): Mean of the temperature difference (axillary and iButton® ºC)**;** Grey dash (- - - -) upper and lower dash line represent the +/- 2 standard deviation (SD) of the mean of the temperature differences

Table 6.4 Lin's concordance correlation between the iButton® and axillary temperature measures for all study participants at different study visits and timepoints

	Lin's concordance correlation							
	4 hours		8 hours		18 to 20 hours		24 hours	
	ρ_c (95%CI)	p-value	ρ_c (95%CI)	p-value	ρ_c (95%CI)	p-value	ρ_c (95%CI)	p-value
2 months	0.161 (0.02-0.320)	0.047	0.390 (0.260-0.515)	<0.0001	0.176 (0.023-0.329)	0.024	0.200 (0.018-0.382)	0.031
3 months	0.153 (-0.006; 0.312)	0.059	0.337 (0.210-0.464)	<0.0001	0.172 (0.026-0.319)	0.021	0.179 (-0.016 – 0.373)	0.071
4 months	0.206 (0.041-0.371)	0.014	0.355 (0.227-0.483)	<0.0001	0.319 (0.191-0.448)	<0.0001	0.315 (0.078-0.552)	0.009
12 months	0.265 (0.081-0.449)	0.005	0.158 (-0.017 - 0.333)	0.077	0.337 (-0.178; 0.496)	<0.0001	0.122 (-0.204; 0.447)	0.463

Considering only the iButton® temperature records, time for the first fever after immunisation, starting with the first minute recorded by the iButton® was determined. No statistically significant differences were found between PCV 2+1 or PCV 1+1 groups at the different timepoints (Figure 6.5). The mean time to the first fever ranged between nine to 10.5 hours and no statistical significant differences were found between visits (repeated measures ANOVA f(3,269)=1.14; *p=0.3*). No statistically significant differences were found between groups regarding the mean temperature at each visit.

Study visits with 4CMenB and paracetamol administration

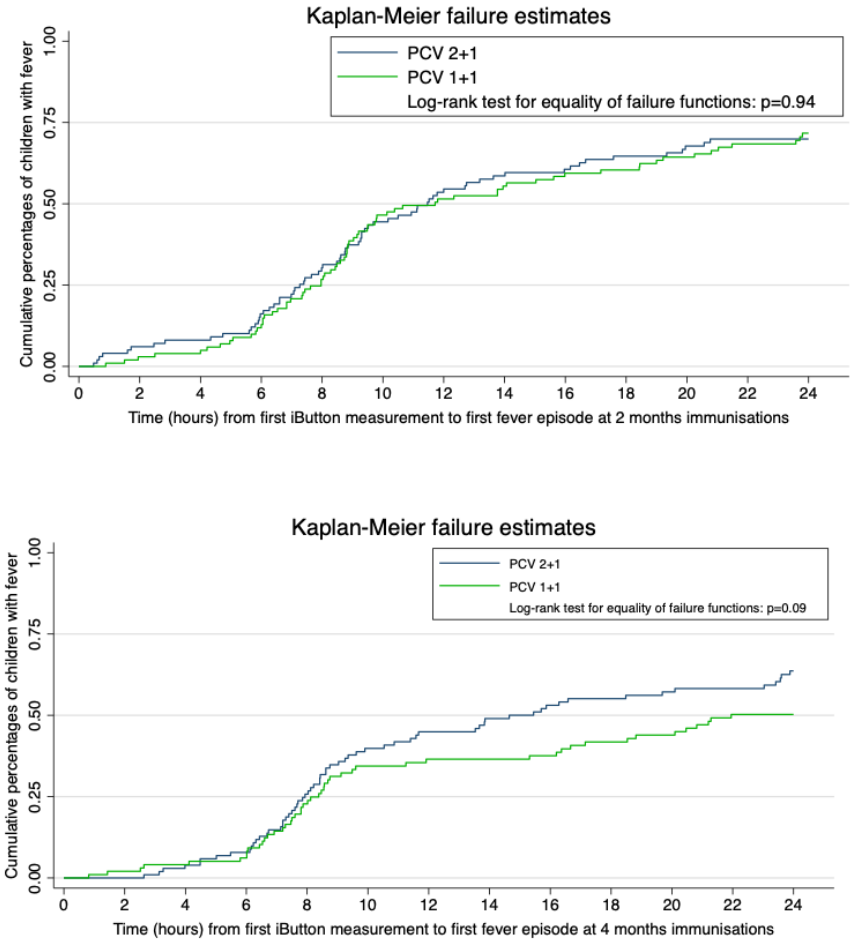


Figure 6.5 Kaplan-Meyer curves with cumulative percentages of individuals with at least one fever episode at 2 and 4 months of age

6.4 DISCUSSION

6.4.1 Summary of the main findings

These studies showed that the use of a continuous method detected a higher number of fever episodes when compared with the conventional intermittent method.

The agreement between methods was considered weak in the majority of the evaluations, although statistically significant.

The continuous method allowed an evaluation of the time for the first fever that was between 7.2 and 10.5 hours in the two studies.

6.4.2 Analysis of the current results based on the available literature

Before implementing a new vaccine in the market one of the main factors that needs to be proved is safety, but safety is not always synonymous of absence of side effects. Despite the comprehensive pre-licensure trials showing vaccine safety, the introduction of 4CMenB is the perfect example of how a high reactogenic vaccine [382,383] can have impact when implemented, with increased parent anxiety, healthcare visits and invasive procedures [354,376].

Although this concept is not new, the challenge in the use of more comprehensive methods, like continuous temperature monitoring, in human clinical trials, is the ability to find a method that is tolerable, non-invasive and can be used in ambulatory. The iButton® fulfils all of these conditions [270,271] and can be easily applied to children.

For this work, the iButton® was applied to two studies, targeting the 4CMenB. The intermittent method of choice was axillary temperature. Although rectal temperatures are acknowledged to be consistently higher [363], this was not considered by the Brighton collaboration, where temperatures ≥38ºC after immunisation were considered to be fever, independently of the method used [277]. No specific recommendations of the best intermittent method in immunisation studies

for different age groups are defined, but the standard practice in pediatrics in the UK is the use of axillary temperature [384,385].

In both EUCLIDS and Sched 3 study the rates of fever determined by intermittent method were lower in the groups that received 4CMenB plus routine (Test group in EUCLIDS study and PCV 2+1 and PCV 1+1 in the sched 3 study) ranging from 10.6%-29.4% at the 4CMenB visits, when compared with previous studies [364,365]. The study from *Gossger et al [365]* that used the same method (axillary thermometer) showed rates of fever between 51 to 61% and in a study from *Vesikari et al [364]* rectal temperatures above 38.5ºC were detected in more than 60% of the infants in the first 6 hours after vaccination. The lower detection in fever rates when using axillary thermometer in infants in the Sched 3 study can be partially due to the use of three doses of prophylactic paracetamol as recommended by NHS [264], although in the EUCLIDS study this medication was only given on demand to around half of the population in the same age group. For the older age group (12 months), the conditions were similar, since prophylactic paracetamol was not recommended. The rates of fever captured by the iButton® were closer to the results from previous studies. It would be relevant to understand if any misuse of the axillary thermometer happened during the study, considering the lower rates of fever detected.

In the EUCLIDS and Sched 3 study, a lack of consistency in the concordance between the two methods (axillary *vs* iButton®) was found, with a maximum Lin's correlation achieved in these studies of ρ_c 0.39. A better correlation between the iButton® and rectal temperatures measurements (r=0.71; *p<0.001*) was shown in a study by *Balla et al* [269], that compared different continuous temperature devices and rectal temperatures collected intermittently in adults (n=15), with a mean age of 68.7 years [269]. Besides previous studies showing a lack of concordance between axillary

and rectal temperatures [363,386], not allowing comparisons, the differences in the age of the participants, with the mechanisms of thermoregulation less matured in infants when compared with adults [387], allows us to hypothesise that the differences between studies can be partially attributed to this finding. Also, in both EUCLIDS and Sched 3 studies, the time that each temperature was collected was registered by the parents in a diary, without supervision of a study staff member, which could lead to inaccurate registries.

To our knowledge application of these methods in immunisation studies in children is scarce and needs wider use to allow further conclusions. In a study by *Drewry et al* a continuous temperature monitoring was used to identify patterns to distinguish septic and non-septic patients and evolution to a more severe disease manifestation [379]. This approach can be taken even further as performed in the EUCLIDS study that was designed to explore molecular pathways and gene signatures associated with reactogenicity after 4CMenB vaccine [2]. It was found that SELL gene, a gene related with neutrophil recruitment and increased neutrophile extravasation, was differently expressed in the participants based on the presence or absence of fever in the first four hours after immunisation [2,388].

6.4.3 Limitations

One of the main limitations of these studies is to rely on the information given by the parents regarding precision of times that the temperatures were collected and when any type of anti-pyretic treatment was administered. Despite the use of recording diaries, the risk of having an estimated time instead of a precise time can limit the interpretation of the minute-to-minute agreement.

Also, regarding the Sched 3 study we made the assumption that all children received the three doses of recommended prophylactic paracetamol as part of the routine immunisations at two and four months of age, but the study team only observed the first administration.

6.4.4 Conclusions

This work shows the relevance and applicability of a novel and alternative method to understand post-immunisation reactogenicity and to characterise fever in a more reliable way. The iButton® has shown to be a good alternative to intermittent temperature measurement methods that can be easily applied in a clinical trial setting and safe in children. For a wider use of this method in vaccination trials it would be ideal to optimise the technique and particularly to improve the record of the timings of the conventional methods to better understand the concordance between them.

The EUCLIDS work [1,2] showed that this method can be also used to better understand the pathways associated with reactogenicity and correlation with immune mechanisms after immunisation, that in the future could help to design vaccines that maintain high levels of immunogenicity, but with lower fever rates, that can greatly contribute for vaccine acceptance. Besides the use of this method to evaluate vaccine reactogenicity, use of continuous temperature monitoring could be applicable in other paediatric settings like patients with suspicion of severe disease, like sepsis (as previously described in adults [379]) or a situation with increased risk of severe disease, like children under chemotherapy or other immunosuppression therapy or admissions at intensive care units. This method can also allow to explore other

parameters, like the speed of rise and stability of the temperature, giving additional information applicable to the high-risk situation described above.

Considering the high relevance of the use of continuous temperature devices, my future work is to apply this method in the AWARE study, as already described (Supplementary data AWARE study).

CHAPTER 7

FINAL DISCUSSION

Whooping cough continues to be a life-threatening disease [4]. Until today, no definitive single CoP for pertussis was determined and accepted widely by the scientific community [184,188]. Although PT IgG antibodies continue to be used as a marker of recent infection according to WHO criteria, since it is specific of *B. pertussis*, no cut-off was found to determine protection against disease [91].

It is acknowledged that pertussis specific antibodies wane after immunisation or natural infection with duration of immunity estimated between 3-4 years to a up to more than two decades depending on the studies and the primary stimulus [176].

The novelty of this work, particularly when considering the BERT study, was the possibility to directly compare populations of different ages, with different immunisation backgrounds, applying a set of traditional and novel functional assays, under the same conditions, evaluating early and long-term responses. Also, the BERT study allowed us to explore the effects of two vaccines, aP and wP, in an unique time and place – after the aP being established for around 15 years and after well recognised outbreaks, with uncontrolled disease transmission being previously experienced by all individuals of this study. This allowed us to develop a wider knowledge of the impact of aP versus wP and how the long-term implementation of aP could influence or not the immune responses in these different age groups.

In this project, at baseline, IgG pertussis specific antibodies were detected in all age groups independently of the immunisation background.

In the UK BERT study before and after booster immunisation, PT and FIM 2/3 were the antibodies with the lowest levels when compared with FHA and Prn. As previously described, the data presented in this thesis is part of a multicentre study

combining population from The Netherlands, Finland and the UK [301]. Contributors for the baseline levels of each one of the antigens include: type of previous immunisation schedules in each country (number of doses, time between each dose, components type and concentration of the vaccines) [283] and epidemiology of the disease [389]. If we look at the incidence rates of pertussis in the last years in the three countries, The Netherlands had the highest disease levels per 100,000 individuals when compared with the other two countries. The Netherlands not only had the highest disease peak of the three countries, in 2011/2012, like in 2014 another disease peak was identified (Figure 7.1).

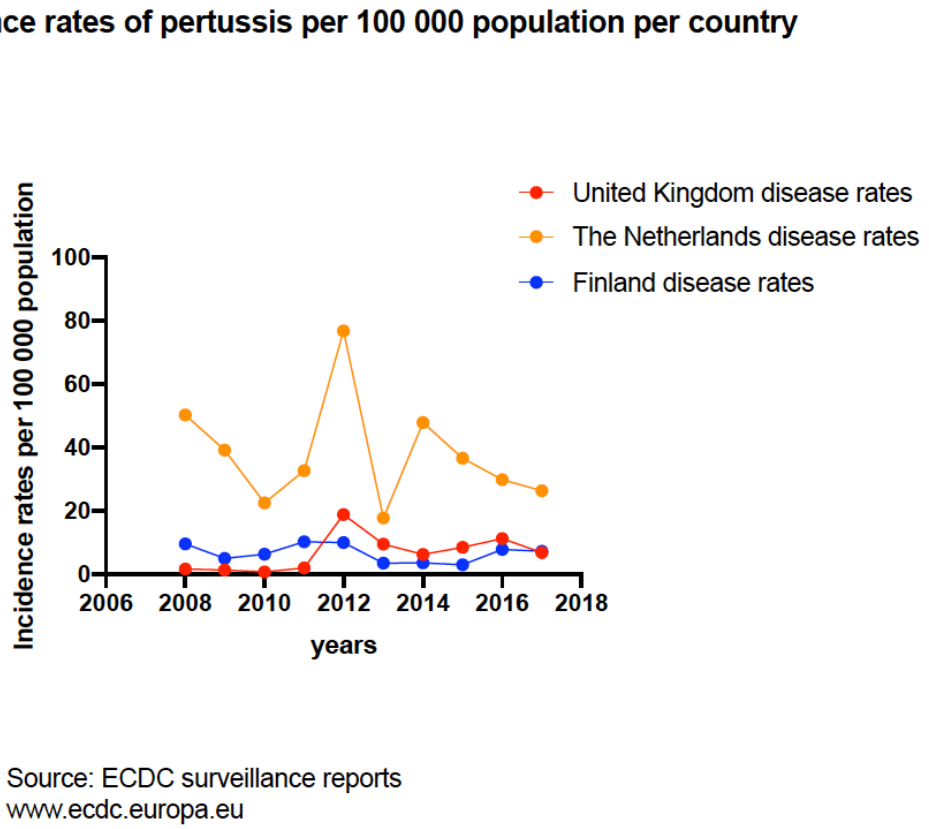


Figure 7.1 Incidence rates of pertussis disease in three European countries

In general, the incidence of pertussis disease is currently low in the UK. Despite an outbreak in 2011/12, likely to have induced a higher natural boosting during that

period, the incidence in the Netherlands was always higher. The disease rates in Finland were continuously low and no outbreak in the years before this study was declared. The introduction of maternal immunisation in the UK in 2012, was considered highly effective, contributing to the decrease of disease rates, and potentially avoiding further outbreaks. In the Netherlands this strategy was not implemented universally at the time of the first outbreak, potentially influencing the subsequent higher number of disease cases [166,167]

These differences could contribute to the higher antibody levels at baseline described in The Netherlands, in the overall BERT study [301]. Although these differences in epidemiology contributed to the magnitude in antibody baseline levels, the patterns were similar between countries, with PT and FIM showing to be the antibodies with the lowest levels. In the older age groups, including individuals with ages between 60 and 70 years, likely to never have been vaccinated or have been vaccinated a long time ago, the detected antibodies in the peripheral blood were probably due to natural boosting with no impact of previous vaccination, showing the circulation of the bacteria in the country.

I showed with this work that after a booster immunisation, independently of the primary immunisation background, a significant increase in all pertussis specific antibody levels for the antigens contained in the vaccine (PT, FHA and Prn) occurred. Although not explored in this thesis we also measured IgG antibodies against tetanus and diphtheria. Evaluation of these antibodies is frequently used to assess humoral immunity as a form to screen a suspected primary immunodeficiency. One of the advantages of testing for those antibodies simultaneously with antibodies against pertussis, is to identify individuals with overall poor serological responses, that are primarily dependent of each individual immune system and not of that specific vaccine.

In this study all recruited individuals were considered healthy and it was assumed that immunologically most of the individuals were able to mount a humoral response as shown by the presented data.

A novelty of this study was to show the behaviour of pertussis specific antibody after a booster dose in very early timepoints (D7/D14), with a quicker increase in the pertussis specific IgG antibody in the younger aP primed cohorts, particularly in children (A) in comparison with the older cohorts. This is an important marker of memory or circulating MBCs confirmed by the fact that, in general, the younger aP groups also had the highest baseline MBCs.

This kinetics can also show the ability of aP primed individuals with least amount of time since the last booster dose to mount a rapid response, although the older cohorts had a slower waning, particularly for Prn IgG, with implications with long term ability to limit the consequences of ongoing contacts with bacteria. This area is poorly explored and further studies evaluating the impact of immunisation in carriage and the association with mucosal antibodies in individuals with different immunisation backgrounds and the influence of time would be relevant.

In general, the elderly individuals had the lowest antibody responses at baseline and after booster immunisation. The same pattern was seen for the MBCs responses and for antibody functionality, particularly SBA and CDA. Considering the DOB, the majority of the elderly were not wP primed in the first years of life but were likely to have had the disease or frequent contact with bacteria. We can attribute the lower responses to the boost in this age group to immunosenescence typical of this age, more than any effect of immunisation background. The booster dose in all groups allowed, after one year, to keep all pertussis antibodies with levels superior to baseline, confirming the advantages of this intervention. Also, the wP primed

individuals had the highest MBCs FC after booster immunisation, showing the long-lasting effects of wP, when compared with aP in terms of long-term protection.

The aP primed adolescents and wP primed adults consistently had the highest antibody responses, with aP primed adolescents showing higher PT IgG responses and wP primed adults showing higher Prn IgG responses.

Prn IgG is an antibody produced against bacterial virulence factor responsible for the adherence of the bacteria. This step is fundamental for the disease to be able to develop [61-63]. I was able to show that Prn IgG was the antibody that best correlated with antibody functionality, including SBA, CDA and OPA. It is described that anti-Prn antibodies in aP are fundamental to induce opsonophagocytosis [290], confirming these results. Also, the best correlation between antibodies and plasma cell and MBCs frequencies 28 days after the booster was against Prn IgG antigen.

From this study two main findings need to be highlighted: 1) Prn IgG antibodies levels, that are easier to measure when compared with other assays, seem to directly correlate with antibody functionality and long-term protection; 2) wP primed individuals showed a better Prn IgG response after aP booster, when compared with aP primed individuals, although correlations with aP were also present.

Although these two assumptions need to be evaluated carefully, Prn IgG should be considered a serious candidate as one of the pertussis CoP, although other factors need to be acknowledged.

IgA subclass responses were lower when compared with IgG, but for pertussis specific responses an age effect was detected in this subclass, not detected in IgG, with higher responses in the older groups. This effect could be related with cumulative contacts with the bacteria and confirms IgA as a good marker for previous bacterial exposure, but not immunisation.

Other players seem to be of relevance in the induction of protection against disease. It is accepted that gene expression induced by specific stimulus can influence responses to vaccination. Previous findings of *Mentzer el al* showed a significant association between IgG PT levels and HLA-DRB1-233thr in wP primed infants, but not aP infants and the same variant being associated with less self-reported cases of pertussis diseases in a European population. In this DPhil project I was able to confirm that this association is not exclusively of the African population, but also found in infants from Asia. Also, this association is not lost with the increase of age, being described in wP adolescents, not recently immunised. This signature is likely to be universal and not ethnicity specific and after early induction can be kept long-term. The only association, besides PT IgG antibodies, were with PT IgG avidity, showing association not only with quantity, but also antibody quality, potentially showing that this variant could also be associated with better memory responses related with PT IgG.

No associations were found with other functionality assays, but the low number of samples needs to be considered, as previously described. In future work, it would be relevant to increase the sample size for each assay to truly determine if this variant could be associated with better bacterial killing or inhibition of bacterial adherence and explore other HLA variants, including the association with Prn IgG levels, an antibody thought to be important for long-term protection. The low sample size was an important limitation of the study, reducing he power to detect an effect. To overcome this issue the BERT study was performed as a multicentre study, to increase the sample size. The serology results of the UK BERT study follow the same pattern in the overall BERT population and between countries differences may relate to the known differences in epidemiology. Also, for the BERT UK study another limitation

was the lack of comparison between the two primary immunisation backgrounds in the same age group (aP vs wP), allowing a better assessment of the influence of age and immunisation background as two different variables. Considering that this is not a possibility in the UK population for now, a follow-up study of clinical trials like AWARE, could be one of the strategies in the future.

The other limitation that we need to consider is use of samples from studies not initially designed to assess pertussis immune responses, with the risk of lacking metadata that in some cases can difficult the interpretation of the results. Although prospective studies allow a better control of the variables to study, the use of biobank samples sometimes is the only way to have samples of individuals with features/exposure not possible at the time of the study, like our adolescent wP primed population. For studies/samples that could enter biobank storage for future use, planning for more comprehensive storage of metadata to anticipate the broader questions that such samples may be used to address in future is an important aim.

One of the bigger impacts of with this work, from immune responses to assay optimisation, is to add knowledge that could improve the design of better vaccine schedules or novel vaccines. The focus should be in preventing colonisation and to achieve better antibody functionality and long-term memory. My work, on this matter shows the relevance of Prn antigen content in acellular pertussis vaccines: and also the relevance of the HLA-II specific variant associated with PT IgG antibody levels in wP-primed individuals, which given its association to protection from disease is an important area to investigate further to understand the better protection of this vaccine when compared with aP. In terms of vaccine policy, particularly the BERT study gave us insight and confirmation that the administration of a booster dose to older age

groups is immunogenic with reasonable persistence of immunity out to 12 months post-booster. It was previously shown that implementation of booster pertussis dose in adolescent population is cost-effective and associated with a decrease morbidity when implemented to an extended age group [279,280]. My DPhil data adds to the current knowledge and supports further evaluation in the UK of replacing the current Td-IPV dose given at 14 years of age [390] with a dTap-IPV, as implemented in other European countries.

It is relevant to highlight at the end of this discussion that my DPhil contributes to the broader scope of knowledge generated by the Periscope consortium. The overall aim, of finding a CoP for pertussis, will be best addressed by an integrated analysis using the perspectives from the variety of studies of immune response to vaccines, infection, and human challenge across the consortium. Although not explored in detail in the consortium human challenge study, this work can be a key component to help determine a CoP [191,192]. The human challenge studies could, by using a controlled intervention, where the time of infection and the pre-existing immune status are both known, allow deep insights into carriage, infection and nature of the immune response needed to protect against these. Some of these aspects have been already evaluated and published [192].

In a broader view of the work produced as part of the Periscope consortium, particularly the development of novel functional antibody assays and the rapid T cell assay presented in this thesis, although pathogen specific, could be used as first step for the development of similar assays applicable to other pathogens.

Finally, the work presented in this thesis showed the relevance of the use of new methods to better address vaccine reactogenicity in general, not pertussis only, fundamental for vaccine development. This method can also be applied in other

settings and directed to severe and acute disease as a tool to predicted disease progression and outcomes. The future work in this area, currently ongoing, is to better explore those difference between aP and wP.

APPENDIX

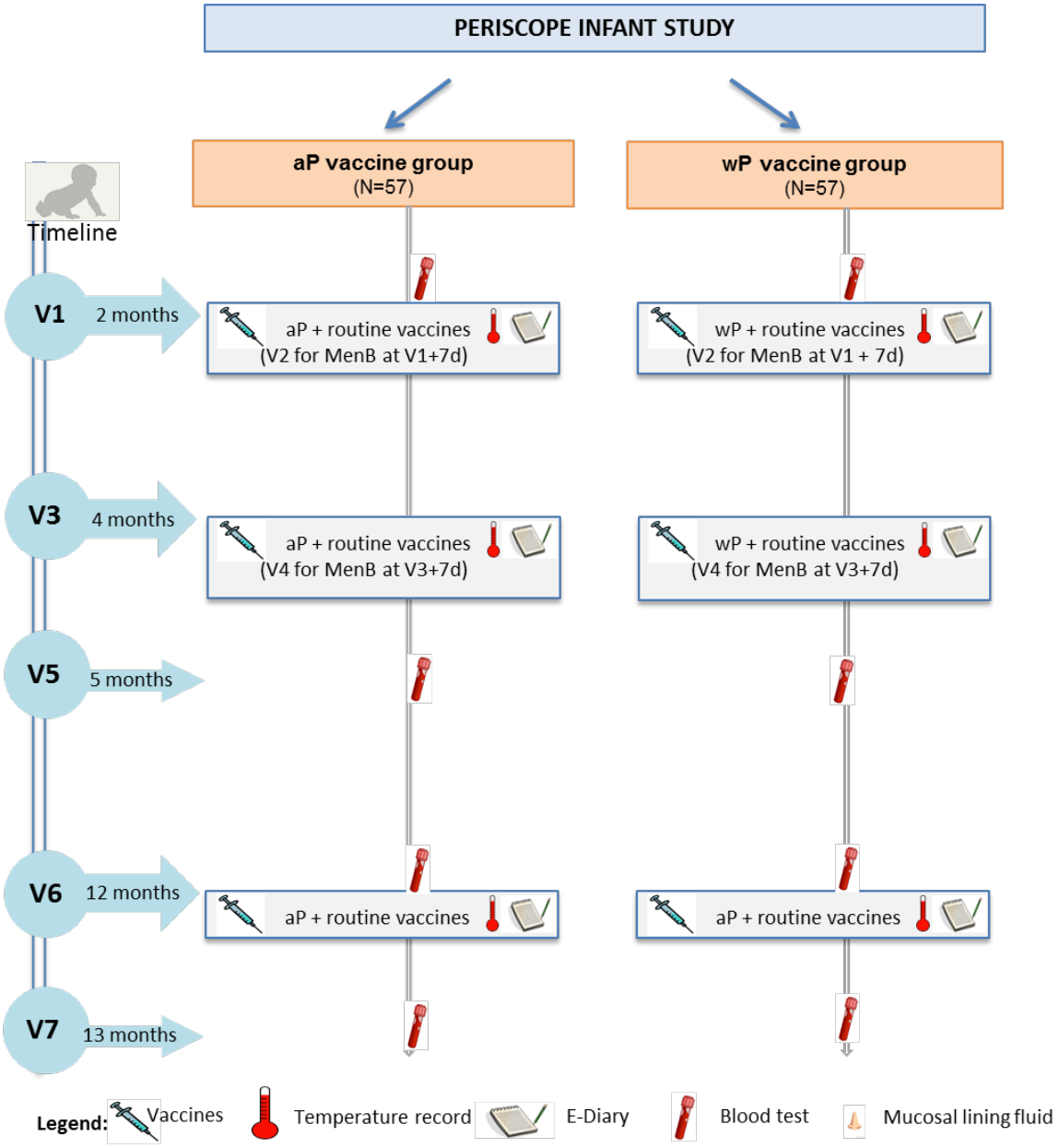
A. Supplementary data AWARE study

AWARE (**Pertussis A**cellular **W**hole cell **A**dvanced **RE**search study): a randomised, open label study, exploring the differences in immunogenicity and reactogenicity of infants after immunisation with either an acellular (aP) or whole cell pertussis (wP) vaccine.

The AWARE study is an open label, randomised controlled trial recruiting infants (aged 8 to 10 weeks), who have not yet received the first immunisations, born to mothers who have received an aP vaccine during pregnancy.

This study will directly compare the immune responses of infants given aP and wP, as part of a 2 dose-schedule of primary immunisation to better understand the immunological features that distinguish a vaccine that we have some evidence generates better long-term protection against pertussis infection and carriage (wP) than aP. A detailed investigation of the immune responses will be possible through the application of cutting-edge novel functional antibody and cellular assays using the technology platforms developed by the PERISCOPE consortium team of experts.

A total of 114 infants, randomised 1:1, are going to be recruited. The design of study is represented in Supplementary figure 1.



Supplementary figure 1 AWARE study design and interventions per vaccine group

In summary the objectives of this study are:

Primary objective: To investigate Pertussis Toxin (PT) specific antibody responses as a marker of memory, following an aP booster given at 12 months of age to infants primed with aP versus wP vaccines

Secondary objectives include:

- To assess the effect of baseline PT, FHA, Prn-specific IgG antibodies on the PT, FHA and PRN antibody responses in infants primed with aP versus wP;
- To compare pertussis antigen-specific MBCs frequencies at 5, 12, and 13 months of age in infants primed with aP versus wP;
- To compare pertussis antigen specific T cell responses at 5 months of age, in infants primed with aP versus wP;
- To compare PT-specific antibody responses in infants primed with aP versus wP;
- To compare other pertussis specific antibody responses including FHA, Prn and FIM specific antibodies in infants primed with aP versus wP;
- To compare serological responses to the non-pertussis vaccines (Hib, diphtheria, tetanus, pneumococcus) in infants primed with aP versus wP;
- Assessment of pertussis specific functional antibodies prior to and after immunisation with aP versus wP;
- To determine the induction and persistence of mucosal antibodies and/or cytokines in mucosal lining fluid before vaccination (2 months) and after vaccination (at 5 and 12 months);
- To assess vaccine reactogenicity in those primed with aP versus wP using continuous body temperature measurements in the first 24 hours after the administration of the study vaccines.

Study Intervention:

In this study the wP vaccine is the Comvac 5 ® (Bharat Biotec). This is a pentavalent vaccine containing DTwP-Hib-Hepatitis B (HepB) antigens. The aP study vaccine is Infanrix hexa® (GSK), a DTaP-IPV_Hib-Hep B.

Temperature monitoring in the AWARE study:

The iButton® device will be issued to the parent/legal guardian for use in the first 24 hours following the 2-, 4- and 12-months vaccinations (V1, V3 and V6). Parents will be given instructions on how to fit the temperature-monitoring device and how to check if it remains in place, and what to do with the device at the end of the monitoring period. Axillary temperatures will be collected by parents using an axillary thermometer at 4, 8, and 24 hours after vaccination and then once daily for 7 days post vaccination at the same timepoints. Temperature monitoring devices can be collected either by a member of a study team if the next visit occurs within 7 days and/or the device can be returned via post by the parents, using pre-paid envelopes provided by the study team.

Current recruitment:

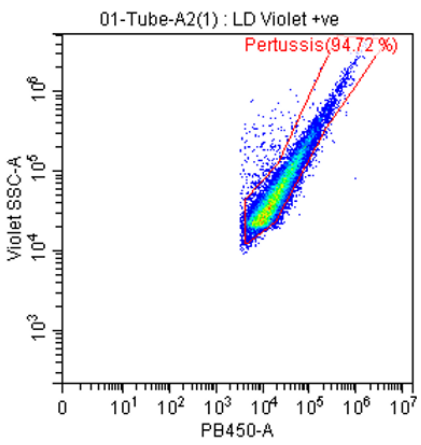
On the 15th of March 2021, a total of 15 participants had been already recruited and V1 performed. Out of those 15, nine participants also had the second visit (V2).

B. Supplementary data from Chapter 2

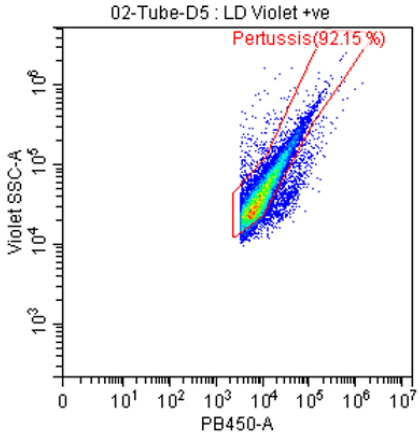
B.1 Complement deposition assay (CDA) sample contamination in Infants_African_wP samples

During the performance of the CDA in the Infants_African_wP samples it was detected a different signal in the flow-cytometry analysis, and it was assumed to be a contamination of the samples. The contamination was identified in 72 of the 124 samples from the Infants_African_wP.

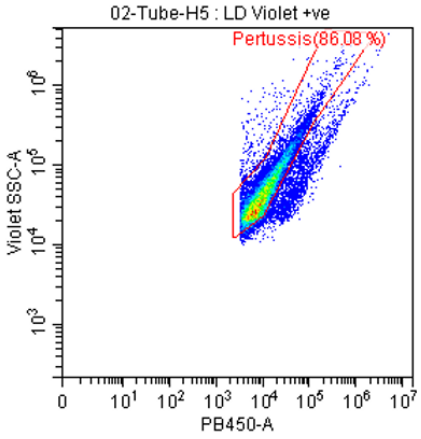
To confirm this finding, a Matrix-Assisted Laser Desorption/Ionization-Time of Flight (MALDI-ToF) was used. It was possible in one of the samples to identify a bacterium from the genus *Leifsonia*, species *aquatica*. This Gram-positive bacterium was described and reclassified by *Evtushenko et al* and although is an environmental microorganism normally present in water sources can be isolated and cause disease in special groups, like septicaemia in immunocompromised individuals [391-393]. For these particular samples collected from healthy infants, it is likely that were contaminated during processing or during storage/transporting from the Burkina Faso laboratory to facilities at the University of Oxford. In order to try to minimise this it was possible to exclude the effect of contamination through careful gating during flow-cytometry analysis (Supplementary figure 2).



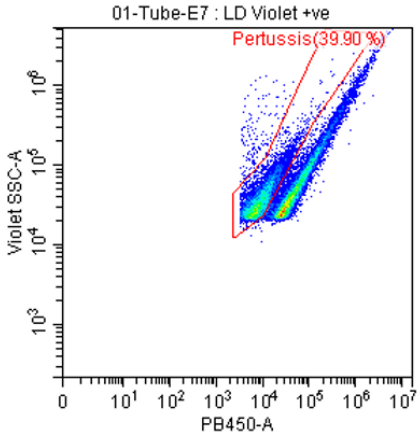
NIBSC 06/140 = 0



Suspected contamination = 1



Suspected contamination = 2



Suspected contamination = 3

Supplementary figure 2 Flow cytometry plots of a mixture of serum and *Bordetella pertussis*

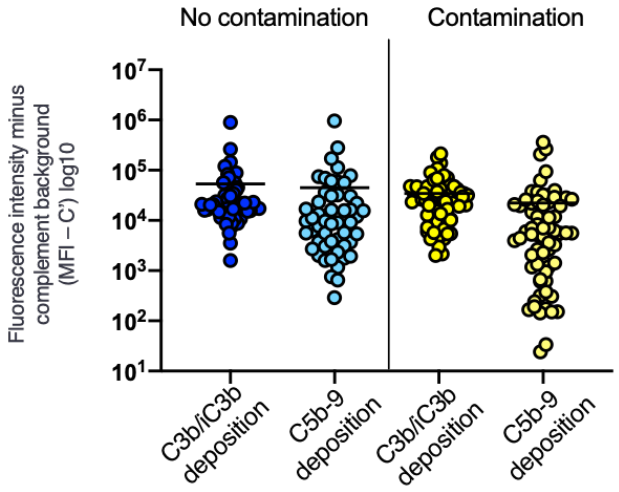
Gating showing a non-contaminated serum sample from NIBSC and samples with different levels of contamination based on the percentage of gated *Bordetella pertussis*

No statistically significant differences were found in pertussis specific antibody levels between contaminated and non-contaminated samples (Wilcoxon rank sum test; PT IgG *p=0.36*; FHA IgG *p=0.48*; Prn IgG *p=0.21*). Although no statistically

significant differences were found between the results regarding C3b-iC3, the MFI-C' C5b-C9 in the non-contaminated samples was higher, with statistically significant differences identified between different contaminated status (Supplementary figure 3).

The interference of contamination in the interpretation of final results is challenging, although we are confident that the gating was performed in order to exclude this effect. When evaluating the correlation (Spearman's correlation coefficient) between pertussis specific antibody levels with the CDA results in non-contaminated samples *versus* the overall samples independently of the status the most relevant difference in rho was found in the correlation between Prn IgG and C3b/iC3 (Supplementary table 1) with other rho behaving in the majority of the cases in a similar way. Therefore, the analysis of the overall samples is presented in this thesis (CHAPTER 5).

A



B

	Non contaminated samples	**Contaminated samples**	**p values** (Wilcoxon rank sum test)
N	52	72	
C3b/iC3 MFI – C' median (IQR)	20,907.1 (15,047.0-46,376.1)	26,265.3 (9911.5-41,978.3)	0.86
C5b-9 MFI – C' median (IQR)	9460.4 (3428.0-30,549.1)	5205.9 (641.2-20,570.9)	**0.014**

Supplementary figure 3 Complement deposition assay results in contaminated and non-contaminated samples from the Infants_African_wP study

A: Dot plot representative of the results of the CDA of the Infants_African_wP study by contamination status; **B:** Table with median (IQR) of MFI-C' of C3b/iC3b and C5b-9 per contamination sample status and comparison between samples using Wilcoxon rank sum test

Legend: IQR: Interquartile range; MFI-C': Mean Fluorescence intensity minus complement background

Supplementary table 1 Spearman's correlation between CDA C3b/iC3 and C5b-C9 and pertussis specific antibodies in the Infants_African_wP study in non-contaminated samples and in all tested samples

Correlations between antibodies and CDA	Non contaminated samples Spearman's correlation rho (p values)	All samples Spearman's correlation rho (p values)
C3b/iC3 and PT IgG	0.26 (0.06)	0.25 (0.006)
C3b/iC3 and FHA IgG	0.31 (0.03)	0.34 (0.0001)
C3b/iC3 and Prn IgG	0.19 (0.17)	0.41 (0.0002)
C5b-9 and PT IgG	0.20 (0.2)	0.16 (0.07)
C5b-9 and FHA IgG	0.17 (0.23)	0.19 (0.03)
C5b-9 and Prn IgG	0.43 (0.0014)	0.39 (<0.0001)

Legend: PT: pertussis toxin; FHA: Filamentous Haemagglutinin; Prn: Pertactin

B.2 Memory B cell ELISpot assay optimisation

SAMPLE 1				SAMPLE 2				SAMPLE 3			
1	2	3	4	5	6	7	8	9	10	11	12
PTX	PTX	PTX 1:2	PT 1:2	PTX	PTX	PTX 1:2	PT 1:2	PTX	PTX	PTX 1:2	PT 1:2
FHA	FHA	FHA 1:2	FHA 1:2	FHA	FHA	FHA 1:2	FHA 1:2	FHA	FHA	FHA 1:2	FHA 1:2
FHA 1:10	FHA 1:10	FIM	FIM	FHA 1:10	FHA 1:10	FIM	FIM	FHA 1:10	FHA 1:10	FIM	FIM
PRN	PRN	FIM 1:2	FIM 1:2	PRN	PRN	FIM 1:2	FIM 1:2	PRN	PRN	FIM 1:2	FIM 1:2
PRN(1:10)	PRN(1:10)	PRN 1:2	PRN 1:2	PRN(1:10)	PRN(1:10)	PRN 1:2	PRN 1:2	PRN(1:10)	PRN(1:10)	PRN 1:2	PRN 1:2
IgG (1:50)	IgG (1:50)	TTOX	TTOX	IgG (1:50)	IgG (1:50)	TTOX	TTOX	IgG (1:50)	IgG (1:50)	TTOX	TTOX
IgG (1:100)	IgG (1:100)	TTOX 1:10	TTOX 1:10	IgG (1:100)	IgG (1:100)	TTOX 1:10	TTOX 1:10	IgG (1:100)	IgG (1:100)	TTOX 1:10	TTOX 1:10
PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS

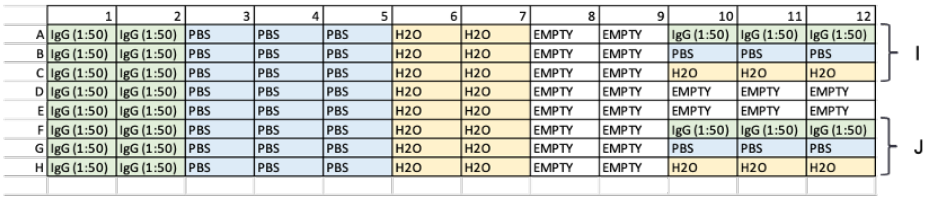
Supplementary figure 4 Memory B cell ELISpot final plate layout

Steps of assay optimisation in the BERT study

During assay development an inconsistency in the background levels between plates, particularly with high background, was detected. This finding was of concern per se, but also due to the low PT responses, with low number of spots, the high background could greatly diminish the final PT counts. To address this issue PBMC's were cultured under Periscope stimulation conditions using AIMV medium with OVG tested FBS (as previously described) or with AIVM medium with RIVM FCS (fetal calf serum, HyClone™, Logan, UT, USA). To try to understand what reagent or step could be related with the high background, several conditions were tested, and this process was performed in two different experiments. In the first experiment only the positive (IgG) and negative control backgrounds (PBS and water) were tested and in the second experiment responses of MBCs to pertussis specific antigens were evaluated (Supplementary figure 5).

For the first experiment and since at the OVG laboratory the PBMC culture media used HI FBS and at The Netherland site (RIVM) used HI FCS (HyClone™, Logan, UT, USA) the first step was to culture the cells from the same participant with the two different media. For each one of the different cultures, a total of ten conditions were tested.

A full description of the 10 conditions tested, including a change in the secondary antibody initially proposed by the Periscope consortium (Sera Care Life Sciences, Milford, CT, USA AP Goat anti-human IgG) to a secondary antibody previously used at OVG in another assay (Calbiochem®, Sigma-Aldrich, St Louis, MO, USA, AP Goat anti-human IgG) is described in Supplementary figure 5.



	1	2	3	4	5	6	7	8	9	10	11	12	
A	IgG (1:50)	IgG (1:50)	PBS	PBS	PBS	H2O	H2O	EMPTY	EMPTY	IgG (1:50)	IgG (1:50)	IgG (1:50)	
B	IgG (1:50)	IgG (1:50)	PBS	PBS	PBS	H2O	H2O	EMPTY	EMPTY	PBS	PBS	PBS	I
C	IgG (1:50)	IgG (1:50)	PBS	PBS	PBS	H2O	H2O	EMPTY	EMPTY	H2O	H2O	H2O	
D	IgG (1:50)	IgG (1:50)	PBS	PBS	PBS	H2O	H2O	EMPTY	EMPTY	EMPTY	EMPTY	EMPTY	
E	IgG (1:50)	IgG (1:50)	PBS	PBS	PBS	H2O	H2O	EMPTY	EMPTY	EMPTY	EMPTY	EMPTY	
F	IgG (1:50)	IgG (1:50)	PBS	PBS	PBS	H2O	H2O	EMPTY	EMPTY	IgG (1:50)	IgG (1:50)	IgG (1:50)	
G	IgG (1:50)	IgG (1:50)	PBS	PBS	PBS	H2O	H2O	EMPTY	EMPTY	PBS	PBS	PBS	J
H	IgG (1:50)	IgG (1:50)	PBS	PBS	PBS	H2O	H2O	EMPTY	EMPTY	H2O	H2O	H2O	

A: PCB + Periscope-Ox SC
B: PCB + Periscope-Ox SC + extra cell wash
C: AB + Periscope-Ox SC
D: AB + Periscope-Ox SC + extra cell wash
E: PCB + OVG CB
F: PCB + OVG CB + extra cell wash
G: PCB + Periscope-RIVM SC
H: PCB + Periscope-RIVM SC + extra cell wash
I: AB + OVG CB + extra cell wash
J: AB + Periscope-RIVM SC + extra cell wash

Description conditions to test

1. Periscope conjugate buffer (PCB):
- PBS
- Goat serum
- Tween
- Secondary antibody

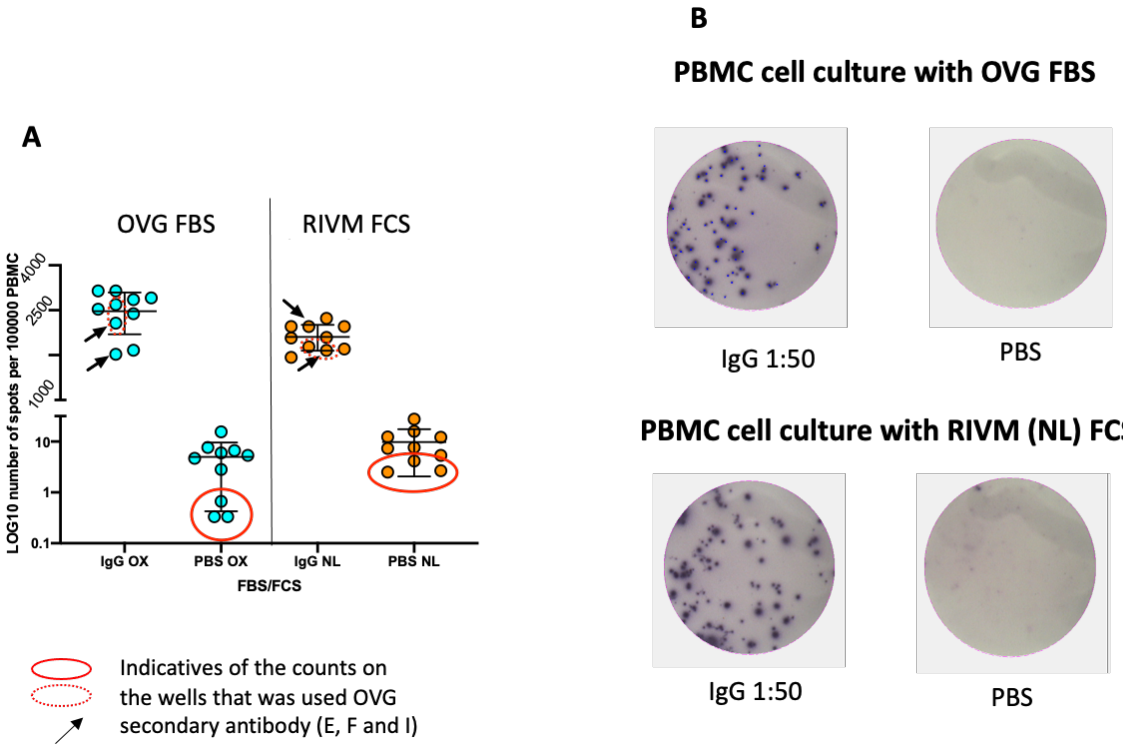
2. Alternative buffer (AB):
- AIMV+FBS
- Secondary antibody

Secondary antibodies for conjugated buffer (AP goat anti-human IgG):
- Periscope secondary antibody purchase by OVG (SeraCare) – **Periscope-Ox SC**
- Periscope secondary antibody sent by RIVM (SeraCare) – **Periscope- RIVM SC**
- OVG secondary antibody (Calbiochem®) – **OVG CB**

Supplementary figure 5 Description of the assay conditions used in experiment one for optimisation of MBCs ELISpot in the BERT study

The HI FBS from OVG used for the cell culture and media showed a similar performance when compared with RIVM FCS, under the same conditions (Supplementary figure 6). After completing the first experiment it was determined that the best condition to achieve the lowest background was to use the Periscope conjugate buffer preparation, but using the OVG secondary antibody from Calbiotech®, adding an extra washing step after the PBMC were harvested from the PBMC cell culture (Supplementary figure 7).

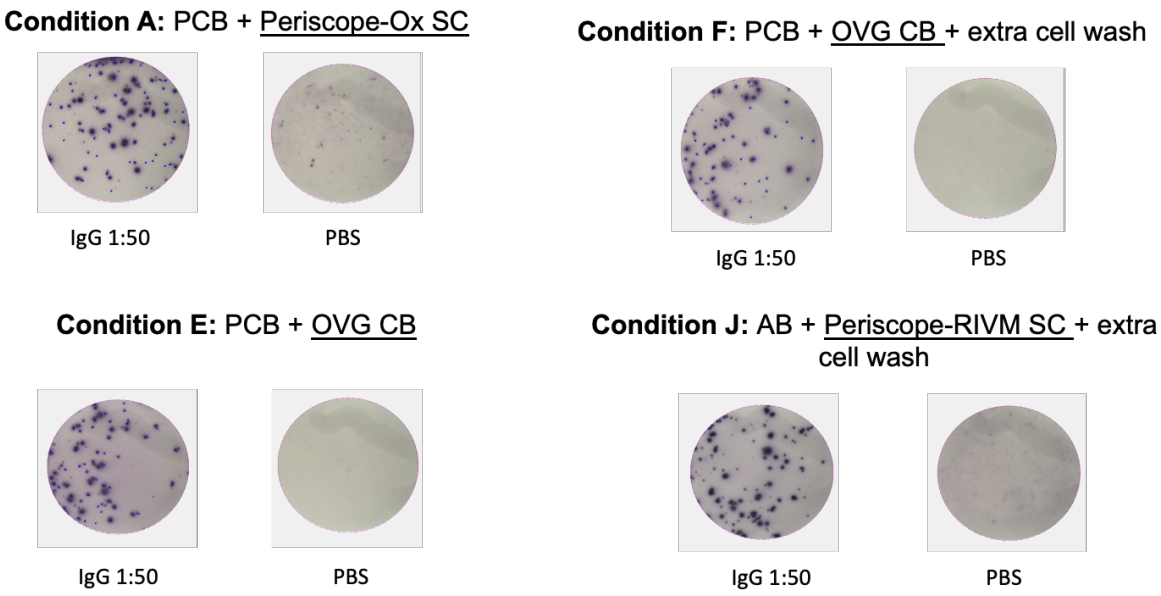
In conclusion, it was required to change the secondary antibody for the OVG Calbiochem® and add a second washing step after harvesting the PBMCs from culture, to decrease significantly background to very low levels, with no interference with the antigen specific MBCs spot count.



Supplementary figure 6 Memory B cell (MBCs) ELISpot experiment one, comparing the number of spots per 100,000 PBMC using OVG fetal bovine serum (FBS) or RIVM (NL) fetal calf serum (FCS)

A: Dot plot of the spots per 100,000 PBMC for the positive (IgG dilution 1:50) and negative control (PBS) in PBMC cultured with two different culture media (OVG FBS and RIVM FCS);

B: Images of the wells after the plates were read by ELISpot reader (Advance Imaging Devices GmbH, Straßberg, Germany) using the two different culture media showing positive control (IgG 1:50) and the negative control (PBS)



Supplementary figure 7 Images of the positive control (IgG diluted 1:50) and negative control (PBS) wells read by ELISpot reader at different conditions (A, E, F and J)

Legend: AB: alternative buffer; OVG CB: conjugated buffer with OVG anti-goat IgG from Calbiotech®: PCB: Periscope conjugated buffer; PBS: Phosphate buffer saline; Periscope-Ox SC: Conjugated buffer with periscope anti-goat IgG from SeraCare purchase by OVG; Periscope-RIVM SC: Conjugated buffer with periscope anti-goat IgG from SeraCare purchase by RIVM.

A second experiment was done to test the performance of the determined conditions in a plate coated with pertussis specific antigens. Due to the high number of samples the volume of the secondary antibody available was not enough to perform all assays, so extra vials from the same company, but from a different batch of the original vial, were ordered. Supplementary figure 8 shows the plate layout. The conditions were tested again using two different cell cultures, with OVG FBS and RIVM FCS, since our collaborators

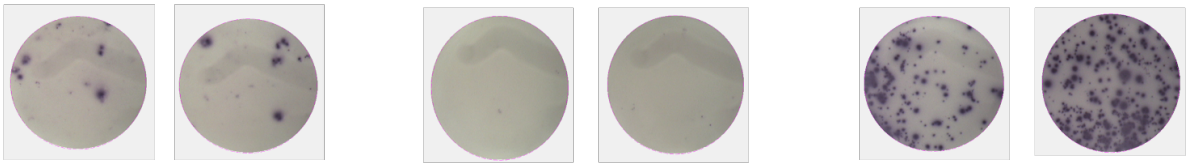
at RIVM were keen to continue to use the FCS already purchased. The results showed a good performance of the specific antigens and positive control tested, maintaining a low background.

A

Old batch A1 | New batch – B1 | New batch – C1

	SAMPLE 1				SAMPLE 2				SAMPLE 3			
	1	2	3	4	5	6	7	8	9	10	11	12
A	PTX	PTX	PTX 1:2	PT 1:2	PTX	PTX	PTX 1:2	PT 1:2	PTX	PTX	PTX 1:2	PT 1:2
B	FHA	FHA	FHA 1:2	FHA 1:2	FHA	FHA	FHA 1:2	FHA 1:2	FHA	FHA	FHA 1:2	FHA 1:2
C	FHA 1:10	FHA 1:10	FIM	FIM	FHA 1:10	FHA 1:10	FIM	FIM			FIM	FIM
D	PRN	PRN	FIM 1:2	FIM 1:2	PRN	PRN	FIM 1:2	FIM 1:2	PRN	PRN	FIM 1:2	FIM 1:2
E	PRN(1:10)	PRN(1:10)	PRN 1:2	PRN 1:2	PRN(1:10)	PRN(1:10)	PRN 1:2	PRN 1:2	PRN(1:10)	PRN(1:10)	PRN 1:2	PRN 1:2
F	IgG (1:50)	IgG (1:50)	TTOX	TTOX	IgG (1:10)	IgG (1:10)	TTOX	TTOX	IgG (1:10)	IgG (1:10)	TTOX	TTOX
G	IgG (1:100)	IgG (1:100)	TTOX 1:10	TTOX 1:10	IgG (1:50)	IgG (1:50)	TTOX 1:10	TTOX 1:10	IgG (1:50)	IgG (1:50)	TTOX 1:10	TTOX 1:10
H	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS

B



PTX IgG old batch | PTX IgG new batch | PBS old batch | PBS new batch | IgG 1:50 old batch | IgG 1:50 new batch

Supplementary figure 8 Experiment 2: summary of the plate layout and results when testing the new ELISpot conditions

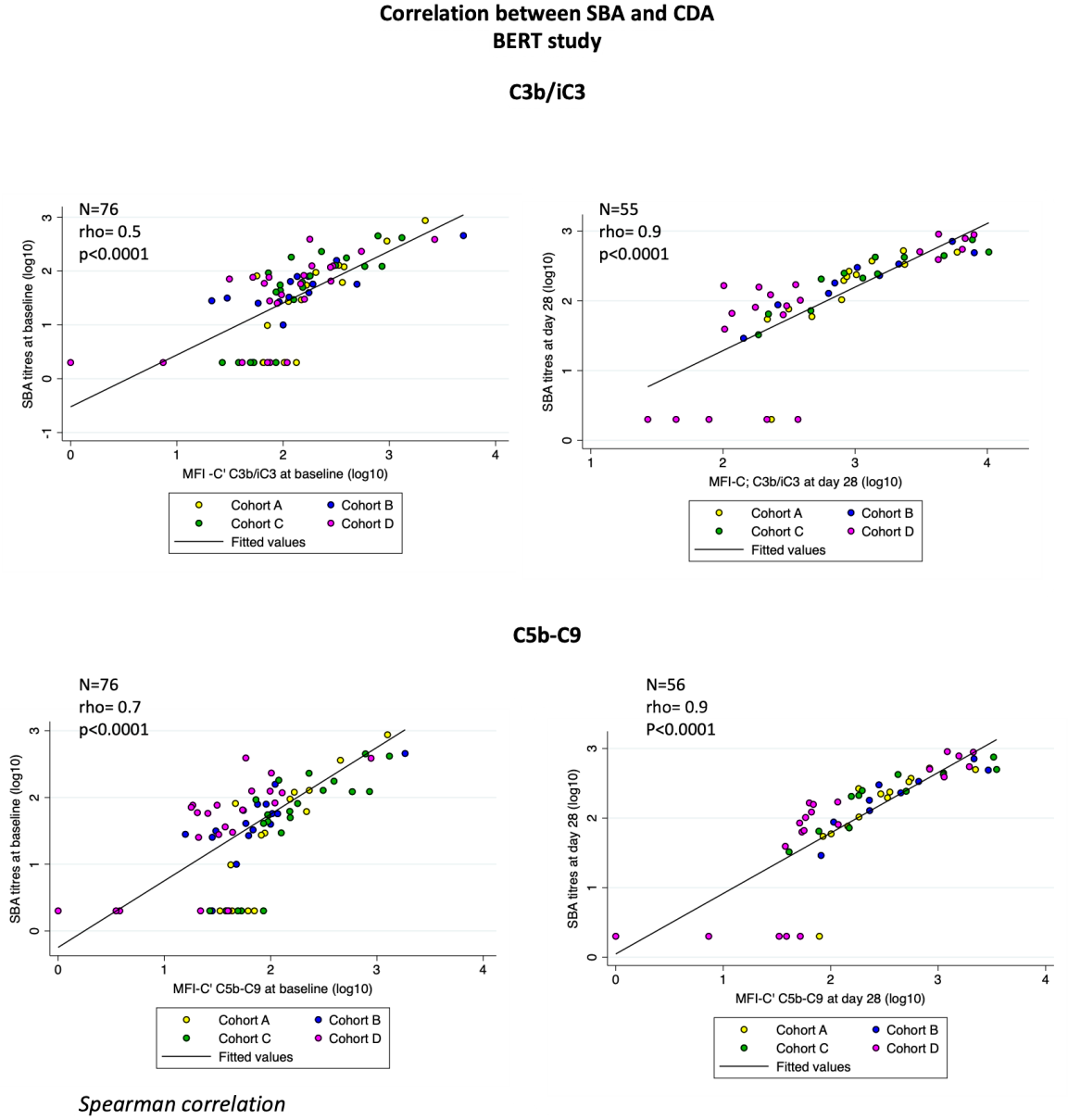
A: Plate layout used to test the old and new batch of secondary antibody (anti-goat IgG Calbiotech®) in the conjugated buffer using OVG FBS in the cell culture; **B:** Picture of the spots on the wells coated with PTX, PBS and IgG after development of the ELISpot plate comparing the results when using the old and new batch of secondary antibody

Legend: PTX: pertussis toxin, FHA: Filamentous Haemagglutinin, PRN: Pertactin; FIM: Fimbriae; IgG: immunoglobulin IgG, TTOX: Tetanus toxin, PBS: Phosphate buffer saline. Cell dilutions: 1:2; 1:10; 1:50 and 1:100.

C. Supplementary data from Chapter 3

Supplementary table 2 Spearman correlation (*p values*) between SBA titres and IgA pertussis specific antibody levels 28 days after booster per study cohort

	PT IgA/SBA D28	FHA IgA/SBA D28	Prn IgA/SBA D28	FIM 2/3 IgA/SBA D28
All individuals N=56 rho (p)	-0.06 (0.7)	-0.02 (0.9)	0.2 (0.1)	0.01 (0.9)
Cohort A N=13 rho (p)	-0.2 (0.5)	0.07 (0.8)	0.4 (0.2)	0.2 (0.5)
Cohort B N=9 rho (p)	-0.03 (0.9)	0.4 (0.3)	0.4 (0.4)	0.2 (0.6)
Cohort C N=9 rho (p)	0.03 (0.9)	-0.01 (1.0)	0.2 (0.6)	0.2 (0.5)
Cohort D N=21 rho (p)	0.2 (0.4)	0.04 (0.8)	0.4 (0.07)	-0.3 (0.2)



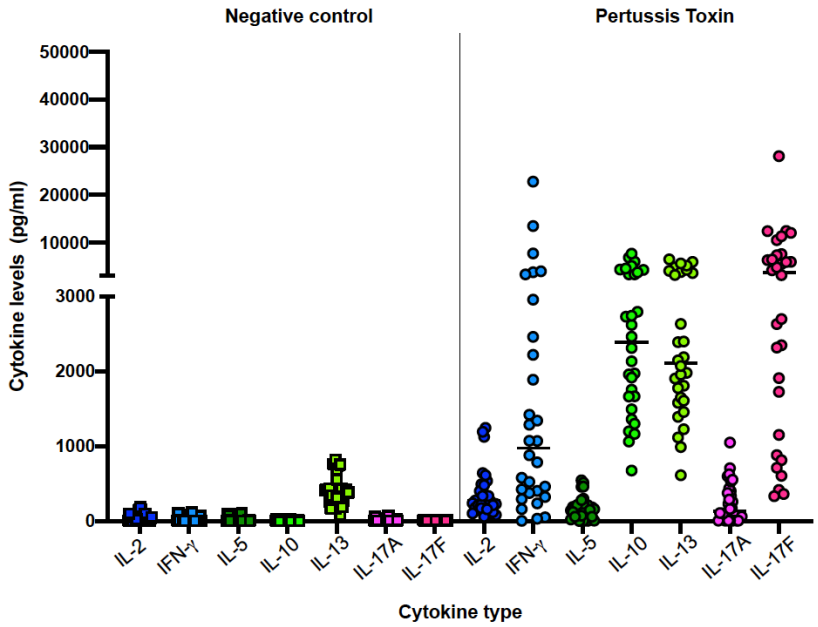
Spearman correlation

Supplementary figure 9 Spearman correlation between SBA titres and CDA results at baseline in the BERT population

D. Supplementary data from Chapter 4

D.1 Whole blood rapid T cell assay – optimisation steps

The steps to determine the final conditions of the whole blood T cell assay are presented below. When performing this assay, a negative control was used for all cultures. The negative control had relatively low cytokine levels (Supplementary figure 10).



Supplementary figure 10 Cytokine levels in the negative control wells and in the pertussis toxin (PT) stimulated cultures in paediatric and adults cohorts

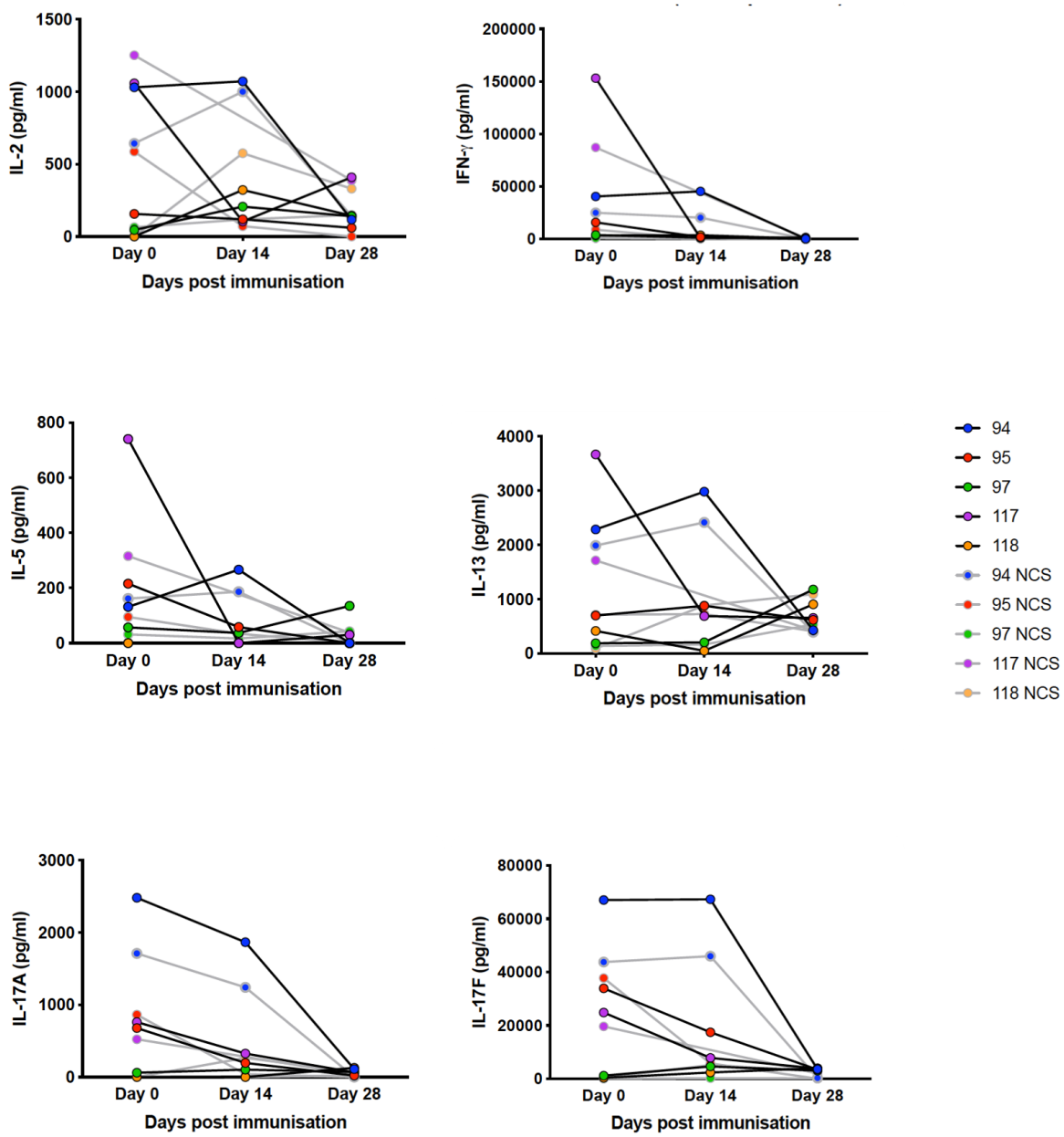
All results showed the cytokine frequencies after stimulation with a specific pertussis antigen (PT and FHA) minus the cytokine levels of the negative control in the same assay.

In all samples, co-stimulants CD28/CD49d were used in the whole blood culture. The option of not using co-stimulants was explored in a sub-set of samples.

In general, the assay using co-stimulants had a better performance, with higher cytokine levels, than the cultures without co-stimulants, for both paediatric and adult cohorts. Supplementary figure 11 shows illustrative graphs of these differences.

No statistically significant differences were found between the cytokine levels against FHA in the adult and paediatric groups measured at 48 and 72 hours when CD28/CD49d co-stimulants were added.

Less consistency was found with the cytokine levels produced as response to PT + co-stimulants with different incubation periods. The cytokines levels produced as part of the Th17 response (IL-17A and IL-17F) were statistically significantly different in both paediatric and adult cohorts (Wilcoxon sign rank test Paediatric cohorts IL-17F D0: *p=0.002* and D28: *p=0.008*; IL-17A D0: *p=0.008* and D28: *p=0.016* /Adult cohorts IL-17F D0: *p=0.016* and D28: *p=0.0016*; IL-17A D0: *p=0.05* and D28: *p=0.015*). No statistically significant differences were found between incubation periods for IL-5 and IL-13 (Th2). The IL-2 and IFN-γ showed in the majority of the cases significant differences at D0 (Wilcoxon sign rank test Paediatric cohorts IL-2 *p=0.04*; IFN-γ: *p=0.02* /Adult cohorts IL-2 *p=0.03*; IFN-γ: *p=0.79*), but not day 28 after booster immunisation (Wilcoxon sign rank test Paediatric cohorts IL-2 *p=0.28*; IFN-γ: *p=0.15* /Adult cohorts IL-2 *p=0.64*; IFN-γ: *p=0.01*).

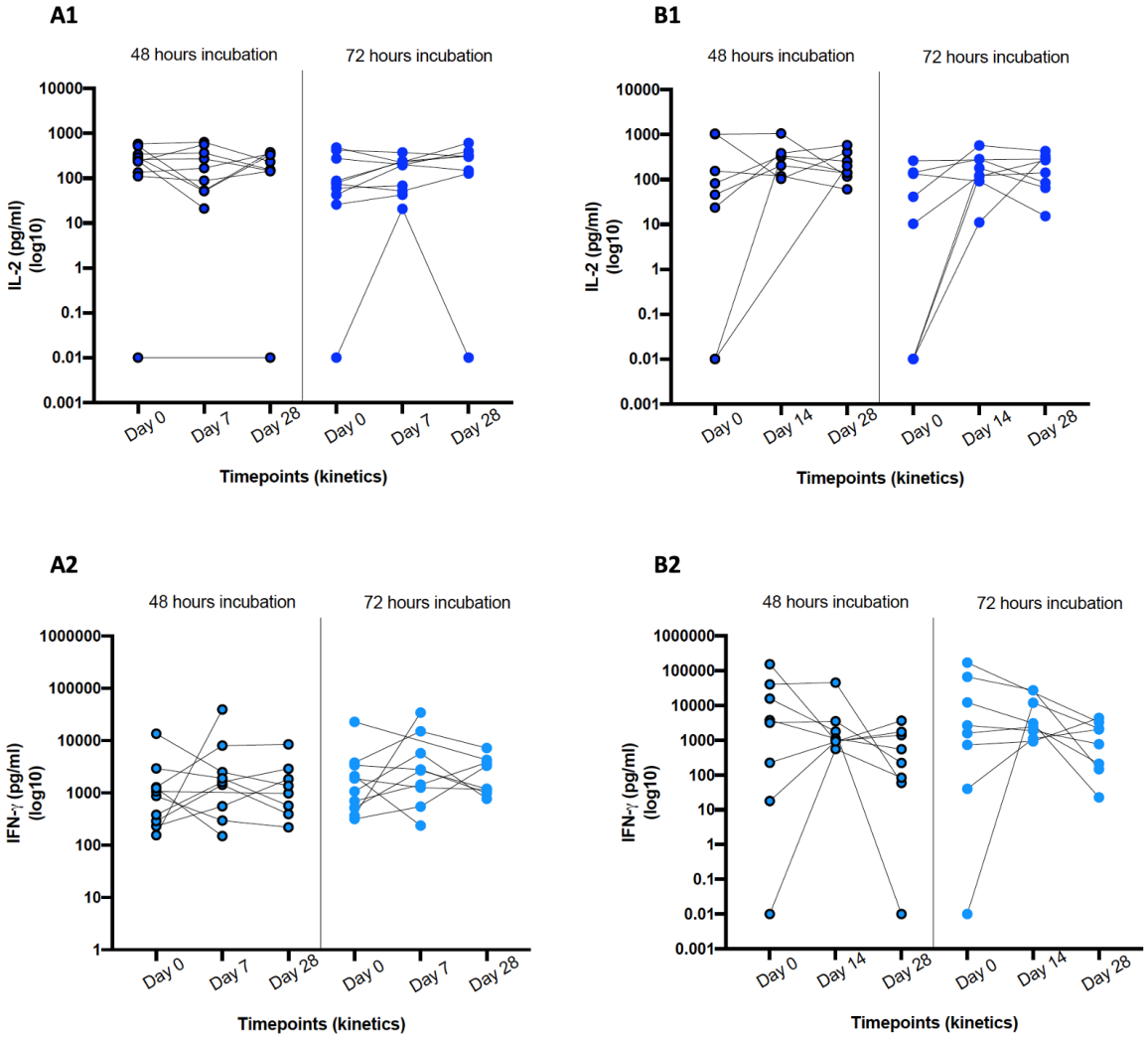


Supplementary figure 11 Cytokine levels in the adult cohorts: pertussis toxin (PT) stimulated cultures in whole blood samples

Legend: CD28/CD49d co-stimulants (black line) and without co-stimulants (NCS) (light grey line)

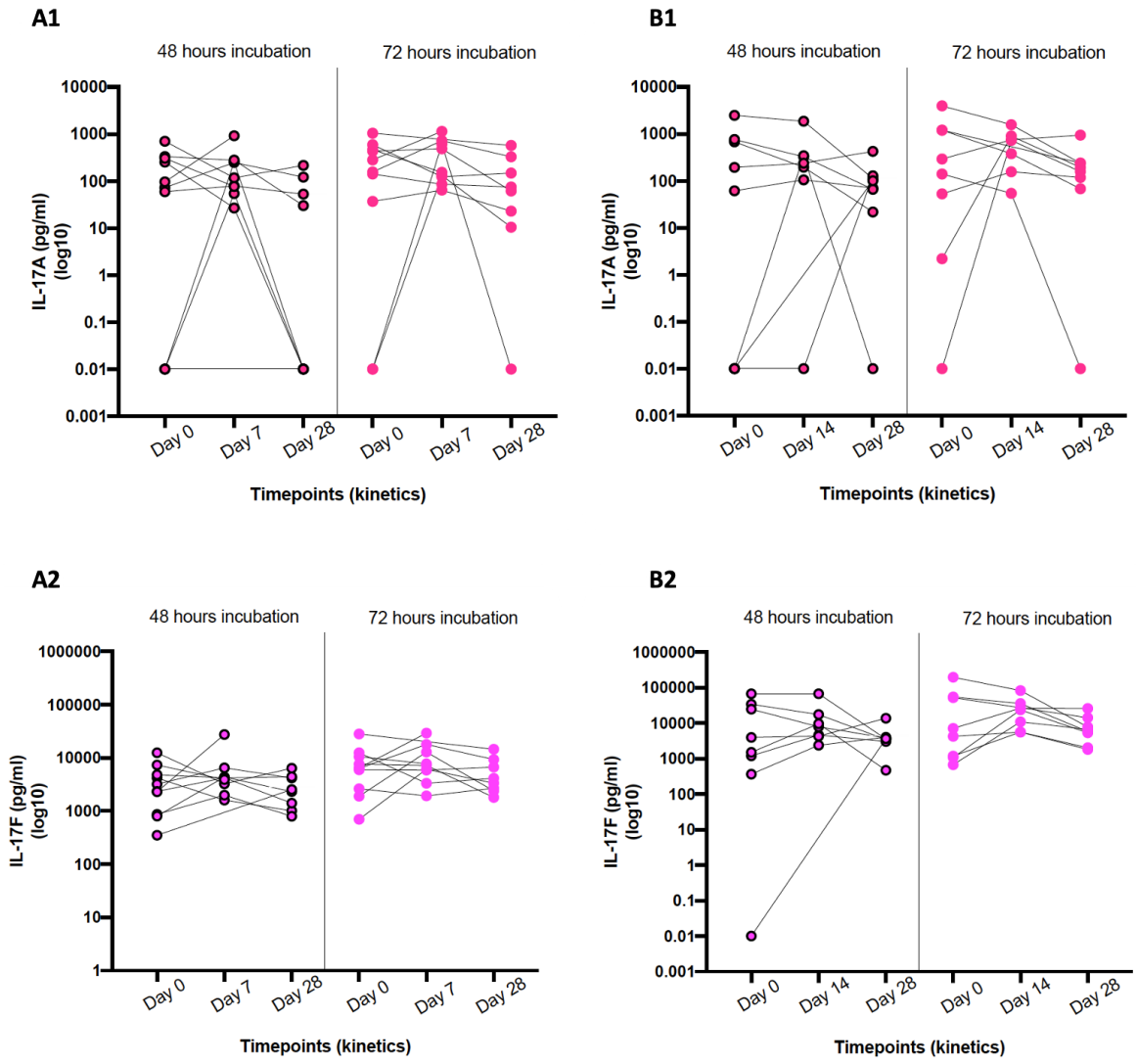
Regarding Th17 responses, IL-17F and IL-17A showed higher levels after 72 hours of incubation. Although IFN-γ showed higher levels after 72 hours of incubation, IL-2 had a better performance at 48 hours incubation period (Supplementary figure 12 and Supplementary figure 13).

Although differences were found in the cytokine levels between incubation periods, the patterns were similar.



Supplementary figure 12 Cytokine patterns of Th1 responses to pertussis toxin (PT) stimulation in paediatric cohorts (A1 and A2) and adult cohorts (B1 and B2)

Legend: A1/B1:IL-2; A2/B2: IFN-γ



Supplementary figure 13 Cytokine patterns of Th17 responses to pertussis toxin (PT) stimulation in paediatric cohorts (A1 and A2) and adult cohorts (B1 and B2)

Legend: A1/B1:IL-17A; **A2/B2**: IL-17F

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