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Immunity to pertussis vaccination

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by

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Abstract

Thailand has implemented universal whole cell pertussis vaccination in children for more than 35 years; nevertheless, the numbers of reported pertussis cases are still on the rise, especially among infants below one year who bear the greatest risk of severe complications. Several countries have implemented a pertussis booster during adolescence. However, data on the appropriate age groups targeted for a booster in the Thai population is limited. The increase in pertussis incidence among Thai infants warrants the need to further investigate the pre-existing maternal antibody against *Bordetella pertussis* (*B. pertussis*) antigens and the potential benefit of Tetanus toxoid-reduced dose of diphtheria and acellular pertussis (Tdap) vaccine administered during pregnancy.

This study aims to investigate IgG antibody to pertussis toxin (anti-PT) among healthy people across all ages after 37 years of universal whole cell pertussis vaccination in Thailand and provide evidence-based recommendation for the age groups that should be targeted for a booster dose. This study also examined the baseline anti-PT, IgG antibody to filamentous hemagglutinin (anti-FHA) and IgG antibody to pertactin (anti-PRN) in Thai pregnant women who did not receive pertussis vaccine during pregnancy. The results showed that the antibody levels to all three *B. pertussis* antigens studied were low, which could result in susceptibility of Thai infants to pertussis. Then, this study evaluated and compared the antibody titers and T cell-mediated immune response induced by currently available acellular (aP) and whole cell (wP)-containing vaccine in infants born to mothers who received Tdap during pregnancy. The results revealed that in the presence of circulating *B. pertussis*-specific maternal antibodies induced by Tdap vaccination, infants who received aP had significantly higher levels of antibodies against all three *B. pertussis* antigens following primary immunization compared to infants who had received wP. However, at one month post booster dose, anti-PT level was similar but anti-FHA and anti-PRN were still higher in the aP group.

This study demonstrated the interference of maternal antibodies with the infant immune responses to wP, as shown by significantly higher antibody levels in the EPI wP group (no maternal immunization) compared to the wP group (with maternal immunization) for all three antigens studied following primary immunization. However, at one month post booster dose, we found that the blunting effect persisted for anti-PT and anti-FHA but not for anti-PRN. The clinical significance of these blunting effects is still unknown. However, monitoring the immunity of this cohort of children should be done in order to understand the long term impact of these immunological findings.

Regarding the T cell-mediated immune response, aP-vaccinated infants had higher Th1 cytokine responses at pre and post pertussis booster than wP-vaccinated children. For Th17 response following primary vaccination, the wP-vaccinated infants appeared to prime a better Th17 response than aP-vaccinated infants. Unlike the rapid waning of humoral immune response found at pre-booster, T cell-mediated immune response to pertussis toxin was still detectable and similar among all groups.

Maternal Tdap immunization can potentially reduce the antibody responses in infants vaccinated with the whole cell pertussis vaccine. Long-term monitoring of pertussis vaccine-induced immunity coupled with improved disease surveillance is warranted especially in countries using whole cell pertussis vaccines in their infant immunization program. However, cellular immune responses after the primary immunization and first booster did not appear to be significantly affected by maternal Tdap vaccination.

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Declaration

No part of the work in this thesis has been submitted for other degrees of other universities apart from the joint degree in Doctor in Philosophy between Chulalongkorn University, Bangkok, Thailand and the University of Liverpool, United Kingdom. The author declared no conflict of interest in any project in the thesis.

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Abbreviations

ACT	Adenelyate Cyclase Toxin
AE	Adverse Events
AIC	Akaike Information Criteria
aP	Acellular pertussis vaccine
B. pertussis	*Bordetella pertussis*
BCG	Bacillus Calmette–Guérin
bOPV	Bivalent OPV
BrkA	*Bordetella* resistance to killing protein
^{o}C	Degrees Celsius
CFSE	Carboxyfluorescein diacetate succinimidyl ester
CI	Confidence Interval
cm	Centimetre (s)
CMIR	Cell-mediated Immune Response
CO_2	Carbon Dioxide
cRPMI	Complete RPMI-1640 culture medium
DMSO	Dimethyl Sulfoxide
DAP12	DNAX activation protein of 12kDa
DNT	Dermonecrotic Toxin
dT	Reduced dose of diphtheria-Tetanus toxoid
DT	Diphtheria Toxoid
DTaP	Diphtheria–Tetanus toxoid–acellular pertussis
DTwP	Diphtheria–Tetanus toxoid–whole-cell pertussis
ELISA	Enzyme-linked Immunosorbent Assay
EPI	Expanded Program on Immunization
FBS	Fetal Bovine Serum
FcRγ	Fc gamma receptor
FHA	Filamentous Hemagglutinin
FIM	Fimbriae
FSC	Forward scatter
GA	Gestational age
gm	Gram (s)
GMC	Geometric Mean Concentration
HB	Hepatitis B
HBsAg	Hepatitis B surface antigen
HELLP	Hemolysis, elevated liver enzyme levels, and low platelet levels
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
Hib	*Haemophilus influenzae* type b
HIV	Human Immunodeficiency Virus
IFN-γ	Gamma Interferon
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IL	Interleukin

IPV	Inactivated Polio Vaccine
IRB	Institutional Review Board
IU	International Units
IVIG	Intravenous Immunoglobulin
JE	Japanese Encephalitis
KCMH	King Chulalongkorn Memorial Hospital
kg	Kilogram (s)
Lf	Limit of flocculation unit
LLOQ	Lower Limit of Quantification
LRI/IAP	Leukocyte-response integrin/integrin-associated protein complex
ml	Millilitre (s)
MMR	Measles-Mumps and Rubella
MyD88	Myeloid differentiation primary response protein
N/D	No data
ng	Nanogram (s)
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PMA	Phorbol 12-myristate 13-acetate
PPROM	Preterm Premature Rupture of Membranes
PRN	Pertactin
PT	Pertussis Toxin
RGD	Arginine-glycine-aspartic tripeptide motif
SAE	Serious adverse events
SD	Standard deviation
SE	Standard Error
SS	Supplement solution
SSC	Side scatter
TCF	Tracheal Colonization Factor
TCT	Tracheal Cytotoxin
Tdap	Tetanus toxoid-reduced dose of diphtheria toxoid and acellular pertussis
Th	T helper
TT	Tetanus Toxoid
vs	*versus*
wP	Whole cell pertussis vaccine
µg	Microgram (s)

Chapter 1

General Introduction and Literature Review

1.1 General background

Pertussis, or more commonly known as whooping cough, is a respiratory disease caused by a pathogenic bacterium, *Bordetella pertussis*. Although pertussis is vaccine-preventable, it remains prevalent in both developed and developing countries despite the implementation of vaccination efforts worldwide for several decades [1-4]. Mortality associated with pertussis often occurred in infants who have not completed primary series of pertussis vaccination [5]. Adult pertussis is increasingly detected despite complete childhood vaccination, suggesting that current pertussis vaccines cannot provide life-long immunity. Although adult pertussis is not fatal, the disease can be transmitted to infants in the household and cause severe diseases in affected infants.

Resurgence of pertussis has been a focus of international concern in recent years. Several factors have been attributed to this resurgence including low childhood vaccination coverage in some countries, waning of vaccine-induced immunity, increased awareness of this disease among physicians and/or increased virulence of circulating *B. pertussis* strains [6, 7]. A number of studies have shown that vaccine-induced immunity during childhood waned over time [8-10]. Since 2006, the Advisory Committee on Immunization Practices (ACIP) has recommended a booster dose of acellular pertussis (aP)-containing vaccine during adolescence in order to prevent adult pertussis [11]. This recommendation also covers postpartum mothers and other family members in order to prevent infant cases but this strategy has achieved only moderate coverage. In 2008, the ACIP recommended that women of child-bearing age and postpartum mothers should receive a booster dose of aP-containing vaccine in order to protect themselves and transmission of pertussis to their children [12]. During that time, experts suggested that vaccination during pregnancy was possible but

physician should discuss the risks and benefits as the data on safety and immunogenicity profile of pertussis vaccine during pregnancy was still limited.

Several reports have currently demonstrated that pertussis vaccination during pregnancy is safe and can elicit high maternal *B. pertussis*-specific antibodies [13, 14]. These antibodies can be transplacentally transferred to fetus and persisted in the infant's circulation during the first months of life [15, 16]. In June 2011, the ACIP made recommendations for use of pertussis vaccine in pregnant women and this strategy has been recommended in an increasing number of countries worldwide [17, 18]. However, follow-up studies suggest that high titers of vaccine-induced maternal *B. pertussis*-specific antibodies may interfere with humoral immune response in aP-vaccinated infants, but data regarding interference in whole cell pertussis (wP)-vaccinated infants is still limited. Since wP vaccine is part of the Expanded Program on Immunization (EPI) in many countries, a possible interference of vaccine-induced maternal antibodies to wP-vaccinated infants should be considered.

1.2 Microbiology of *Bordetella pertussis*

*Bordetella pertus*sis is a small Gram-negative aerobic coccobacillus that grows optimally on Bordet-Gengou or Regan-Lowe agar at 35-37 °C. There are other *Bordetella* species apart from *B. pertussis* as demonstrated in Table 1.2.1 [19]. Pertussis is mainly caused by *B. pertussis*, a strict human pathogen with no known animal reservoir. However, infection by other *Bordetella* species can produce clinically milder "pertussis-like illnesses". Pertussis occurs as a result of several virulence factors including pertussis toxin (PT), filamentous hemagglutinin (FHA), fimbriae (FIM), pertactin (PRN), adenylate cyclase toxin (ACT), tracheal cytotoxin (TCT) and dermonecrotic toxin (DNT).

Table 1.2.1: *Bordetella* species and their associated hosts.

Bordetella species	Associated host organism(s)
B. pertussis	Only human
B. parapertussis	Human, sheep, goat, pig
Bovine-associated *B. parapertussis*	Cattle
B. bronchiseptica	Human, pig, cat, dog, rabbit
B. avium	Human, bird
B. hinzii	Human, bird
B. holmesii	Human
B. trematum	Human
B. petrii	Human
B. ansorpii	Human

1.3 Pathogenesis of pertussis

The pathogenesis of pertussis involves several virulence factors in which their productions are affected by the environment [20]. The system that controls these changes is the *bvgA/S* operon. Infection begins with adhesion of the bacteria to the human respiratory epithelium using surface adhesins including FHA, PRN, FIM and TCF. At 37 ^{0}C, the *bvgA/S* genes are activated resulting in transcription and translation of *B. pertussis* virulence genes. The bacteria multiply locally and cause local damage to the respiratory tract. The infection can cause systemic manifestations such as lymphocytosis, hypoglycemia and encephalopathy [21, 22]. Recent studies have found that, in infants, the bacteria can descend from upper to lower respiratory tract and produce necrotizing bronchitis, diffuse alveolar damage, intra-alveolar hemorrhage, fibrinous edema, macrophage-rich alveolar infiltrates, lymphangiectasia, neutrophilic bronchopneumonia, and fibrin thrombi [23, 24]. This pathology can lead to pulmonary hypertension, respiratory failure, and even death.

1.4 Clinical manifestations

The typical symptoms of pertussis can be divided into three phases. The first is often called the catarrhal phase when patients have sneezing, runny nose, sore throat, lacrimation and mild progressive dry cough. Fever might not be present. This phase can go on for one week or up to two weeks and then the severe coughing starts. Whooping cough gets its name from this stage, known as paroxysmal stage, when patients develop severe, persistent, and progressive coughing that continues for months. A "whoop" is the sound caused by forced inhalation after rapid and violent coughing. Some patients may experience vomiting (post-tussive vomiting), cyanosis, eye proptosis, tongue protrusion, salivation, fatigue and respiratory exhaustion. This stage lasts for 2-3 weeks then it turns to convalescent phase when the severity of coughing gradually declines.

Nevertheless, the range of symptoms that occur after *B. pertussis* infection can vary from asymptomatic to fatal disease. In adults and adolescents, symptoms are usually milder compared to children. Young infants bear the greatest risk of severe complications and death. It was estimated that more than half of infant pertussis required hospitalization and in 1% of the affected infants die due to severe complications [(19)].

1.5 Whole cell pertussis vaccine

The wP vaccine consists of killed *B. pertussis* and was integrated as part of routine infant vaccination in the United States in 1948 [(25)]. Many countries have implemented this vaccine into their EPI. Although the vaccine was proven to be effective because the morbidity and mortality from pertussis disease significantly decreased after its implementation in many countries, it was associated with several adverse local and systemic reactions, such as pain, redness and swelling at injection sites, high-grade fever, persistent crying, hypotonic–hyporesponsive episodes, and seizures [(26, 27)]. Whole cell pertussis vaccine

is usually administered in combination with Diphtheria toxoid, tetanus toxoid and sometimes Hepatitis B surface antigens and *Haemophilus influenzae* type b (Hib) polysaccharide. This vaccine was adsorbed onto an aluminum salt to enhance the immunogenicity. Some of the commercially available whole cell pertussis vaccines are shown in Table 1.5.1.

Table 1.5.1: Some of the commercially available wP-containing vaccines and their compositions.

Trade name	Composition	Amount of *B. pertussis*	Manufacturers
Quadrovax	DTwP+Hib	≥ 4 IU	Serum Institute of India
Quinvaxem	DTwP+Hib+HB	≥ 4 IU	Novartis

DTwP, Diphtheria–Tetanus toxoid–whole-cell pertussis; Hib, *Haemophilus influenzae* type b; HB, hepatitis B

1.6 Acellular pertussis vaccine

Due to the reactogenicity of the wP vaccine, the new generation of pertussis vaccine or aP vaccine was developed in 1980's. Acellular vaccine contains 2-5 purified inactivated *B. pertussis* antigens. It is given to infants in the form of combined Diphtheria–Tetanus toxoid–acellular pertussis (DTaP) vaccine adjuvanted with aluminium salt. DTaP or DTwP are recommended for children up to seven years of age, while reduced dose of diphtheria-Tetanus toxoid (dT) or Tetanus toxoid-reduced dose of diphtheria and acellular pertussis (Tdap) vaccine are recommended in older children and adults as booster doses. The DTaP contains a higher dose of diphtheria toxoid and *B. pertussis* antigens to ensure adequate immune response in children, but if used in adults, there could be an increased risk of adverse reaction.

There are many different brands of combined aP-containing vaccines that are now available in the market as demonstrated in Table 1.6.1. Each brand differs in number and amount of *B. pertussis* antigens. DTaP for infants are usually combined with Inactivated Polio Vaccine (IPV), *Haemophilus influenzae* type b and/or hepatitis B vaccine. The major *B. pertussis* antigens present in every brand are PT and FHA. Some also contains PRN and a few contain FIM 2 and 3. Clinical trials have shown that aP-containing vaccines are effective against pertussis with less adverse reactions compared to wP-containing vaccines [28, 29].

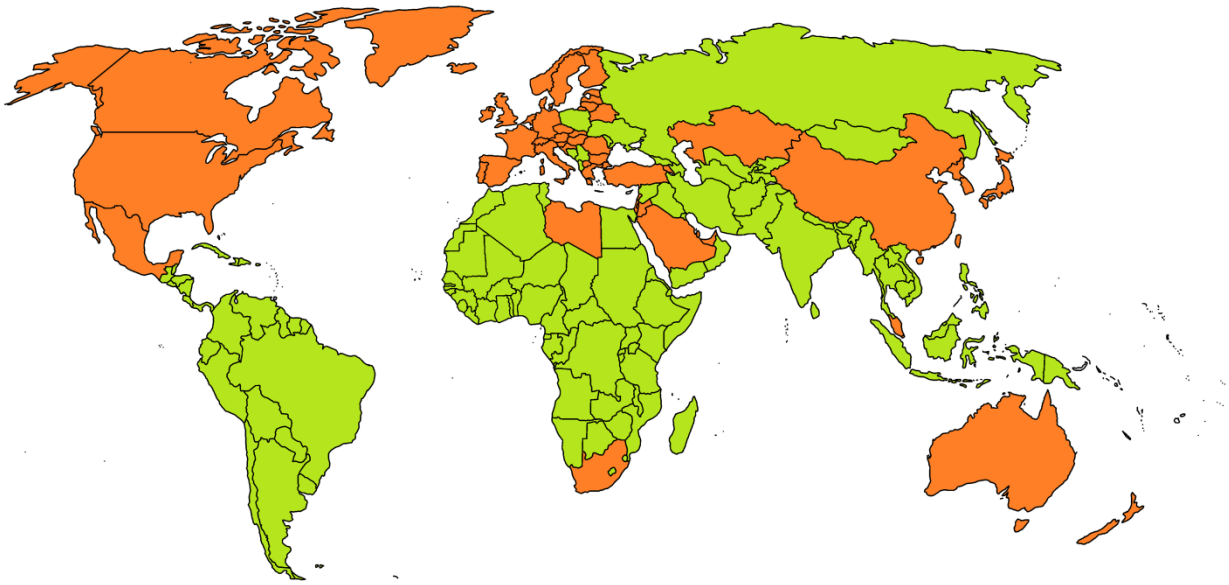
Table 1.6.1: Some of the commercially available aP-containing vaccines and their compositions.

Trade name	Composition	Antigenic components of *B. pertussis* in µg				Manufacturer
		PT	FHA	PRN	FIM 2 and 3	
Acelluvax	DTaP	5	2.5	2.5	-	Biocine
Tripacel	DTaP	10	5	3	5	Sanofi Pasteur
Actacel	DTaP+Hib	10	5	3	5	
Pediacel/Pentacel	DTaP+IPV+Hib	20	20	3	5	
Infanrix	DTaP	25	25	8	-	GlaxoSmithKline (GSK)
Infanrix-IPV	DTaP+IPV+Hib	25	25	8	-	
Infanrix-Hexa	DTaP+IPV+Hib+HB	25	25	8	-	
Tetraxim	DTaP+IPV	25	26	-	-	Sanofi Pasteur
Pentaxim	DTaP+IPV+Hib	25	26	-	-	
Hexaxim	DTaP+IPV+Hib+HB	25	26	-	-	
Boostrix	Tdap	8	8	2.5	-	GlaxoSmithKline (GSK)
Adacel	Tdap	2.5	5	3	5	Sanofi Pasteur

Hib, *Haemophilus influenzae* type B; IPV, inactivated polio vaccine; HB, hepatitis B

The safety and efficacy profile of aP vaccine encouraged many developed countries to replace the reactogenic wP with aP vaccine. Currently, the aP-containing vaccine are routinely used in the infant immunization programs in several countries (Figure 1.6.2), however, in many other parts of the world especially in developing countries, wP is still in the infant EPI program [30].

Figure 1.6.2: World map showing acellular pertussis-vaccinating countries (orange) and whole-cell pertussis-vaccinating countries (green) given to infants as part of the EPI program (data retrieved from WHO, 2014).



There are two *B. pertussis* antigens that are in the components of every aP-containing vaccine available in the market. First is PT which composes of five subunits S1 to S5. PT is the key virulence factor that mediates severe disease and it is specific to *B. pertussis* only. PT is also important for the development of protective immunity against pertussis. The five subunits are formed in an A-B structure, in which the A protomer (S1) expresses ADP-ribosyltransferase activity and the B moiety (S2-S5) is responsible for binding to the target cells.

The second *B. pertussis* antigens in all aP vaccine is FHA, the hairpin-shaped, surface-associated protein that mediates initial adhesion of *B. pertussis* to the respiratory epithelium [32]. FHA also involves in the progression of infection from upper to lower respiratory tract and promotes phagocytosis of *B. pertussis* by macrophages and polymorphonuclear neutrophils [33, 34].

Innate immunity is the first line defense that interacts with microbial pathogens and foreign antigens. Phongsisay et al. investigated the interaction between PT and innate immune components. They found that PT targets the innate immunity through DNAX activation protein of 12kDa (DAP12), Fc gamma receptor (FcRγ) and Myeloid differentiation primary response protein (MyD88) adaptor molecules [35]. FHA possesses the arg-gly-asp (RGD) tripeptide motif that interacts with the leukocyte-response integrin/integrin-associated protein (LRI/IAP) complex on monocytes and macrophages, and with very late antigen-5 on epithelial cells, it stimulates the up-regulation of intercellular adhesion molecule-1 via an NF-κB signaling pathway [36]. PRN is another common antigen present in most acellular vaccines. PRN, similar to FHA, also has the RGD tripeptide motif that support cell adhesion [37]. However, a receptor has not yet been identified for PRN [38].

1.7 Immune responses to *B. pertussis* infection, aP and wP vaccines

1.7.1 Murine model

Immune response to *B. pertussis* infection and vaccination in murine models has been extensively studied for many years. Mills et al. found that T cells induced by natural infection are largely confined to CD4+ T cells that secrete interleukin-2 (IL-2) and gamma interferon (IFN-γ) but not IL-4 [39]. Redhead et al. conducted a study comparing immunity induced by natural infection, aP (in the form of chemically detoxified PT, FHA and PRN plus aluminum sulfate) and wP (killed *B. pertussis*) vaccination. The results indicated that spleen cells from convalescent mice elicited extensive *in vitro* T-cell proliferation and secreted high levels of IL-2 and IFN-γ but not IL-4 or IL-5, suggestive of a Th1 response. However, serum from convalescent mice showed low anti-*B. pertussis* antibody levels. Immunization with wP vaccine also induced a Th1 response together with the high antibody levels. In contrast, aP vaccine induced high levels of *B. pertussis*-specific antibodies and spleen cells secreting IL-5 but not IFN-γ, suggestive of a Th2 response. Following *B. pertussis* challenge among convalescent and vaccinated mice, Redhead et al. found that convalescent and wP-vaccinated mice clear infection faster than aP-vaccinated counterparts, suggesting that Th1-mediated immune response is an essential component of immunity against pertussis [40]. Further studies confirmed the better efficacy of wP vaccine as they reported wP-vaccinated mice can clear the infection faster than aP-vaccinated mice, however, they also address the importance of humoral immunity by providing evidence that passive transfer of antibodies confers protection against experimental challenge of *B. pertussis* [41].

Recent discovery of a new Th subtype, the Th17, and its role in protection against extracellular bacteria has brought a lot of attention to its contribution to prevention against pertussis [42]. Ross et al. found that previous infection and wP vaccination induced Th1 and

Th17 response whereas aP vaccination induced Th2 and Th17 cells, but weak Th1 response. Th1 and Th17 cells are important in clearing the bacteria but Th2 is not necessary. Th17 plays an important role by promoting neutrophil recruitment to the lungs [43].

1.7.2 Baboon model

The difference in immune response to pertussis vaccination and previous infection in baboons has been examined. Warfel et al. found that aP (DTaP) -vaccinated baboons had respiratory colonization after *B. pertussis* challenge and can transmit the infection to their naïve contacts, while none of the previously infected baboons had colonization after the challenge [9]. Whole cell (DTwP)-vaccinated baboons had respiratory colonization but were able to clear infection more rapidly than the aP-vaccinated group. Whole cell-vaccinated, aP-vaccinated, and convalescent animals did not cough and showed no reduction of activity, loss of appetite, or other outward signs of disease, suggesting that previous infection or vaccination conveys protection against pertussis disease.

Examination of the immunological mechanisms responsible for protection against *B. pertussis* in baboons showed that the inability to prevent *B. pertussis* colonization was not due to low antibody titers because wP, aP, and natural infection induced high antibody titers to all *B. pertussis* antigens studied. Peripheral blood mononuclear cells (PBMC) were collected one week before *B. pertussis* challenge in naïve, aP-vaccinated, wP-vaccinated and convalescent baboons. IL-5 (represented Th2 response), IL-17A (represented Th17 response) and IFN-γ (represented Th1 response) were measured from the supernatants after 36 hours of PBMC culture with heat-killed *B. pertussis*. The results showed that cells from convalescent animals secreted very high levels of IL-17A, high level of IFN-γ and low level of IL-5. Cells from aP-vaccinated animals secreted very high level of IL-5 and cells from wP-vaccinated animals secreted cytokines similar to convalescent animals but the response was weaker. It

was concluded that aP vaccination induces Th2/Th1 immune memory responses while infection, and to a lesser extent wP vaccination, induces Th17/Th1 memory. This observation further confirmed the importance of Th1 and Th17 cells in the host mechanisms conveying protection against *B. pertussis* colonization.

1.7.3 Human studies

There have been several clinical trials that examined the immunogenicity and efficacy of aP and wP vaccine in infants, children and adults. A systematic review involving more than 100,000 participants showed that the efficacy of multicomponent DTaP vaccine against severe and mild pertussis were 85% and 71-78%, respectively. However, the efficacy of wP vaccine varied between studies, ranging from 37-92% [44, 45].

Both aP and wP vaccines induced high antibody levels to *B. pertussis* antigens in the vaccine components but the geometric mean concentrations at post vaccination varied among different studies [46-48]. Studies that compared the antibody responses after DTaP and DTwP vaccination demostrated that most DTaP vaccine stimulated comparable or higher serum antibody levels compared to DTwP [28, 49]. Regarding the cellular immune response, Mascart et al. found that in infants as young as two months of age, previous *B. pertussis* infection and wP (DTwP-IPV) vaccination can induce Th1 response [50]. Mascart et al. also further compared the cellular immunity in aP (DTaP-IPV-Hib) and wP-vaccinated infants (DTwP-IPV-Hib) up to one year post vaccination. They found that the wP vaccine induced mostly Th1-type cytokine secretions, whereas aP vaccine is associated with mixed Th1/Th2 responses. These findings highlight the difference in cellular immunity induced by aP and wP vaccine [51, 52].

1.8 Epidemiology of pertussis

1.8.1 Global

Despite the widespread use of vaccines, pertussis remains endemic and affects more than 130,000 people worldwide in 2016 [53], with frequent outbreaks occurring in different regions around the world. In the United States, the Center of Disease Control and Prevention reported 32,971 cases and 13 deaths from pertussis in 2014, of which nearly 70% of fatal cases were infants younger than one year [54]. In the California pertussis epidemic 2010, all deaths and most hospitalizations occurred in infants younger than 3 months. Adolescents who were completely vaccinated according to the national recommendation also contracted the disease [3]. Outbreaks were also reported in the Netherlands, starting since 1996 with increasing incidence rates in adolescent and adults. Although pertussis is not severe in these age groups, it could result in increased transmission to the infants who are at risk for severe disease [2]. Octavia et al. reported the epidemic of pertussis affecting more than 35,000 Australian in 2009, and a large number of pertussis-related deaths (more than 50,000) were reported in Africa in 2008 [1, 55].

There are several limitations in estimating the global pertussis disease burden. Many countries have different clinical criteria and surveillance reporting system, along with the limited laboratory infrastructure to perform molecular diagnostic tests for *B. pertussis* in some developing countries. Under-reporting is common in affected adolescent and adults, for whom the cough pattern may be atypical. This can lead to delay in seeking medical care, physician unawareness and missed diagnosis.

1.8.2 Thailand

Although the number of pertussis cases from passive surveillance in Thailand was low between 2009 and 2014 (ranging from 0.01 to 0.11 cases per 100,000 population) with no report of major outbreaks, these numbers are likely to be underestimated due to physician unawareness and limited laboratory confirmation of suspected cases [56]. However, in 2015-2016, there was a rise in incidence of infant pertussis as demonstrated in Table 1.8.2 [57]. There were 2 pertussis-related deaths in 2015 and both cases occurred in infants younger than one year old.

Table 1.8.2: Pertussis incidence in Thailand between 2011 and 2016 by age group. Data were retrieved from the annual epidemiology surveillance report by the Bureau of Epidemiology, Department of Disease Control, Ministry of Public Health, Thailand. The numbers represented suspected, probable and confirmed cases* reported annually.

Year	Age group in year (s)									
	0-1	1-4	5-9	10-14	15-24	25-34	35-44	45-54	55-64	>65
2016	27	16	9	4	3	5	3	0	0	5
2015	35	8	3	0	0	1	0	1	2	1
2014	11	1	1	1	0	1	0	0	0	1
2013	11	4	1	1	0	1	2	2	1	1
2012	6	2	3	3	1	0	1	1	0	0
2011	7	2	1	1	0	0	1	0	0	0

*Pertussis case definitions; a suspected case is a patient presenting with a cough illness lasting ≥2 weeks with at least one of the following signs or symptoms: paroxysms of coughing; or inspiratory "whoop"; or post-tussive vomiting. A probable case is defined as a suspected case with epidemiologic linkage to a laboratory-confirmed case. A confirmed case is defined as a suspected case with laboratory confirmation by polymerase chain reaction (PCR) or bacterial culture.

Recently, there has been growing interest in active surveillance of pertussis in Thai pediatric and adult patients presenting with prolonged cough. Siriyakorn et al. conducted a prospective study to determine the prevalence of pertussis in 74 Thai adults presenting with cough of more than two weeks between 2010 and 2011 [58]. Nasopharyngeal swabs were obtained and *B. pertussis* DNA was detected by real-time PCR. Paired serum samples were collected and tested for anti-PT IgG. The criteria for serological diagnosis were the four-fold rising of anti-PT IgG in acute and convalescent sera or the agglutinin titer of ≥ 3 standard

deviation (SD) in single serum. The results showed that 18.4% (14 patients) had laboratory evidence of pertussis infection. One case was diagnosed by real-time PCR and the rest was diagnosed by serological evidence. Another study evaluated the prevalence of serologically confirmed pertussis in 147 Thai adults with prolonged cough of more than two weeks between 2012 and 2013 [(4)]. The cut-off value for serological diagnosis was anti-PT $\geq$ 62.5 IU/ml by ELISA. They found that 6.1% (9/147) of Thai patients had serological evidence of recent pertussis infection. Suntarattiwong (2013) investigated the incidence of pertussis in 143 Thai children aged 1 month to 16 years (median age 8.1 months) presenting with cough of more than 7 days. Pertussis was diagnosed by detection of *B. pertussis* DNA by PCR from nasopharyngeal swab samples. The results showed that 19.6% of children had pertussis. The majority of the cases occurred in infants below 6 months of age [(59)].

1.9 Rationale of maternal immunization

Unvaccinated or incompletely-vaccinated infants are at the highest risk for severe outcomes, including respiratory failure, encephalopathy and death. Pre-existing maternal *B. pertussis*-specific antibody showed low concentrations in newborns and their presence in infants was short-lived, despite an active transplacental antibody transfer [15]. A previous study found that pertussis vaccination during pregnancy elicits a high *B. pertussis*-specific antibody in infant cord sera and these antibodies persisted at a higher level at two months of age compared to infants born to unvaccinated mothers [14, 16]. Therefore, vaccination during pregnancy could offer protection in infants during this crucial susceptibility gap. In 2011, the ACIP made recommendations for use to Tdap vaccine in pregnant women and this strategy has been recommended in an increasing number of countries worldwide [18, 60, 61]. Effectiveness of Tdap vaccine during pregnancy in prevention of infant pertussis has been confirmed by many studies [62-64].

1.10 Current pertussis vaccination strategy in Thailand

Thailand implemented a routine infant immunization program with two doses of the DTwP vaccine for all infants beginning in 1977. This recommendation was changed to three doses of DTwP in 1982 and four doses (at 2, 4, 6, and 18 months) in 1987. Since 1992, the Thai national vaccine policy recommends five doses of DTwP vaccine for children at the ages of 2, 4, 6, 18, and 48 months [65]. DTwP vaccine given to infants and children in Thailand were combined with HB vaccine. In addition, oral polio vaccine is also given at the same vaccination visits. As a consequence of changes in the Thai polio vaccine recommendations when the World Health Organization switched from trivalent to bivalent oral polio vaccine (bOPV) in April 2016, all infants who reached the age of 4 months by 1 December 2015 also received monovalent IPV (IMOVAX polio, Sanofi Pasteur, Lyon,

France) vaccine at four-month vaccination visits. This vaccine was injected separately into the anterolateral thigh. A full list of current infant immunization program (as of 2016) in Thailand is provided in Table 1.10.1.

Table 1.10.1: Current infant immunization program in Thailand (2016)

Age	Vaccines
Birth	BCG, HB
2 months	DTwP-HB, bOPV
4 months	DTwP-HB, bOPV, IPV (since Dec 2015)
6 months	DTwP-HB, bOPV
9-12 months	MMR 1
12 months	Live JE 1
18 months	DTwP, bOPV
2 years	Live JE 2
2.5 years	MMR 2 – since 2014
4 years	DTwP, bOPV
Every 10 years	dT

Recent studies demonstrated that vaccine-induced immunity against *B. pertussis* is not life-long [10, 66]. In 2006, the ACIP recommended that in order to reduce susceptibility to pertussis later in life, a booster dose of the Tdap vaccine should be given during adolescence [11]. However, Thailand has integrated only the diphtheria–tetanus vaccine (dT) into its EPI for this group. It recommends the dT vaccine at age 12 years as part of a school-based program since 2008 and a dT booster every ten years thereafter. In addition, pregnant women visiting the antenatal care clinic are asked by their health care providers regarding the history of tetanus immunization during childhood. If they had not received any tetanus toxoid vaccine or are unsure about their vaccination history, dT will be given at the first visit and

subsequently at 1 and 6 months apart. If they had received some doses or had not yet completed the recommended vaccine series, they will be provided a booster vaccination. Although Tdap vaccination is better as the additional *B. pertussis* antigens component offers protection from whooping cough, Thailand has not yet implemented such vaccine into the national vaccination program.

1.11 Current gap in knowledge

Thailand has implemented universal whole cell pertussis vaccination in children for more than 35 years; nevertheless, the numbers of reported pertussis cases are still on the rise, especially among infants below one year who bear the greatest risk of severe complications. Several countries have implemented a pertussis booster during adolescence. However, data on the appropriate age groups targeted for a booster in the Thai population is limited. The increase in pertussis incidence among Thai infants warrants the need to further investigate the pre-existing maternal antibody against *B. pertussis* antigens and the potential benefit of Tdap vaccine administered during pregnancy. Many developed countries implemented Tdap vaccination during pregnancy and reported the blunting effect of antibody to *B. pertussis* antigens in DTaP-vaccinated infants born to vaccinated mothers, but data regarding interference in DTwP-vaccinated infants are limited. In addition, the cytokine responses including Th1/Th2 and Th17 cytokines among aP *versus* wP-vaccinated infants needs to be further studied in order to understand the kinetics of both humeral and cellular immune responses in infants born to Tdap-vaccinated mothers and help determine the optimal timing of pertussis booster vaccination in these infant groups.

1.12 Aims of study

The aims of this thesis are

1. To investigate the anti-PT IgG among healthy people across all ages after 37 years of universal whole-cell pertussis vaccination in Thailand and provide evidence-based recommendation for the age group that should be targeted for a booster dose.

2. To examine the anti-PT, anti-FHA and anti-PRN IgG in Thai pregnant women who did not receive pertussis vaccine during pregnancy.

3. To assess the potential benefit and reactogenicity of a booster dose of Tdap vaccine during pregnancy when tetanus vaccine is currently in the EPI program.

4. To assess the humoral interference of maternal antibody in aP *vs* wP-vaccinated infants born to mothers who received Tdap vaccine during pregnancy in Thailand.

5. To investigate the difference in T cell-mediated immune responses among aP *vs* wP-vaccinated infants following primary series vaccination and first booster.

Chapter 2

Materials and Methods

2.1 Processing and storage of samples

Peripheral blood samples were collected from healthy volunteers or infants after the written informed consent was obtained. Venous blood samples (2.5 ml from infants and 10 ml from adults) were collected in the red-top BD Vacutainer Tubes containing no anticoagulants. Then, samples which were kept at 4°C refrigerator and then centrifuged according to the standard operating procedures within 24 hours from the collection time. Sera were collected and stored at -20°C at the Center of Excellence in Clinical Virology, Department of Pediatrics, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand until tested.

In a subset of infants whose additional informed consent were obtained from their legal representatives for cell-mediated immune response study, additional four ml of venous blood samples were obtained at 7, 18 and 19 months of age. Samples were collected in a green-top BD Vacutainer tubes coated with heparin. Heparinized blood samples were processed within four hours after collection. Isolation of PBMC was performed according to the following steps in 2.1.1.

2.1.1 Protocol for isolation and cryopreservation of PBMC from children

Step 1: Plasma collection

- Centrifuge 4 ml heparinized blood at 400 × *g* for 5 minutes, room temperature, accelerate 8, decelerate 8.

- Collect approximately 2 ml of plasma and store at -20 ^{o}C.

- Replace plasma volume with 2 ml of complete RPMI-1640 culture medium (cRPMI).

Step 2: Lymphocyte separation

- Samples from step 1 were further diluted with 4 ml cRPMI.

- Four ml of this diluted blood samples were gently layered and isolated by density centrifugation on 3 ml Ficoll solution (Ficoll-Paque PREMIUM, GE Healthcare) using centrifugation programs of 30 minutes at 400 × *g*, accelerate 8, decelerate 0 (no brake) at room temperature.

- PBMC layer was collected into a 15 ml conical tube using 3 ml sterile Pasteur pipette.

Step 3: Wash

- Complete RPMI was added to the PBMC up to 10-12 ml and the diluted PBMC preparation was centrifuged for 10 minutes at 260 × *g*, accelerate 8, decelerate 8 at 4 ^{o}C.

- Supernatant was discarded and cells were washed again using the same protocol.

Step 4: Cell counting

- Resuspend pallet in 1 ml cRPMI and transfer cells to 2 corning cryotubes (0.5 ml of PBMC/vial)

- Prepare 180 µl PBS in a 1.5 ml tube and pipette 20 µl of PBMC suspension (1:10)

- Pipette 10 µl of PBMC (1:10) and load into haemocytometer and count cells in the central large square

Total cells/ml = no.of cells ×10 × 10^4

- Freshly isolated cells were resuspended to 2 x10^6/ml and processed according to the protocols of the experiments. Leftover PBMC were cryopreserved according to the following steps.

Step 5: Cryopreservation of PBMC

- Add dropwise 4°C freezing medium to double the volume of the cell suspension and immediately place the tube on ice. Avoid any further mixing or agitation of the cells.

- Place the cryovials in a 4 ^{o}C freezing container.

- Place the freezing container at –80 ^{o}C for 3 days, then transfer samples to liquid nitrogen for long term storage.

2.1.2 Composition of complete RPMI-1640 culture medium

Complete RPMI-1640 medium is composed of 10% Fetal Bovine Serum (FBS) (Hyclone, GE Healthcare, Brondby, Denmark) and 5% Supplement Solution (SS) in RPMI-1640 Culture Medium supplemented with 2.05 mM L-glutamine (Hyclone; catalog number 30027.02).

The 600 ml of SS contains the following components

1. RPMI-1640 320 ml

2. Non-essential amino acids 100 ml

3. Penicillin/Streptomycin (10,000 units/ml of penicillin and 10,000 µg/ml of streptomycin) 80 ml

4. 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) 11.9 gm

5. L-glutamine 100 ml

6. Beta-mercaptoethanol 35 µl

Reagents no. 2-6 were filtered through 0.2 µm filter. SS was kept at -20 ^{o}C.

2.1.3 Composition of freezing medium

The freezing medium contained 90% FBS and 10% Dimethyl Sulfoxide (DMSO) (Applichem). Freezing medium was kept in -20 ^{o}C and once thawed, it was kept in the 4 ^{o}C prior to use.

2.2 Enzyme-linked Immunosorbent Assay (ELISA)

Anti-PT anti-FHA and anti-PRN IgG were analyzed quantitatively using a commercial ELISA kit (EUROIMMUN, Lübeck, Germany) according to the manufacturer's instructions. The standards were calibrated based on the World Health Organization (WHO) international standards. Sera were diluted 1:101 as written in the manufacturer's protocol. Further dilution to achieve values within the standard curves was performed as necessary. The lower limit of quantification (LLOQ) was 5 International Units (IU) per ml of serum for anti-PT, anti-FHA and anti-PRN. Samples with values below the LLOQ were calculated as 50% of the LLOQ.

2.3 Analysis of cytokine levels in cell culture supernatants

To characterize the cell-mediated immune response, PBMC from infants were stimulated with heat-inactivated PT and assessed for cytokine production. PBMC were isolated using Ficoll density gradient centrifugation (GE Healthcare) and washed twice in cRPMI. Cells were resuspended at 2 x 10^6 cells/ml and seeded into 96-well flat-bottom plates. PT (Calbiochem, Merck, Germany) was heat-inactivated at 80 ^{o}C for 15 minutes and used at a final concentration of 5 µg/ml as previously described [67]. Cells were incubated at 37 ^{o}C with 5% CO_2 for 88 hours. Culture supernatants from PT-treated and untreated (medium control) cells were then harvested and stored at -80 ^{o}C. Th1 (IFN-γ, IL-12p70, TNF-α), Th2 (IL-4, IL-5, IL-6) and Th17 (IL-17A) cytokines were analyzed by commercial

multiplex bead-based ELISA (ProcartaPlex®, Thermofisher scientific, Vienna, Austria) according to the manufacturer's instructions. Standard Mix A provided with the kit was used as reference standard. Data were acquired using Bio-plex instruments with Luminex xMap Technology (Biorad). Values below the LLOQ were quantified as half of the LLOQ. For IL-6 values that exceeded the upper limit of quantification in some samples, 50-fold dilution was performed and IL-6 levels were evaluated using a human IL-6 simplex kit (ProcartaPlex®, Thermofisher scientific, Vienna, Austria). There were 21 samples with limited amount of cell culture supernatant (Table 9.2.7), therefore, we estimated the IL-6 levels using the upper limit of quantification.

2.4 Intracellular cytokine staining assay

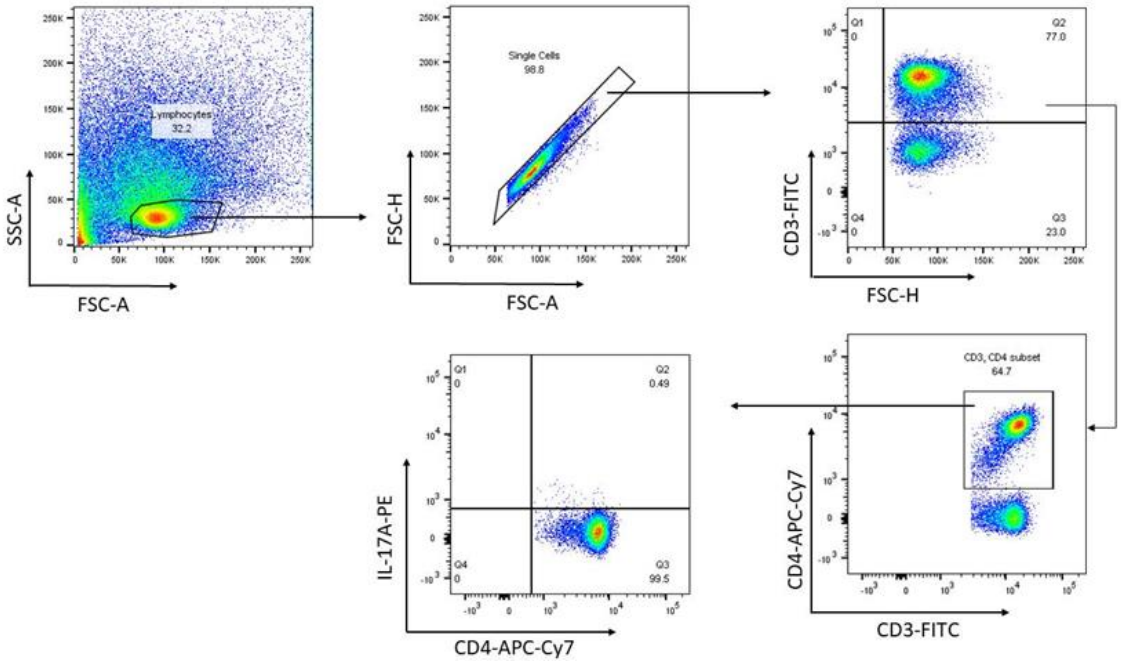
After cell culture supernatants were harvested, Brefeldin A solution (eBioscience, Thermo Fisher Scientific) was added to cells at the final concentration of 3 µg/ml for 4 hours prior to cell staining. Cells were then fixed using fixation buffer (eBioscience) and stained with surface markers including anti-CD3-PE/Cy7 (Biolegend, San Diego, California, USA; clone UCHT1) and anti-CD4-APC/Cy7 or anti-CD4-APC/Fire750 (Biolegend, San Diego, California, USA; clone SK3). Afterwards, stained cells were permeabilized using permeabilization buffer (eBioscience) and stained with anti- IFN-γ-APC (eBioscience; clone 4S.B3) and anti-IL-17A-PE (BD Bioscience; clone SCPL1362).

2.5 Flow cytometric analysis of cells

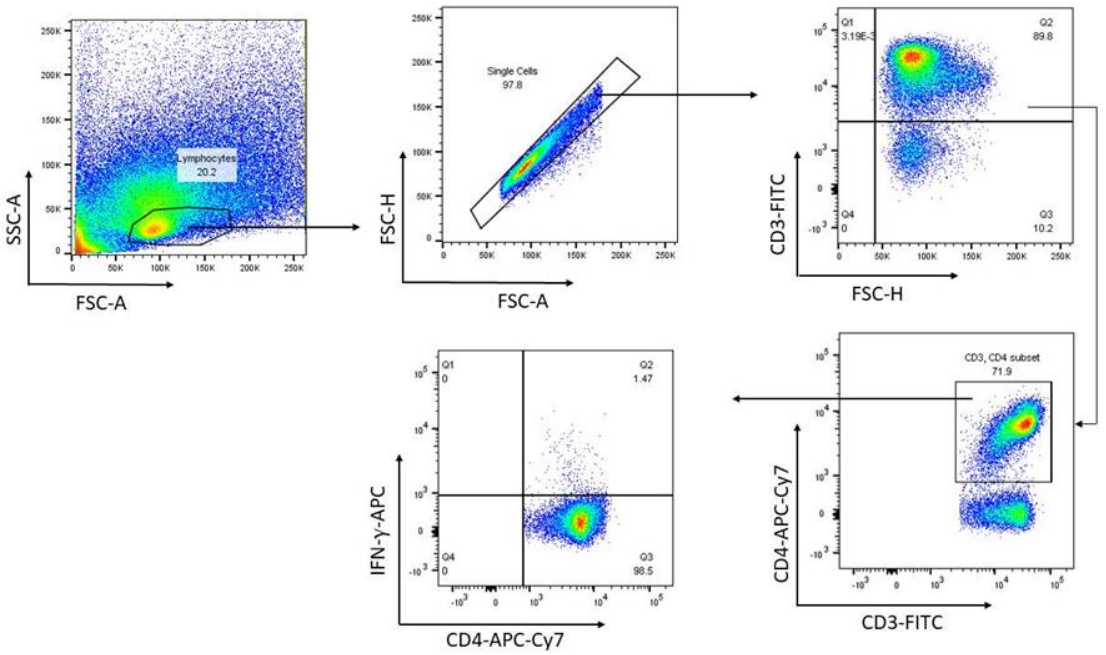
The flow cytometer analyses the optical properties of the fluorocrome-labelled cells. Cells scatter the laser signals as the forward and side scattered light. Forward scatter (FSC) is related to cell size, whereas side scatter (SSC) represents cell internal complexity or granularity. Data acquisition was performed on BD LSRII analyzer (BD Biosciences). Gating strategies were demonstrated in Figure 2.5.1. BD FACSDIVA software (BD Biosciences) was used for data acquisition and Flowjo software (Tree Star, Oregon, USA) was used for data analysis. Percentages of cytokine producing CD4+ T cells were recorded.

Figure 2.5.1: Sample gating strategy for intracellular cytokine staining assay. A, IL-17A; B, IFN-γ.

A.



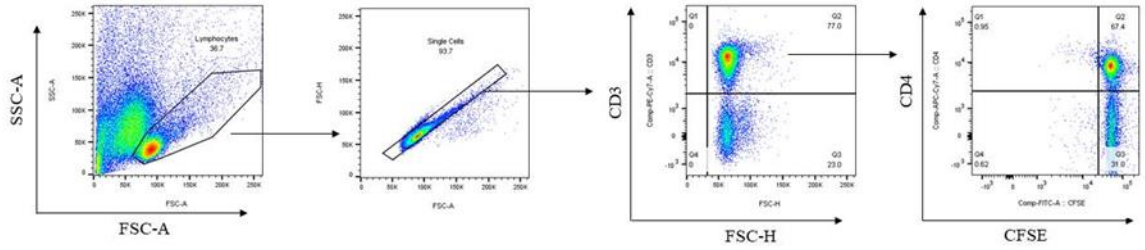
B.



2.6 T cell proliferation assay

Infant PBMC were isolated and resuspended at the concentration of 2 x 10^6 cells/ml in a 15 ml conical tube at a total volume of 1 ml. Carboxyfluorescein diacetate succinimidyl ester (CFSE) (Biolegend, Oregon, USA) at a volume of 1.1 µl was added on top of 110 µl sterile phosphate-buffered saline (PBS) on the side of the tube. Cell suspension were mixed thoroughly by Vortexing at high speed to make the final working concentration of 5 µM CFSE. Cells were then incubated at 37°C for 4 minutes in the dark. Then, the reaction was stopped by adding 10 ml of cRPMI. Thereafter, cells were centrifuged at 300 × g for 5 minutes, and the pellet was resuspended in cRPMI at the concentration of 1 x 10^6 cells/ml. Cells were seeded into 96-well flat bottom plates at the concentration of 200,000 cells/well and incubated with or without stimulation reagents at 37°C in 5% CO_2 for 6 days. Under stimulated condition, pertussis toxin (Calbiochem, Merck, Germany) at the final concentration of 5 µg/ml was heat-inactivated at 80 ^{0}C for 15 minutes prior to use. Positive control for this experiment was anti-CD3 (Biolegend, Oregon, USA) at the concentration of 1 µg/ml. After the cells uptake CFSE, the dye was evenly distributed among the dividing daughter cells. This dilution of fluorescence in the offspring cells was used to measure the cell proliferation by a flow cytometer. After six days of incubation, cells were harvested and stained with anti-CD3-PE/Cy7 (Biolegend, Oregon, USA; clone UCHT1) and anti-CD4-APC/Cy7 (Biolegend, Oregon, USA; clone RPA-T4). Data acquisition was performed on BD LSRII analyzer (BD Biosciences). Gating strategies were demonstrated in Figure 2.6.1. BD FACSDIVA software (BD Biosciences) was used for data acquisition and Flowjo software (Tree Star, Oregon, USA) was used for data analysis. CD4+ and CD3+ T cell proliferation were recorded. Proliferating CD4+ T cells was calculated from the percentages of CD4+CFSEdim cells among CD4+ T cells. Proliferating CD3+ T cells was calculated from the percentages of CD3+CFSEdim cells among CD3+ T cells.

Figure 2.6.1: Sample gating strategy for cell proliferation assay.



2.7 Statistical analysis

Baseline characteristics were reported as mean and standard deviation (SD). The IgG levels were expressed as geometric mean concentrations (GMC) with 95% confidence interval. GMC were calculated using the log transformation of antibody levels and taking the antilog mean of the transformed values. Data were analyzed using SPSS software version 20 (IBM Inc., Armonk, NY, USA) and R statistical software version 3.3.3. The conventional *t*-test was used to compare parameters in the baseline characteristics and the GMC between groups using the antibody logarithmic scales. The paired *t*-test was used to compare the antibody titers on the logarithmic scale in order to make inference about the difference in GMC between month 2 and month 7 or month 18 and month 19 infant sera. Wilcoxon signed ranks test was used to compare the differences in cytokine levels and CD4+ T cell responses before and after stimulation. Mann-Whitney U test was used to compare the post-stimulation cytokine levels and CD4+ T cell responses between groups.

Chapter 3

Seroprevalence of antibody to pertussis toxin among Thai population

3.1 Introduction

Pertussis is a respiratory disease characterized by a severe protracted paroxysmal cough that sometimes ends with a whooping sound, also known as a whooping cough. It is caused mainly by *Bordetella pertussis* and spread by direct contact or breathing aerosolized secretions of infected individuals. Despite the high coverage of prophylactic vaccine in many countries, pertussis remains a common vaccine-preventable disease. Whole cell pertussis (wP) vaccine was first licensed as part of a routine infant vaccination in 1950s in the United States and afterwards, many countries implemented this vaccine into their EPI [(19)]. Although the vaccine was proven to be effective as the morbidity and mortality from pertussis decreased after vaccine implementation in many countries, it was associated with many adverse local and systemic reactions such as pain, redness and swelling at injection sites, high-grade fever, persistent crying, hypotonic-hyporesponsive episode and seizure [(68)].

In the 1980s, a less reactogenic acellular pertussis (aP) vaccine was introduced and it was also proven to be effective with less adverse effects. Therefore, many industrialized countries replaced wP with aP vaccine. A few decades after aP vaccine implementation in childhood immunization program, the incidence of pertussis gradually increased. Sporadic outbreaks were reported in many regions of the world [(2, 3)]. Recent research found that aP and wP-vaccine induced immunity is not lifelong, with some evidences pointing out that immunity induced by aP wanes faster than wP vaccine [(8, 10, 66)]. Thailand implemented a routine infant immunization program with wP vaccine starting in 1977, and since 1992, the EPI has included five doses of DTwP vaccine for children at the ages of 2, 4, 6, 18, and 48 months. The vaccine coverage of the first three doses achieved > 95% since 1999 and the

coverage of the fourth dose reached > 95% in 2008. The coverage with five doses has just successfully reached > 95% in 2013 [65]. Although a booster dose of Tdap is recommended during adolescence in many developed countries, Thailand has not yet integrated Tdap in the EPI for this age group. Pertussis toxin (PT) is the major *B. pertussis* antigen that mediates severe disease. PT is the major component in the aP vaccine and is also presents in the wP vaccine at variable concentrations [69]. This study aims to evaluate the level of pre-existing antibodies to PT in Thai people across all ages and provide an evidence-based recommendation for a booster dose in the Thai population.

3.2 Aim of the study

This study aims to explore the serological profiles of anti-pertussis toxin IgG among Thai population, the population which received only whole-cell vaccine for more than 37 years. The results could help determine the level of pre-existing immunity and identify which age group was the target for a booster dose.

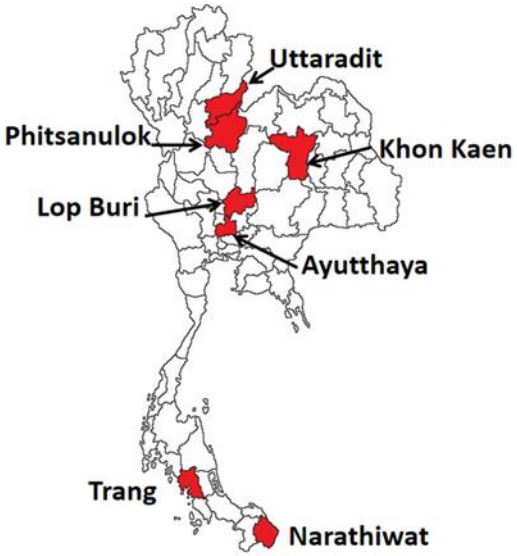
3.3 Study design and laboratory methods

3.3.1 Study design

The study was approved by the Institutional Review Board of the Faculty of Medicine, Chulalongkorn University (IRB No. 154/58), and was conducted in accordance with the principles of the Helsinki Declaration II. The sera analyzed in this study were collected between March and October 2014 for research on the impact of universal hepatitis B immunization in newborns as part of the EPI (IRB No. 419/56). All subjects had previously been informed about the spin-off study, and had provided written informed consent for use of their sera. All information and patient identifiers were kept anonymous in order to strictly protect patient confidentiality. Permission for specimen utilization was granted by the Director of King Chulalongkorn Memorial Hospital.

In total, 900 serum samples from healthy Thai individuals were randomly selected from people residing in seven provinces of the four regions of the country; Uttaradit and Phitsanulok province representing the north, Lop Buri and Ayutthaya province representing the central, Trang and Narathiwat province representing the south, and Khon Kaen province representing the northeastern part of Thailand. A map of the seven provinces is shown in Figure 3.3.1.1. There were 252 samples from Khon Kaen province, and 108 samples from each of the other provinces.

Figure 3.3.1.1: The map of Thailand showing the seven provinces from which serum samples were taken.



3.3.2 Laboratory methods

The sera were processed at the regional hospitals and stored in -20^{o}C freezer. They were transferred with dry ice to be stored at -20^{o}C at the Center of Excellence in Clinical Virology, Department of Pediatrics, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand until tested.

The presence of anti-PT IgG was detected by quantitative analysis using a commercially available ELISA kit (EUROIMMUN, Lübeck, Germany) according to the

provided instructions. Results were also interpreted according to the instructions. Levels >100 IU/ml indicated acute pertussis infection or recent vaccination. Level 40-100 IU/ml were interpreted as probable past exposure to pertussis. Level 5-40 IU/ml indicated no evidence of recent infection, and <5 IU/ml indicated seronegativity.

3.3.3 Statistical analysis

The IgG level was expressed as the geometric mean concentrations (GMC) with 95% confidence interval. Data were analyzed using SPSS software (version 20; IBM Inc., Armonk, NY, USA). One-way analysis of variance was used to evaluate statistically significant differences in the GMC between different age groups. The $\chi 2$ test was used to compare the proportion of different antibody levels between individuals. Any differences were considered statistically significant at $p < 0.05$.

3.4 Results

3.4.1 Demographic characteristics

A total of 900 sera samples were analyzed in this study. Participants' ages ranged from 12 days to 64 years. There were 61.6% and 38.4% male and female participants, respectively. Samples were divided into six age groups (0–10, 11–20, 21–30, 31–40, 41–50, and >50 years). The numbers of participants in each age group were demonstrated in Table 3.4.1.1.

3.4.2 GMC among different age groups

The overall GMC of anti-PT IgG in the population was 5.83 IU/ml. The highest GMC was found in the 0–10 years age group and the lowest in the 31–40 years age group (Table 3.4.1.1). The statistical difference was observed between the GMC from group 0–10 years and those from 21 years and older.

Table 3.4.1.1: Age-specific GMC of anti-PT IgG in Thai population (n=900).

Age in year (s)	Sample size	GMC (IU/ml)	95% CI of GMC		ANOVA *p*-value
			Lower	Upper	
0-10	154	12.81	10.31	15.91	Reference
11-20	147	5.22	4.01	6.80	0.449
21-30	147	4.53	3.47	5.91	0.007*
31-40	153	3.76	2.87	4.93	0.004*
41-50	149	6.04	4.78	7.63	0.011*
>50	150	5.57	4.47	6.94	0.001*
Total	900	5.83	5.26	6.46	

*denotes statistical significance

3.4.3 Percentages of population with different antibody levels

When samples were classified according to the antibody levels (<5 IU/ml, 5–40 IU/ml, 40–100 IU/ml, and >100 IU/ml), it was found that the majority of the population had antibody levels of 5–40 IU/ml (48.5%), followed by < 5 IU/ml (42.1%) (Table 3.4.3.1). However, 23 samples (2.6%) had antibody levels >100 IU/ml indicating a recent infection or vaccination. Of these 23 samples, 9 were aged between 6 months and 5 years, 6 were aged 11–16 years, and the remaining were >21 years. Among 61 subjects with antibody levels between 40 and 100 IU/ml, 18 were between 0–10 years and 43 were > 11 years.

Table 3.4.3.1: Percentages of population with different antibody levels

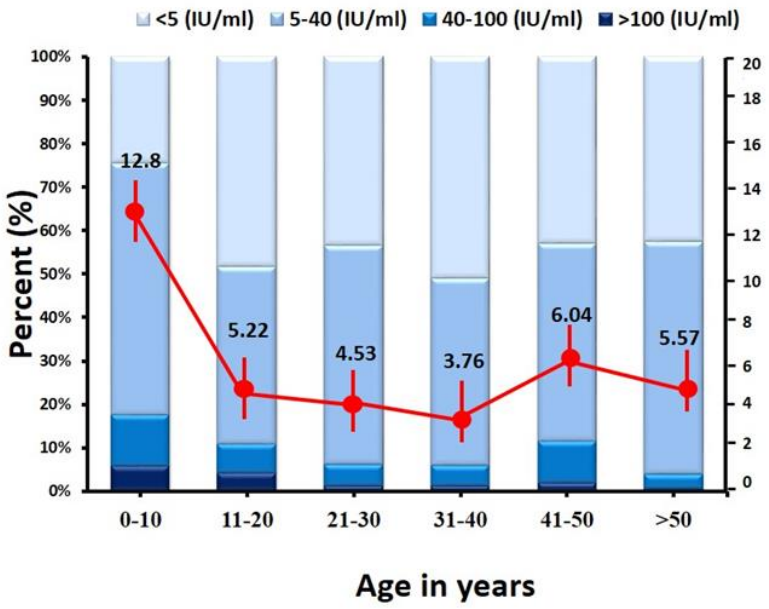
Anti-PT IgG (IU/ml)	No. of participants	Percent (%)	95% CI of Percent	
			Lower	Upper
<5	379	42.1	38.9	45.4
5-40	437	48.5	45.2	51.9
40-100	61	6.8	5.2	8.6
>100	23	2.6	1.6	3.8

3.4.4 Seronegativity rates by age groups

Comparison of the seronegativity rates by age group (Fig 3.4.4.1) revealed that children aged <10 years had the lowest proportion of seronegativity (24.7%), which was significantly different compared with the other age groups (p < 0.01). The highest

seronegativity rate was observed in the 31–40 years age group (51%), followed by the 11–20 years (48.3%), 21–30 years (43.5%), 41–50 years (43%), and >50 years (42.7%) age groups. The seronegativity rates among people residing in different provinces were not statistically different.

Figure 3.4.4.1: Age-specific anti-PT level and GMC. Scale on the left represented the percentage of population with different antibody levels. Scale on the right represented the GMC in each age group with the means indicated as red dots (red vertical lines denote 95% CI). Antibody measurements were <5 IU/ml (very light blue), 5–40 IU/ml (light blue), 40–100 IU/ml (blue) and >100 IU/ml (dark blue).



3.5 Discussion

The result of this population-based cross-sectional seroepidemiology survey of anti-PT in the Thai population showed that the lowest seronegativity rate and the highest GMC were found in the 0–10 year age group, corresponding to the most recent pertussis vaccinations. The proportion of seronegativity rate increased from 11 years of age onwards, reflecting that wP vaccine-induced antibody to PT also do not last long. In addition, among elderly population who were born before pertussis vaccine was integrated into the EPI, the

seronegative rate was similar to adolescents. These observations suggest that even in the pre-vaccination era when the population was exposed to *B. pertussis*, anti-PT induced by natural infection was not life-long.

Serosurveys in countries using wP vaccine have also reported waning of antibody levels. A study in Iran involving 640 students aged 6–17 years showed that 53% were seronegative and susceptible to pertussis despite five-dose wP vaccination during childhood [(70)]. Another study in Pakistan demonstrated that 77.4% of children 10–12 year-old who were vaccinated with wP vaccine at 6, 10, and 14 months of age were seronegative [(71)]. However, antibody level alone may not reflect the overall protection against pertussis. Previous studies have shown that, despite the waning of antibody, the clinical efficacy of wP vaccine was as high as 92% at six years after the last dose [(72)].

Recent clinical studies reported that both aP and wP vaccine-induced immunity is not life-long [(8, 66)]. It was estimated that the protection obtained from wP vaccine is 5-14 years [(73-75)], and the protection obtained from aP vaccine lasts only 4-7 years [(76, 77)]. The Advisory Committee on Immunization Practices (AICP) recommends a booster dose of the Tdap vaccine during adolescence since 2006, regardless of the type of pertussis vaccine received during infancy [(11)]. This study provided evidence of low anti-PT IgG level in the Thai population after 11 years of age and suggested an inclusion of a pertussis booster dose for Thai adolescents in order to increase antibody level and provide better protection against pertussis.

This study demonstrated that there were 23 samples with anti-PT IgG >100 IU/ml. Of these 23 participants, 9 were aged between 6 months and 5 years, indicating the effect of childhood vaccination, while the remaining 14 participants were aged above 11 years. As the accurate vaccination data of the participants were not available, a strong conclusion cannot be made as to whether the high anti-PT titers resulted from recent vaccination or recent exposure

to *B. pertussis*. However, due to the very low coverage of Tdap vaccination among Thai adolescents and the last doses of wP according to the Thai EPI were given before six years of age, it is possible that 14 participants may have been exposed to *B. pertussis*, resulting in the high antibody titers. In addition, among 61 participants whose antibody concentrations were between 40–100 IU/ml, 43 were aged > 11 years and likely represented exposure to *B. pertussis* in the past years. These observations suggest that despite the high coverage of wP vaccine in Thailand for many years, *B. pertussis* continued to circulate in the Thai population.

In conclusions, a booster dose during adolescence should be considered for better protection against pertussis among the Thai population. Further studies should also be conducted to explore how long the booster vaccine can provide protective immunity.

Chapter 4

Antibody to *Bordetella pertussis* antigens in Thai pregnant women without pertussis vaccination during pregnancy

4.1 Introduction

Countries with universal pertussis vaccination have experienced pertussis resurgence in recent years especially those implementing the acellular pertussis (aP) vaccines [1-3]. Recent evidence suggests that this was partly due to the waning immunity as a result of the aP-vaccine use [10]. Infants are at highest risk for severe complications and pertussis-related death. Maternal vaccination during pregnancy is an effective strategy to prevent pertussis-related morbidity and mortality in newborns [78]. Vaccination during pregnancy boosts the immune response against *B. pertussis* in expectant mothers and affords the transplacental transfer of antibodies to the baby, thus conferring protection to pertussis in infants during the first few months of life [15, 62, 63].

Thailand implemented a routine infant immunization program with two doses of the DTwP vaccine beginning in 1977. From 1992 onward, the country's Expanded Program on Immunization (EPI) offers five doses of DTwP to infants at 2, 4, 6, 18 and 48 months of age. A booster dose of Tetanus Toxoid (TT), which has since been replaced by diphtheria–tetanus vaccine (dT) in 2012, is also recommended during adolescence and every 10 years thereafter. Since then, data from the national passive surveillance showed a relatively low burden of pertussis in the general population ranging between 0.01-0.04 per 100,000 individuals between 2007-2014 [56]. In 2015 and 2016, however, there was an increased incidence of 51 cases (0.08/100,000) and 72 cases (0.11/100,000), respectively [57]. Significant numbers of morbidity belonged to children one year of age or younger and this age group bears the

greatest risk of pertussis-related morbidity. Between 2013 and 2015, 3 pertussis-related deaths were reported.

During the antenatal care visits, pregnant women are asked by their health care providers regarding the history of tetanus immunization during childhood. If they had not received any tetanus toxoid vaccine or are unsure about their vaccination history, dT will be given at the first visit and subsequently at 1 and 6 months apart. If they had received some doses or had not yet completed the recommended vaccine series, they will be provided a booster vaccination. Although Tdap vaccination is better as the additional acellular pertussis (aP) component offers protection from whooping cough, Thailand has not yet implemented such vaccine into the national vaccination program due to the budget constraint and the limited data on the interference of maternal antibody in wP-vaccinated infants.

4.2 Aim of study

The resurgence of pertussis among Thai infants in recent years warrants the need to examine the pre-existing antibodies to *B. pertussis* antigens in pregnant women. This study aims to determine the baseline concentration of anti-PT, anti-FHA and anti-PRN in pregnant women who did not receive pertussis vaccination during pregnancy. Data from this study may be important in providing evidence-based consideration for a pertussis booster during pregnancy.

4.3 Study design and laboratory methods

4.3.1 Study population

This study was approved by the Institutional Review Board of the Faculty of Medicine of Chulalongkorn University (IRB No. 154/58). The serum samples were archived residual samples from 90 mother-cord blood pairs collected between July 2011 and August 2012 to examine serological protection against tetanus among pregnant women at King

Chulalongkorn Memorial Hospital in Bangkok. All samples were deidentified and anonymous, therefore no consent was required and the permission to use these samples was granted by the Director of King Chulalongkorn Memorial Hospital. Inclusion criteria were healthy pregnant Thai women between 15 and 45 years who sought antenatal care at King Chulalongkorn Memorial Hospital in 2011 and 2012. During their first visits, history of tetanus immunization and blood samples were obtained. Cord blood samples which served as a surrogate for infant blood samples were collected at birth. None of the mothers in this study were able to provide their vaccination records, therefore women born prior to 1977 were assumed to have never received DTwP, while those born after 1977 were assumed to have had between 2 and 5 doses of DTwP.

4.3.2 Measurement of antibodies

Anti-PT, anti-FHA and anti-PRN IgG were analyzed quantitatively using commercial ELISA kits (EUROIMMUN, Lübeck, Germany) according to the manufacturer's instructions. The antibody level was quantified in international unit per milliliter (IU/ml). Sera were initially diluted 1:101 for the test and higher dilutions were performed as necessary. The lower limit of quantification (LLOQ) for anti-PT, anti-FHA and anti-PRN IgG is 5 International Units (IU) per ml of serum. Values below LLOQ were calculated as half of the LLOQ. For anti-PT, Values <5 IU/ml were interpreted as seronegative, 5-40 IU/ml as no evidence of recent acute infection, 40–100 IU/ml as probable past exposure to pertussis, and >100 IU/ml as acute pertussis infection or recent vaccination.

4.3.3 Statistical analysis

The IgG level was expressed as geometric mean concentrations (GMC) with standard error of the mean (SE). Data were analyzed using SPSS software (IBM Inc., Armonk, NY, USA), SigmaPlot (Systat Software, San Jose, CA) and R statistical software. Chi square and Fisher's exact test were used for statistical comparisons of seronegativity rates of pregnant

women born before or after the pertussis inclusion in the EPI. Linear regression model was used to predict antibody levels in cord sera. A simple and multivariable regression models were used to analyze the predictors affecting the antibody level in the cord blood.

4.4 Results

4.4.1 Demographic characteristics

There were 90 mother-infant blood pairs tested. The majority of maternal blood samples were obtained during the first trimester of pregnancy as defined by the gestational age (GA) at less than 12 weeks (Table 4.4.1.1). All babies except one were born healthy. One baby born at GA of 23 weeks died hours after birth due to multiple congenital anomalies.

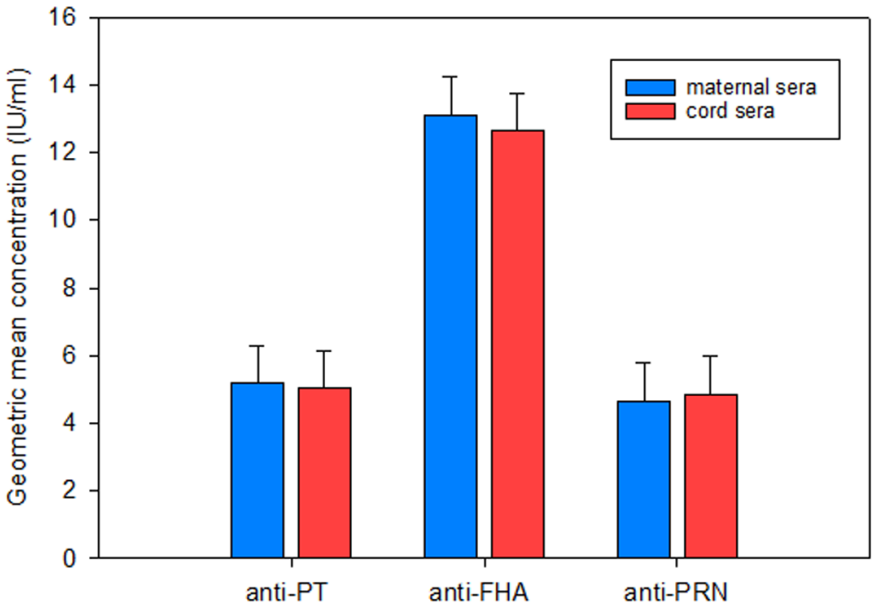
Table 4.4.1.1: Demographic characteristics of pregnant women and infants in the study.

Characteristics	
Number of pregnant women	90
Mean age in years (range)	30.9 (19-42)
No. of participants whose maternal sera were collected during	
- First trimester (GA≤12 weeks)	65 (72%)
- Second trimester (GA13-28 weeks)	24 (27%)
- Third trimester (GA≥29 weeks)	1 (1%)
Mean GA at maternal blood collection in weeks (range)	11 (5-29)
Mean GA at delivery in weeks (range)	37.6 (23-40)
Infant birth weight in grams (range)	2,952 (980-4,060)
Percentage of premature delivery (GA<37 weeks)	11.1%
Percentage of twins	3.3%

4.4.2 GMC of anti-PT, anti-FHA and anti-PRN IgG

The GMC of anti-PT, anti-FHA and anti-PRN IgG in maternal and cord sera were demonstrated in Figure 4.4.2.1. Maternal and cord sera samples demonstrated similar anti-PT and anti-PRN levels, both of which were lower than that of anti-FHA. When values of > 10 IU/ml were accepted as potential protective concentrations, the percentages of unprotected infants were 73.3%, 43.3% and 75.5% for anti-PT, anti-FHA and anti-PRN IgG, respectively.

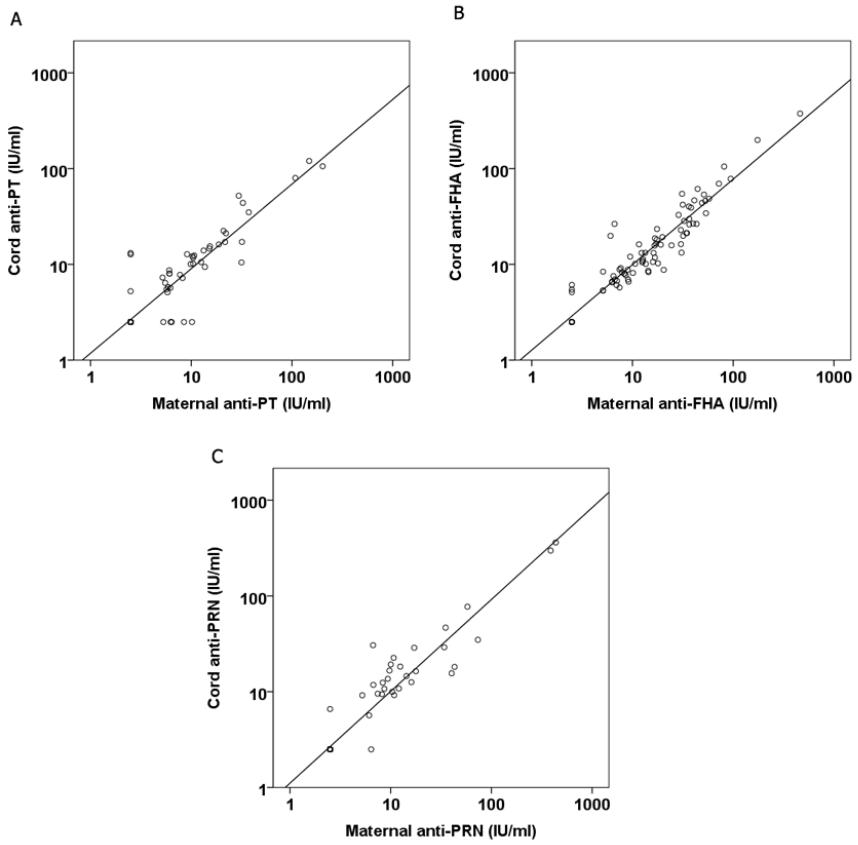
Figure 4.4.2.1: Maternal and cord anti-PT, anti-FHA and anti-PRN IgG. Values are expressed as GMC in IU/ml. Error bars indicated the standard error (SE) of the mean. Mean±SE (IU/ml) for each concentration were: anti-PT in maternal sera (5.19±1.11), anti-PT in cord sera (5.05±1.11), anti-FHA in maternal sera (13.10±1.13), anti-FHA in cord sera (12.64±1.12), anti-PRN in maternal sera (4.65±1.12), and anti-PRN in cord sera (4.84±1.12).



4.4.3 Correlation and effect of maternal on cord antibody levels

Comparison of all 90 mother-infant paired samples showed that maternal anti-PT, anti-FHA and anti-PRN IgG correlated significantly with cord blood values (Figure 4.4.3.1). To determine factors affecting the antibody level in the cord sera, two models were tested. Using the bivariable model, which assumed that levels of anti-PT, anti-FHA and anti-PRN in cord sera were entirely attributed to the maternal sera, it was found that antibody levels in maternal sera significantly affected the levels in cord sera ($p < 0.001$). This model would account for 88.4, 89.2 and 95.9% of the variations in cord anti-PT, anti-FHA and anti-PRN IgG, respectively. Using the multivariable model, which took the gestational age at delivery and the interval between maternal and cord sera collection into consideration, neither parameters were significantly associated with the level in cord sera and did not confound the effect of maternal sera.

Figure 4.4.3.1: Simple linear regression model of IgG to (A) PT, (B) FHA and (C) PRN in maternal and cord sera. R^2 for anti-PT = 0.854, $p < 0.001$; R^2 for anti-FHA = 0.894, $p < 0.001$; R^2 for anti-PRN = 0.913, $p < 0.001$.



4.4.4 Proportion of seronegative rates among women born before and after EPI

Anti-PT IgG levels can reflect not only the circulating antibody from past vaccination but also the recent exposure to *B. pertussis*. When the levels were classified as <5 IU/ml (seronegative), 5–40 IU/ml (no evidence of recent infection), 40–100 IU/ml (probable past exposure to pertussis) and >100 IU/ml (acute or recent infection or vaccination). It was found that 56.7% of pregnant women were seronegative for anti-PT. Forty percent of the maternal serum samples did not show evidence of recent infection, as defined by antibody levels between 5-40 IU/ml. Three women (aged 19, 28, and 40 years) possessed anti-PT IgG

titers of >100 IU/ml, which represented 3.3% of the samples. Two-thirds of these high anti-PT IgG samples also possessed >350 IU/ml of anti-PRN IgG, while one-third had >350 IU/ml of anti-FHA IgG, suggesting possible recent infection. There were no statistically significant differences in the proportion of anti-PT IgG seronegativity or recently infected rates in women born before or after the EPI program of pertussis vaccine (Table 4.4.4.1).

Table 4.4.4.1: Anti-PT IgG in pregnant women born before or after the implementation of pertussis vaccination.

Anti-PT IgG (IU/ml)	Before EPI (aged ≥ 34 years) (n=30)	After EPI (aged < 34 years) (n=60)	*p-value*
<5	16	35	0.660
5-40	13	23	0.656
40-100	0	0	-
>100	1	2	1.000

4.5 Discussion

The increase in the prevalence of reported pertussis morbidity among infants in Thailand in recent years warrants the need to examine the level of antibody to *B. pertussis* antigens in maternal and cord blood paired samples in order to determine serological baseline. Despite receiving wP vaccination as children, most pregnant women in this study demonstrated low anti-PT, anti-FHA and anti-PRN IgG levels. Similar low titers were found in corresponding cord serum samples. This finding suggests that a significant number of women and their newborn are susceptible to pertussis.

Previous studies examining level of anti-PT IgG in pregnant women revealed that the GMC of anti-PT detected at delivery ranged between 2.4-19.39 ELISA Units or IU/ml [79-88]. Although the GMC of 5.18 IU/ml in this study was towards the low side of this range, it was

similar to the values reported from the nationwide seroprevalence survey of anti-PT IgG in 2014 [65]. In that study, the GMCs of anti-PT IgG were 4.53 and 3.76 IU/ml among 21-30 and 31-40 year-olds, respectively. This reflects the susceptibility to pertussis among women of child-bearing age in Thailand and supports the potential benefit of a booster during pregnancy.

FHA is a cell surface protein found in many bacteria including *Bordetella pertussis*. It plays a key role in bacterial adhesion to epithelial cells and thus contributes to its pathogenesis. Previous studies have shown that women who did not receive pertussis vaccination during pregnancy had anti-FHA IgG ranging from 6.9 to 26.6 ELISA units or IU/ml [79-82, 84, 86, 88]. The results of this study were consistent with those previously reported values. In addition to PT, PRN is another virulence factor which promotes bacterial adhesion to epithelial cells and antibody to PRN facilitates phagocytosis by polymorphonuclear cells [89]. Anti-PRN IgG level were found to be correlated with protection against pertussis. The levels of anti-PRN IgG in this study were consistent with those reported in recent studies, which found that the levels in maternal sera were between 4.09-13.5 ELISA units or IU/ml [79, 81, 86-88].

It has been reported that approximately 1.8-6.3% of pregnant women possessed anti-PT IgG levels of >100 IU/ml, which indicated recent pertussis infection [90-92]. These women were presumed to be protected from pertussis during pregnancy and were able to transfer anti-PT IgG to their fetus. With the assumption of vaccination based on age, but without available vaccination record or clinical confirmation of pertussis, it can be concluded that 3.3% of mothers with anti-PT IgG titer >100 IU/ml in this study had recently been exposed to *B. pertussis*. For 30-40% of pregnant women who had anti-PT IgG titers between 5-40 IU/ml, the possibility of waning immunity associated with *B. pertussis* exposure sometime in the past few years cannot be eliminated. If this was indeed representative of the whole

population, then it is plausible that *B. pertussis* exposure is not uncommon despite universal childhood vaccination in Thailand.

Previous studies suggested that transplacental transfer of antibodies occurs via active transport, which result in the increased level of antibodies in the cord blood at term when compared to maternal sera [80, 84, 93]. During early infancy, protection from diseases often relies on the passively transferred maternal antibodies to newborn infants. In this study, it was demonstrated that the majority of Thai infants were unlikely to be protected from pertussis despite the implementation of childhood pertussis vaccination program in the mothers. Although the increase in reported pertussis incidence seen in very young children may reflect better disease awareness by clinicians, it may also reflect the under-diagnosis of pertussis in adults and the waning of the immunity against pertussis in the general population.

Future work to assess the effect of a booster dose of pertussis vaccination among pregnant women in terms of antibody to *B. pertussis* antigens, the extent of maternal-fetal antibody transfer, and long term immunity in Thai infants induced by whole cell or acellular pertussis vaccine is ongoing. Nevertheless, the results of this study explained the susceptibility for pertussis among newborn infants in Thailand and supported the requirement for a pertussis booster vaccine during pregnancy which may provide passive seroprotection in newborns during the first months of life.

Chapter 5

Evaluating reactogenicity profile and antibody to *Bordetella pertussis* antigens at delivery in Tdap-vaccinated pregnant women

5.1 Introduction

Pertussis is a respiratory disease caused by the Gram negative bacterium *Bordetella pertussis (B. pertussis)*. Although pertussis is a vaccine-preventable disease, it remains prevalent in both developed and developing countries despite the implementation of vaccination efforts worldwide. Severe morbidity and mortality associated with pertussis often occurs in infants and young children [(5)]. Current pertussis immunization strategies fail to protect infants who are too young to complete primary series of pertussis vaccination. These infants are susceptible to severe pertussis-related complications and even death due to the lack of protective immunity.

Passively acquired maternal *B. pertussis*-specific antibodies show low concentrations in newborns and their presence in infants is short-lived, despite an active transplacental transport [(79)]. A previous study suggested that significantly higher titers of *B. pertussis*-specific antibodies in cord blood were detected in infants born to Tdap–vaccinated women compared to unvaccinated women [(16)]. As a consequence, a number of countries including, but not limited to, the UK, the USA, Spain, Italy, Belgium and Argentina have implemented Tdap vaccination during pregnancy in their national immunization programs in order to increase the maternal *B. pertussis*-specific antibody level that will be transplacentally-transferred to protect the newborn [(18, 60, 94)].

Low antibody titers to *B. pertussis* antigens among Thai pregnant women warrant the need to assess the effect of a booster dose of tetanus, diphtheria and acellular pertussis vaccination (Tdap) during pregnancy. In addition, the local and systemic adverse events

following Tdap vaccination in women who recently received tetanus vaccination are evaluated.

5.2 Aim of study

This study aims to evaluate the reactogenicity of Tdap vaccine from the randomized controlled clinical trial involving Tdap-vaccinated Thai mothers, and describe the amount of antibodies to *B. pertussis* antigens in sera and paired cord blood samples. The adverse events and pregnancy outcomes when multiple tetanus vaccines are administered were also assessed.

5.3 Study design and laboratory methods

5.3.1 Study design

This study was conducted according to the Declaration of Helsinki and Good Clinical Practice Guidelines (ICH-GCP) with the approval of the Institutional Review Board of the Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand (IRB no. 604/57) and the ethical committee of the University of Antwerp, Belgium. Written informed consent was obtained from all pregnant women prior to enrolment.

A randomized controlled clinical study involved Thai pregnant women who were offered a Tdap vaccine (Boostrix®, GlaxoSmithKline Biologicals, Rixensart, Belgium) between 27 and 36 weeks of gestation following the ACIP recommendation [17]. Healthy pregnant women aged 18-45 years with low obstetrical risks were recruited during routine antenatal visits at King Chulalongkorn Memorial Hospital, Bangkok, Thailand during April 2015-September 2016. Full inclusion and exclusion criteria are listed in section 9.1. All healthy infants born after 36 weeks of gestation and with birth weight greater than 2,500 grams were included in the follow up study (ClinicalTrial.gov NCT02408926). Blood samples (10 ml) were collected from the participating women at delivery to measure vaccine-induced antibody to *B. pertussis* antigens. Cord blood (10 ml) were also collected after

delivery. Maternal and cord samples were kept for no more than 24 hours in the refrigerator until centrifugation and freezing of serum samples were performed.

5.3.2 Study vaccine

The Tdap vaccine (Boostrix®) contains 2.5 limit of flocculation units (Lf) of diphtheria toxoid, 5 Lf of tetanus toxoid, 8 µg of inactivated Pertussis Toxin (PT), 8 µg of formaldehyde-treated Filamentous Hemagglutinin (FHA) and 2.5 µg of formaldehyde-treated Pertactin (PRN). Each 0.5-ml dose contains aluminium hydroxide as adjuvant. The vaccine was given intramuscularly in the musculus deltoideus to each pregnant woman by a nurse or medical doctor. Three hundred and twenty seven women were given the Boostrix vaccine lot number AC37B175AQ. Twenty nine, nine, four and one pregnant women received the boostrix vaccine lot number AC37B175AI, AC37B211AH, AC37B218BN and AC37B164AF, respectively. One vaccine lot for all pregnant women was planned but it was not possible because the recruitment period was beyond the vaccine expiry date.

5.3.3 Safety and reactogenicity assessment

Injection site and systemic reactions were assessed in all women by 30-minute observation and a 7-day telephone call follow-up. The trained research nurses made phone calls on day 2 and day 7 after vaccination to ask for the presence of solicited local (redness, pain, induration) and systemic (fever) adverse events (AE). Other AE and the general well-being of participants after vaccination were documented. In addition, participants were informed that they can call back anytime when any AE occurred. If the team captured any AE, information regarding severity and duration was recorded and daily phone call follow up made until symptom resolution. Serious adverse events (SAE) and pregnancy outcome were recorded in all participants. AE and SAE relationship to Tdap vaccine were determined by the investigators and the data safety monitoring board.

5.3.4 Laboratory testing

Anti-PT, anti-FHA and anti-PRN IgG were analyzed using a commercial ELISA kit (EUROIMMUN, Lübeck, Germany) according to the manufacturer's instructions. The ELISA kits used in this study were calibrated based on the World Health Organization (WHO) international standards. The values were expressed in International Units (IU) per milliliter. Sera were initially diluted 1:101 as written in the manufacturer's protocol. However, sera samples with titers above upper limit of detection were further diluted until they were within ranges of detection. Samples with values below the lower limit of detection (< 5 IU/ml) were calculated as 50% of the cut-off values (2.5 IU/ml). There were some samples that were not processed within 24 hours after collection, and the data were analysed, therefore, in two separate sets; according to the protocol *versus* all available data. Since there were only trivial differences in the two data set, only all data (intention-to-treat analysis) are shown.

5.3.5 Statistical analysis

The number of pregnant women in this study was calculated based on the estimation of possible interference of maternal anti-PT in wP-vaccinated children [(95)] (significance level=0.05, power =0.90). The interference of maternal antibodies in wP-vaccinated children was assessed and results were provided in the next chapter. The IgG levels were expressed as geometric mean concentrations (GMC) with 95% confidence interval. Data were analyzed using SPSS software version 24 (IBM Inc., Armonk, NY, USA) and R statistical software version 3.3.3. Graphs were created in Sigma plot software version 13. Pearson's correlation was used to show the relationship between maternal and cord antibody titers. The conventional *t*-test was performed on the antibody logarithmic scales to compare the GMC and cord/maternal ratios in pregnant women who received Tdap before and after 30 weeks

gestation. Mann-Whitney U test was used to compare the duration of solicited AE in women with or without prior tetanus immunization. Assessment of factors affecting cord antibody levels was performed using a regression approach with the log-transformed values as the outcome. There were three steps: (1) variable selection using random forest; (2) backward model selection based on Akaike Information Criteria (AIC) using multiple linear regression and (3) further model reduction using likelihood ratio tests. This procedure was used for model building [96]. Antibody titers below the detection limit (censoring observations) and two extreme outliers of anti-PRN cord-to maternal ratios were excluded.

5.4 Results

5.4.1 Demographic characteristics of pregnant women and infants

A total of 631 pregnant women were screened, of whom 370 were enrolled and vaccinated with Tdap vaccine. There were 297 (80.3%) maternal blood samples collected at delivery, of which 278 samples were processed as stated in the protocol. There were 284 (76.7%) cord blood collected at delivery, of which 270 samples were processed as stated in the protocol. Demographic and baseline characteristics of the women in the study can be found in Table 5.4.1.1.

Table 5.4.1.1: Descriptive characteristics of participants in this study.

Characteristics	Pregnant women (n=370)
Mean age (SD)	28.9 years (5.5)
Mean gestational age at vaccination (SD)	30.7 weeks (2.3)
Mode of delivery	
Vaginal	209 (56.5%)
Cesarean	158 (42.7%)
No information	3 (0.8%)
Average days between vaccination and delivery (SD)	54.1 days (18.8)
Gestational age at delivery	
< 37 weeks	25 (6.7%)
≥ 37 weeks	344 (93.0%)
No information	1 (0.3)
Mean infant birth weight (SD)	3087.6 grams (416.2)

5.4.2 Reactogenicity profile and pregnancy outcome after Tdap

No immediate reactions were observed during the 30-minute post vaccination. Pain was the most common AE after Tdap vaccination as it was reported in 76.2% of pregnant women. Most of the women reported mild pain which resolved in a few days. Low grade fever was the second most common AE which was reported in 5.1% of women. Swelling and redness were infrequent and resolved within a few days. Duration of each solicited AE can be found in section 9.2.1-9.2.4. Table 5.4.2.1 summarizes the AE and SAE recorded in this study. Regarding the SAE, 37 obstetrical, 4 fetal and 47 neonatal SAE were recorded. Of the 6.7 % preterm deliveries, 84 % were late preterm deliveries (GA 34-37 weeks). There were 2

exclusions due to fetal demise. None of the AE was related to the study procedures, in the opinion of the investigators and the data safety monitoring board of the study.

Table 5.4.2.1: Summary of the adverse events (AE) and the severe adverse events (SAE) reported among Tdap-vaccinated pregnant women and neonates in this study.

	Type of reaction	Description	No. (%)	Reported among 4,636 deliveries at KCMH in 2015 (%)
AE	Localized to the injection site	- Pain total	282 (76.2)	
		Mild	231	
		Moderate	51	
		Severe	0	
		- Swelling total	15 (4.1)	
		Mild	15	
		Moderate	0	
		Severe	0	
		- Redness total	5 (1.4)	
		Mild	5	
		Moderate	0	
		Severe	0	
	Systemic	- Low grade fever	19 (5.1)	
		- Upper respiratory tract infection	1 (0.3)	
		- Uterine contraction	1 (0.3)	
		- Rash	1 (0.3)	
		- Itchiness	1 (0.3)	
		- Vertigo	1 (0.3)	
		- Vomiting	1 (0.3)	
		- Chest discomfort	1 (0.3)	
	Others	-Gestational diabetes mellitus	10 (2.7)	8.8
		-Gestational hypertension	5 (1.4)	1.9
		-Thrombocytopenia	1 (0.3)	0.1
		-Oligohydramnios	3 (0.8)	0.8

SAE	Obstetrical	- Premature delivery	25 (6.7)	12.8
		- Premature contractions resulting in hospitalization	3 (0.8)	N/D
		- Chorioamnionitis	2 (0.5)	0.4
		- Psychosis at delivery	1 (0.3)	N/D
		- Severe pre-eclampsia	4 (1.1)	2.1
		- HELLP[a] syndrome	1 (0.3)	2.1
		- Urinary tract infection	1 (0.3)	2.0
	Fetal	- Fetal death	2 (0.5)	0.8
		- Congenital defects[b]	2 (0.5)	2.1[c]
	Neonatal	Neonatal birth asphyxia, No. (%)		
		- Severe birth asphyxia (APGAR score at 1 minute = 0-3)	2 (0.5)	1.3
		- Mild to moderate birth asphyxia (APGAR score at 1 minute = 4-7)	10 (2.7)	6.1
		- Prolonged hospitalization after birth[d]	35 (9.5)	21.0

KCMH = King Chulalongkorn Memorial Hospital; AE = adverse events; SAE = severe adverse events; and N/D = no data.

[a]Hemolysis, elevated liver enzyme levels, and low platelet levels.

[b]One cleft lip and cleft palate and one imperforated anus.

[c]Conditions of significant morbidity, which required medical and/or surgical treatment.

[d]Defined as hospitalization >5 days after birth as a result of an illness.

5.4.3 Prior tetanus vaccination and risk of AE

There were 181 women who had received at least one dose of a tetanus vaccine within the past five years prior to this study Tdap vaccination. Among these women, 51, 94 and 36 women received one, two and three doses, respectively. Ninety eight women received at least one extra dose of tetanus vaccine during this current pregnancy (1 dose in 37 women, 2 doses in 60 women, 3 doses in 1 woman). Administration of prior tetanus vaccine within the previous 5 years (N=181 women) or during the current pregnancy (N= 98 women) did not elicit increased incidence and severity of any solicited AE (pain, redness, swelling and fever) or result in prolonged duration of the symptoms. There was also no observed increase in the occurrence of other AE or premature delivery compared to women without prior vaccination (Table 5.4.3.1).

Table 5.4.3.1: Comparisons of AE after Tdap in women who have or have not had prior immunization with tetanus vaccine.

		Receiving tetanus vaccine in the last 5 years (N=181)*, n (%)	Not receiving tetanus vaccine in the last 5 years (N=189), n (%)
Injection site reaction	- Pain		
	Mild	113 (62.4)	118 (62.4)
	Moderate	24 (13.3)	27 (14.3)
	Severe	0	0
	- Swelling		
	Mild	9 (5.0)	6 (3.2)
	Moderate	0	0
	Severe	0	0
	- Redness		
	Mild	1 (0.6)	4 (2.1)
	Moderate	0	0
	Severe	0	0
Systemic symptoms	- Low grade fever	10 (5.5)	9 (4.8)
	- Upper respiratory tract infection	1 (0.6)	0 (0.0)
	- Uterine contraction	0 (0.0)	1 (0.5)
	- Rash	1 (0.6)	0 (0.0)
	- Itchiness	1 (0.6)	0 (0.0)
	- Vertigo	0 (0.0)	1 (0.5)
	- Vomiting	0 (0.0)	1 (0.5)
	- Chest discomfort	0 (0.0)	1 (0.5)
Serious adverse events	Premature delivery	11 (6.1)	14 (7.4)

*Including women receiving multiple doses of tetanus vaccine in this current pregnancy

5.4.4 Antibody to *B. pertussis* antigens at delivery

The GMC of anti-PT, anti-FHA and anti-PRN IgG in maternal-cord paired sera including the transplacental transferred ratios (cord/maternal) are demonstrated in Table 5.4.4.1. Anti-PT GMC was 42.9 IU/ml (95% CI 36.5-49.2) in maternal sera and 48.6 IU/ml (95% CI 42.0-55.2) in cord sera. The ratio of transplacental transfer (cord/maternal ratio) of anti-PT IgG was 118%. Anti-FHA and anti-PRN IgG level were higher than anti-PT,

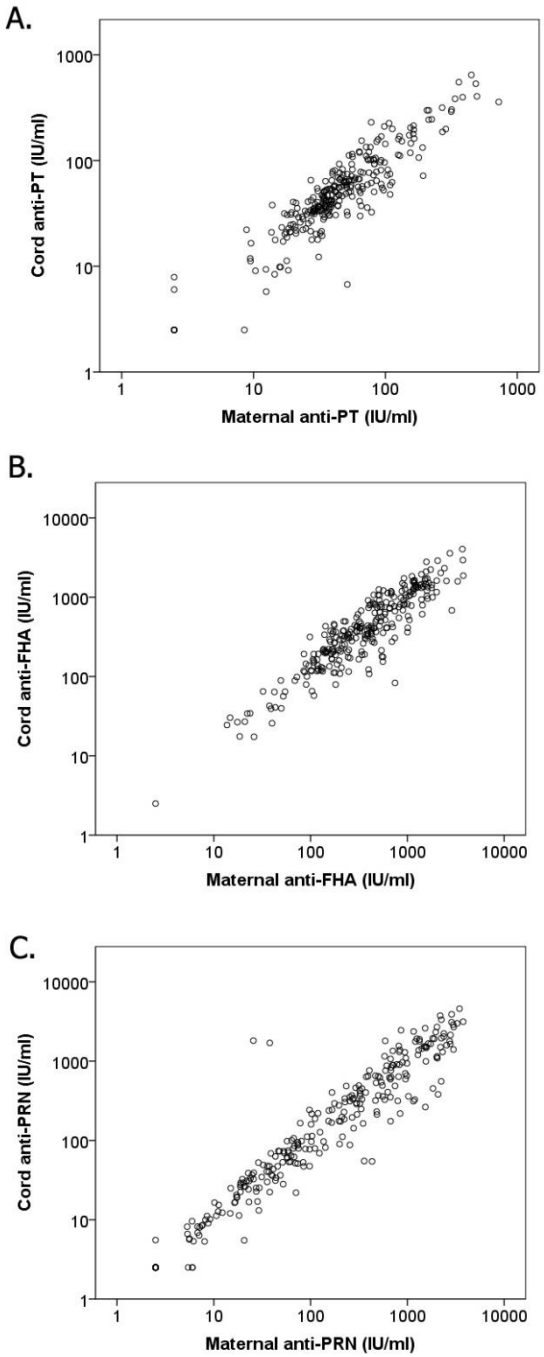
however, the ratio of transplacental transfer of anti-FHA was the same as anti-PT whereas it appeared to be lower for anti-PRN. In addition, we also observed that maternal anti-PT, anti-FHA and anti-PRN IgG significantly correlated with cord values (Fig 5.4.4.2).

Table 5.4.4.1: GMC of anti-PT, anti-FHA and anti-PRN IgG in maternal and cord blood from Tdap-vaccinated women

IgG antibodies	Maternal, IU/ml (95% CI)	Cord, IU/ml (95% CI)	Ratio cord/maternal (95% CI)
Anti-PT	42.9 (36.5-49.2)	48.6 (42.0-55.2)	1.18 (0.3-2.0)
Anti-FHA	347.4 (337.7-357.1)	383.0 (374.3-391.7)	1.18 (0.19-2.18)
Anti-PRN	125.3 (68.4-182.1)	128.8 (76.2-181.3)	1.07 (0.19-1.97)*
Total	297	284	278

* After exclusion of two extreme outliers

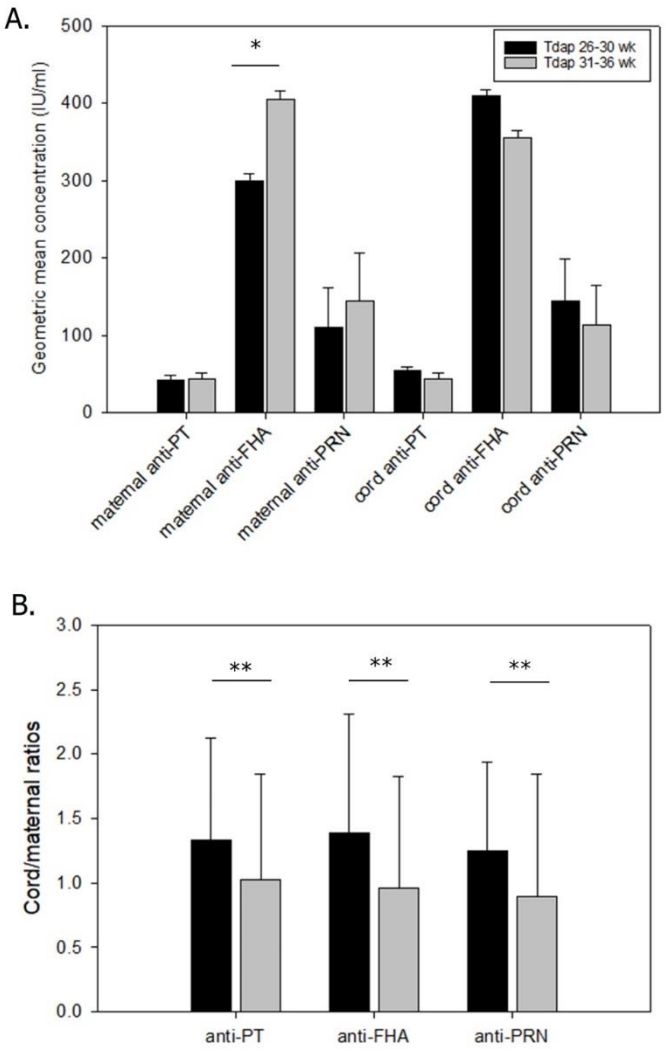
Figure 5.4.4.2: Correlations of (A) anti-PT, (B) anti-FHA and (C) anti-PRN IgG in maternal and cord sera. Pearson's correlation coefficient (r) for anti-PT = 0.89, $p < 0.001$; for anti-FHA=0.85, $p < 0.001$; and for anti-PRN = 0.86, $p < 0.001$.



5.4.5 Early *versus* late trimester vaccination

When pregnant women were divided into two groups based on their GA at vaccination. The first group (early Tdap group) received Tdap between 26-30 weeks (n=194) and the second group (late Tdap group), received Tdap between 31-36 weeks (n=175). Anti-FHA in maternal sera were significantly higher in late Tdap group compared to early Tdap group (p = 0.024, *t*-test), however, the cord/maternal ratio were significantly higher for all three *B. pertussis*-specific antibodies tested among early Tdap group as shown in Figure 5.4.5.1.

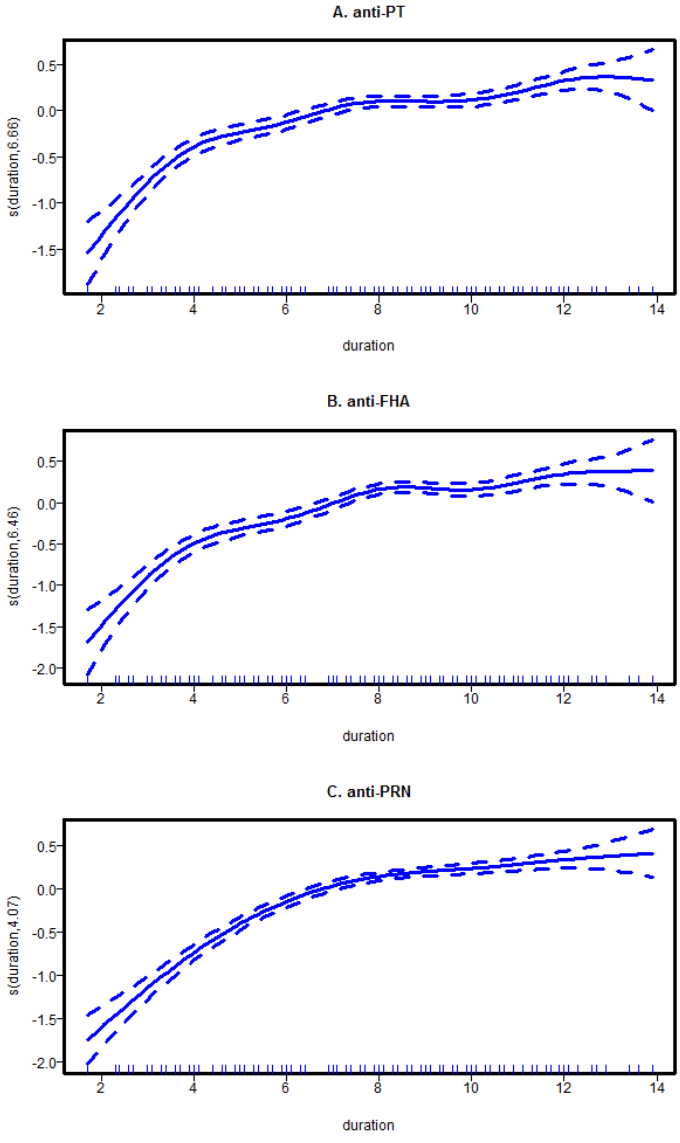
Figure 5.4.5.1: Antibody levels and cord-to-maternal ratios between early Tdap-vaccinated and late Tdap-vaccinated pregnant women. (A) Comparison of antibodies to PT, FHA and PRN. Maternal anti-FHA was significantly higher in the late Tdap group (*p = 0.024, *t*-test). Maternal and cord sera in the early Tdap group, n = 152; maternal sera in the late Tdap group, n=145; cord sera in the late Tdap group, n =132. (B) cord-to-maternal ratios were significantly higher in all three *B. pertussis*-specific antibodies among early the Tdap group (**p < 0.001, *t*- test). Sample size in the early Tdap group, n = 147; sample size in the late Tdap group, n = 131. Error bars represented upper 95% confidence intervals of each value.



5.4.6 Factors affecting cord anti-PT, anti-PRN and anti-FHA in cord sera

The random forest approach showed that the maternal antibody titers at delivery as well as the interval between Tdap vaccination and delivery (in weeks) were the most predictive factors for cord titers. The backward model selection and model reduction using likelihood ratio tests gave the same conclusion. Three separate generalized additive models (GAM) for anti-PT, anti-FHA, and anti-PRN IgG, respectively were fitted. The results showed that the maternal antibody titers at delivery significantly affect the cord titers. High maternal antibodies were related to high cord titers. Longer interval between vaccination and delivery led to higher titers in the cord blood as demonstrated in Figure 5.4.6.1. This finding suggests that vaccination at least eight weeks prior to delivery maximized antibody titers to all three *B. pertussis* antigens in the cord blood.

Figure 5.4.6.1: The relationship between the interval of vaccination and delivery (in weeks) and smoothed function of duration in generalized additive models reflecting cord (A) anti-PT, (B) anti-FHA and (C) anti-PRN. The increase in the time interval leads to the increase in the smoothed function; thus leading to higher antibody titers. It was observed that the smooth lines were steep when the interval increased between 2 and 8 weeks, but the steepness decreased between 8 and 14 weeks for all three antibodies tested. It was concluded from the graph that the best timing to maximize the cord titers was at least eight weeks prior to delivery.



5.5 Discussion

This study reported the first results of a randomized controlled clinical trial that aims to investigate the effect of Tdap vaccination during pregnancy on infant immune responses to aP and wP vaccines. The most important finding from the maternal and cord results is that Tdap vaccination at early third trimester (26-30 weeks) resulted in significantly higher cord to maternal GMC ratios compared to late third trimester vaccination (31-36 weeks). This is in agreement with other studies that show the benefit of early vaccination. A study from Switzerland reported that anti-PT and anti-FHA in cord sera were higher in second trimester vaccination (up to 26 weeks of gestation) [(97)] compared to third trimester vaccination. Abu raya et al. also investigated the potential difference of antibody levels among women vaccinated at 27-30 weeks *versus* more than 31 or 36 weeks. They also observed significantly higher anti-PT and anti-FHA in cord sera when vaccinating at 27-30 weeks gestation [(98)]. This study did not demonstrate the significant difference in cord titers among early and late Tdap group but highlighted the monotonic non-linear relationship between interval of vaccination-delivery and cord titers and demonstrated that the maximum antibody transfer occurs when women were vaccinated at least eight weeks prior to delivery.

This study demonstrated a lesser gradient of transplacental transferred ratios of all *B. pertussis*-specific antibodies tested when compared to the study by Muñoz et al from the USA [(15)] and Maertens et al from Belgium [(14)], but comparable anti-FHA cord/maternal ratios were reported from the study in Vietnam, a neighboring country of Thailand [(88)]. In the Belgian and Vietnamese pregnant women cohorts, researchers found higher avidity of anti-PT IgG in maternal and cord sera from Belgian mother-infant pairs compared to Vietnamese mother-infant pairs. Thus, the efficiency of placental transfer may increase as a result of higher antibody avidity [(99)]. Avidity of the antibodies can be influenced by several factors such as number of previous vaccine doses, types of vaccine and natural exposure to the

bacteria, and this might influence the transport rate through the placenta and result in different cord/maternal ratios between different cohorts. In addition, this study demonstrated that timing of vaccination also has an impact on cord/maternal antibody ratios, with significantly higher values similar to the US study in early third trimester vaccination [15]. There are other factors such as genetics, maternal age and existing comorbidities that can affect placental function and influence the transplancental transport. However, there were no major morbidities present in this Thai cohort.

The variation in transport ratios for the different antigens has been observed in this study as the anti-PRN cord/maternal ratio was lowest among the three *B. pertussis*-specific antibodies tested. This is in agreement with the previous studies from the USA and Belgium [14, 15]. It is possibly due to the production of variable proportions of IgG subclasses after exposure to different antigens [100]. Future studies that evaluate IgG subclasses in response to different *B. pertussis* antigens will probably shed some lights to the difference in transplacental-transferred ratios of the antibodies.

Apart from the antibody levels at delivery, the results of this study depict that Tdap vaccination given after prior tetanus-containing immunizations in the same pregnancy does not elicit more local or systemic adverse reactions nor affects the pregnancy outcomes compared to women without prior tetanus immunizations. Moreover, local injection site reactions and systemic symptoms reported in this pregnant women cohort were mild and resolved within a few days. Previous literature also supports that Tdap vaccine receipt during pregnancy is not associated with increased risk of acute local and systemic reactions or adverse pregnancy outcomes in women receiving multiple doses of tetanus vaccine in a short period of time [101, 102].

Regarding the effect of Tdap during pregnancy on obstetrical and neonatal complications in general, several studies have found that it did not increase the risk of

complications [13, 103-107], although two studies demonstrated a slight increase risk of chorioamnionitis in Tdap-vaccinated group [108, 109]. There was one study from the US which found that non-vaccinated women had higher preterm birth rates, incidence of small for gestational age, and length of neonatal hospitalization when compared to Tdap-vaccinated cohort [101]. This study also recorded the obstetrical and neonatal outcomes of Tdap-vaccinated pregnant women compared to the general background rate at the study site. It was observed that rates of obstetrical, neonatal and fetal SAE in Tdap-vaccinated pregnant women were similar or even lower compared to the general rate of AE and SAE reported at King Chulalongkorn Memorial Hospital. This is due to the fact that the baseline characteristics of enrolled Tdap-vaccinated women and other women seeking antenatal care at the study site were different. The inclusion criteria of this study strictly recruited women with low risk of obstetrical complications, and this could explain why the observed rates of adverse events were generally lower in Tdap-vaccinated group. As a tertiary care hospital and a referral center for complicated pregnancy, the incidence of obstetrical complications at this hospital is likely to be higher compared to the general Thai population.

In conclusion, this study demonstrated that Tdap vaccination during pregnancy was safe and well-tolerated even after recent tetanus immunization. The results also highlight that early vaccination resulted in high transplacental transferred antibody, which may help to protect newborn infants from pertussis during the first few months of life.

Chapter 6

Humoral immune responses in acellular *versus* whole cell pertussis vaccinated infants born to mothers who received Tdap vaccine during pregnancy

6.1 Introduction

Pertussis is a respiratory disease that is difficult to control despite the implementation of vaccination worldwide for several decades. Unvaccinated or incompletely-vaccinated infants are at the highest risk for severe outcomes including respiratory failure, encephalopathy and death. Current effort to protect young infants is maternal immunization during pregnancy. Pertussis vaccination in pregnant women induces high level of *Bordetella pertussis*-specific antibodies detectable in cord sera of the newborns and these antibodies persist at a higher level in two-month-old infants compared to those born to unvaccinated mothers [14, 15, 88]. The effectiveness of maternal immunization in protecting newborn against pertussis has been confirmed in baboon model and many human studies [62-64, 110]. Since 2012, the ACIP made recommendations to vaccinate pregnant women with Tdap during the third trimester in every pregnancy [78], and this strategy has been implemented in an increasing number of countries worldwide [18, 60, 94].

It remains unclear, however, to what extent the presence of pre-existing passive maternal antibodies may blunt the infant immune response to childhood pertussis vaccination. In a murine model, maternal acellular pertussis vaccination (aP) interfered with aP-induced immunity in the pups, but this effect was reduced when the first vaccine dose was postponed [111]. In human studies, high titers of naturally-acquired maternally-derived antibodies to pertussis toxin (PT) interfered with infant humoral immune responses to wP [112, 113], but not to aP vaccine [95] .In contrast, US researcher found that infants born to Tdap-vaccinated mothers manifested lower anti-FHA following receipt of the third dose DTaP-containing

vaccine but the effect disappeared after the administration of the fourth dose at 12 months of age [15]. Another study from Belgium also reported that there was blunting in anti-PT response after primary immunization with DTaP-containing vaccine and the blunting still persisted after the fourth dose [14, 114]. A study in Vietnam found significant interference in anti-PRN response, not to anti-PT and anti-FHA, after primary immunization but the effect disappeared after the fourth DTaP-containing vaccination [88, 115].

At the April 2014 World Health Organization meeting by the Strategic Advisory Group of Experts on immunization to prevent early mortality due to pertussis, it was concluded that findings of interference in infant humoral immunity induced by aP vaccine cannot be extrapolated to the situation of wP vaccine without additional immunogenicity data. In many countries where wP vaccines are used in infants within the Expanded Programme on Immunization (EPI), possible interference of vaccine-induced maternal antibodies has to be considered.

6.2 Aim of study

To investigate the effect of maternal immunization on humoral immune responses in wP and in aP vaccinated infants after primary series and first booster vaccination.

6.3 Study design and laboratory methods

6.3.1 Study design

This study was conducted according to the Declaration of Helsinki and Good Clinical Practice Guidelines (ICH-GCP). Written informed consent was obtained from the parents prior to infant enrolment. The Institutional Review Board of the Faculty of Medicine at Chulalongkorn University (IRB no. 604/57) and the ethical committee of the University of Antwerp (IRB no. 14/49/511) approved this study. This study enrolled 370 pregnant Thai women who visited the antenatal care clinic at King Chulalongkorn Memorial Hospital and

consented to Tdap vaccination (Boostrix®, GSK, Rixensart, Belgium) during their third trimester of pregnancy (ClinicalTrial.gov NCT02408926). Pregnant women were recruited between April 2015 and September 2016. The inclusion and exclusion criteria of pregnant women, vaccine reactogenicity, and *B. pertussis*-specific antibody titers in maternal and cord blood were previously described [(116)]. Healthy full-term and late preterm infants born at gestational age of 36 weeks with birth weight greater than 2,500 grams were randomized to receive either the aP-containing vaccine (Infanrix hexa®, GlaxoSmithKline Biologicals, Rixensart, Belgium) or wP-containing vaccine (Quinvaxem®, Crucell-Janssen, Incheon, South Korea) vaccine. Randomization was performed 1:1 with a block-size of four using a computer-generated sequence (www.randomization.com). Participants were randomized when they met inclusion criteria and parents signed the informed consent. This study was not blinded as it was impossible to do so. The wP-vaccinated infants received oral polio vaccine whereas the aP-vaccinated infants did not. Eighty full-term infants born to non-Tdap-vaccinated women were also recruited and this group received wP-containing vaccine (Quinvaxem) and was designated as EPI wP group. The EPI wP group represented infants who received pertussis vaccine according to the current Thai Expanded Program on Immunization (EPI), as Thailand has not yet implemented maternal pertussis vaccination and currently uses wP routinely in the infant immunization program.

6.3.2 Study vaccine

All infants received pertussis-containing vaccine at 2, 4, 6 and 18 months of age according to the current recommendation in Thailand. Infanrix hexa administered to infants in the aP group contains 25 µg pertussis toxoid, 25 µg filamentous hemagglutinin (FHA), 8 µg pertactin (PRN), 10 µg hepatitis B surface antigen (HBsAg), 30 IU diphtheria toxoid (DT), 40 IU tetanus toxoid (TT), 10 µg *Haemophilus influenzae* type b (Hib) polysaccharide conjugated to 25 µg of TT and 40, 8, and 32 D-antigen units of inactivated polioviruses type 1, 2, and 3, respectively. PT, FHA and PRN were adsorbed on aluminium hydroxide (0.5 milligrams Al^{3+}). HBsAg were adsorbed on aluminium phosphate (0.32 milligrams Al^{3+}). This vaccine was given intramuscularly at the anterolateral thigh.

Quinvaxem administered to infants in the wP and EPI wP groups contains inactivated *B. pertussis* at more than 4 IU/dose of potency against pertussis, 10 µg HBsAg, 30 IU of DT, 60 IU of TT and 10 µg Hib oligosaccharide conjugated to 25 µg of CRM-197, a non-toxic mutant derivative of diphtheria toxin. Aluminium phosphate (0.3 milligrams Al^{3+}) is the adjuvant. Infants in the wP and EPI wP groups received bivalent oral polio vaccine (Biofarma, Bandung Jawa Barat, Indonesia) containing $\geq 10^{6.0}$ $CCID_{50}$ poliovirus type 1 and $\geq 10^{5.8}$ $CCID_{50}$ poliovirus type 3 per 0.1 ml oral dose. Infants in the wP and EPI wP groups also received the monovalent IPV vaccine (IMOVAX polio, Sanofi Pasteur, Lyon, France) containing 40, 8, and 32 D-antigen units of inactivated polioviruses type 1, 2, and 3, respectively, at four-month vaccination visits, except two children in the wP group who reached 4 months before the national polio vaccination policy changed. This vaccine was injected separately at the anterolateral thigh.

All infants received an intradermal bacille Calmette-Guerin (BCG) vaccine and an intramuscular monovalent hepatitis B vaccine at birth. They also received Measles-Mumps-Rubella (MMR) vaccine (Priorix®, GlaxoSmithKline Biologicals, Rixensart, Belgium or M-

M-R®II, Merck & Co., Inc., New Jersey, USA) at 9 months of age and Japanese Encephalitis (JE) (CD.JEVAX®, Chengdu Institute of Biological Products, Chengdu, China) vaccine at 12 and 19 months of age according to the current national vaccine program. They also received trivalent influenza vaccine (Influvac®, Abbott Biologicals, Olst, The Netherlands) at 7 and 9 months of age. Some infants received the optional rotavirus vaccine given orally, pneumococcal vaccine or varicella zoster vaccine administered at a separate injection site. It was the parents' decision to buy rotavirus, pneumococcal and varicella zoster vaccines for their children.

6.3.3 Sample collection

In the aP and wP groups, venous blood samples (2.5 ml) were collected from the infants at two months of age before they received the first pertussis-containing vaccine, one month after the last dose of the primary vaccination series (7 months of age), at 18 months of age before they received the first pertussis booster, and one month after the first booster (19 months of age). Cord sera which were collected at delivery were also analysed together with the results of infant sera. Intervals between birth and month 2, month 2 and 4, and month 4 and 6 vaccination visits were 45 to 60 days according to the protocol. Intervals between the last dose of the primary vaccination series or first booster and blood sampling were between 28 to 35 days. In the EPI wP group, blood samples (2.5 ml) were taken from infants only at month 7 and 19.

6.3.4 ELISA for antibody to *B. pertussis* antigens

Anti-PT, anti-FHA and anti-PRN IgG were analyzed using a commercial ELISA (EUROIMMUN, Lübeck, Germany) according to the manufacturer's instructions. Experiments were performed as described in the previous chapter. Samples with values below the lower limit of quantification (LLOQ), which was 5 IU/ml, were calculated as 50%

of the LLOQ. The percentages of values below LLOQ ranged from 0.3% to 12% depending on types of antibodies and sample collection time point. Fifty percent of LLOQ (2.5 IU/ml) was used in the calculation of Geometric Mean Concentration (GMC) and the *t*-test.

6.3.5 Outcome

The primary outcome was to compare *B. pertussis*-specific antibody response after primary series and first booster vaccination among aP and wP-vaccinated infants born to mothers who received Tdap during pregnancy. The secondary outcome was to measure the effect of the pre-existing maternal antibody in aP and wP-vaccinated children.

6.3.6 Statistical analysis

Baseline characteristics of all data were reported as means and standard deviations (SD). Antibody titers were presented as GMC with 95% Confidence Interval (CI). The conventional *t*-test was used to compare parameters in the baseline characteristics and the GMC between groups using the antibody logarithmic scales. The paired *t*-test was used to compare the antibody titers on the logarithmic scale in order to make inference about the difference in GMC between month 2-7 and month 18-19 (pre-vaccination *versus* post-vaccination) infant sera.

Multiple linear regression was used to determine factors associated with the antibodies to *B. pertussis* antigens observed in infants at two months of age. Data points with both censoring (values less than the limit of detection) at cord and at month 2 were excluded from the regression analysis (2.2%, 0.3% and 3.8% for anti-PT, -FHA and, -PRN, respectively). Twelve, three and three outliers were excluded from the final multiple linear regression model for anti-PT, -FHA and -PRN antibody data, respectively, in order to satisfy the assumption of homoscedasticity (equal variances) and normality of the residuals (Table

9.2.5). A *p*-value < 0.05 was considered statistically significant. The intention-to-treat analysis in samples at all time-points was performed.

6.4 Results

6.4.1 Demographic characteristics of infants and children in the study

The consort flow diagram of the study is depicted in Figure 6.4.1.1. Healthy full-term (n=311) and late preterm infants (n=4) born at gestational age of 36 weeks with birth weight greater than 2,500 grams were randomized to receive either the aP vaccine in the form of DTaP-Hib-HB-IPV (Infanrix hexa®) or wP vaccine in the form of DTwP-Hib-HB + OPV. After enrollment, 158 infants (156 term and 2 preterm) received Infanrix hexa (aP group) and 157 infants (155 term and 2 preterm) received Quinvaxem (wP group). Seventy-nine full-term infants born to non Tdap-vaccinated women were also recruited and this group received Quinvaxem (EPI wP group). Baseline characteristics of infants in all groups are presented in Table 6.4.1.1. There were no differences in the parameters of infants between the aP and wP groups. However, the mean weight at birth of infants born in the EPI wP group was slightly larger than that of infants in the aP ($p = 0.046$) and the wP ($p = 0.019$) groups. Statistically significant differences in the mean weight were also observed for infants at 4 months of age between the EPI wP and the aP groups ($p = 0.035$). Some infants were not vaccinated according to the protocol (Table 9.2.6) as a result of illness or delayed follow-up visits by parents. Thus, some infant blood samples were not collected according to the defined timeframe in the protocol. Differences between all available data and data from infants with full protocol adherence were not significant. All available data (intention-to-treat analysis) were presented and discussed, except in the analysis of the multiple linear regression model where the adapted database was used.

Figure 6.4.1.1: The consort flow diagram. Tdap, Tetanus- diphtheria and acellular pertussis; GA, Gestational age; aP, acellular pertussis vaccine; wP, whole cell pertussis vaccine; mo, month (s). *One wP child received Quinvaxem at month 7 which was not according to the protocol. **One wP child and one EPI wP child had seizure related to high grade fever following Quinvaxem injection, therefore, wP was replaced by the less reactogenic aP.

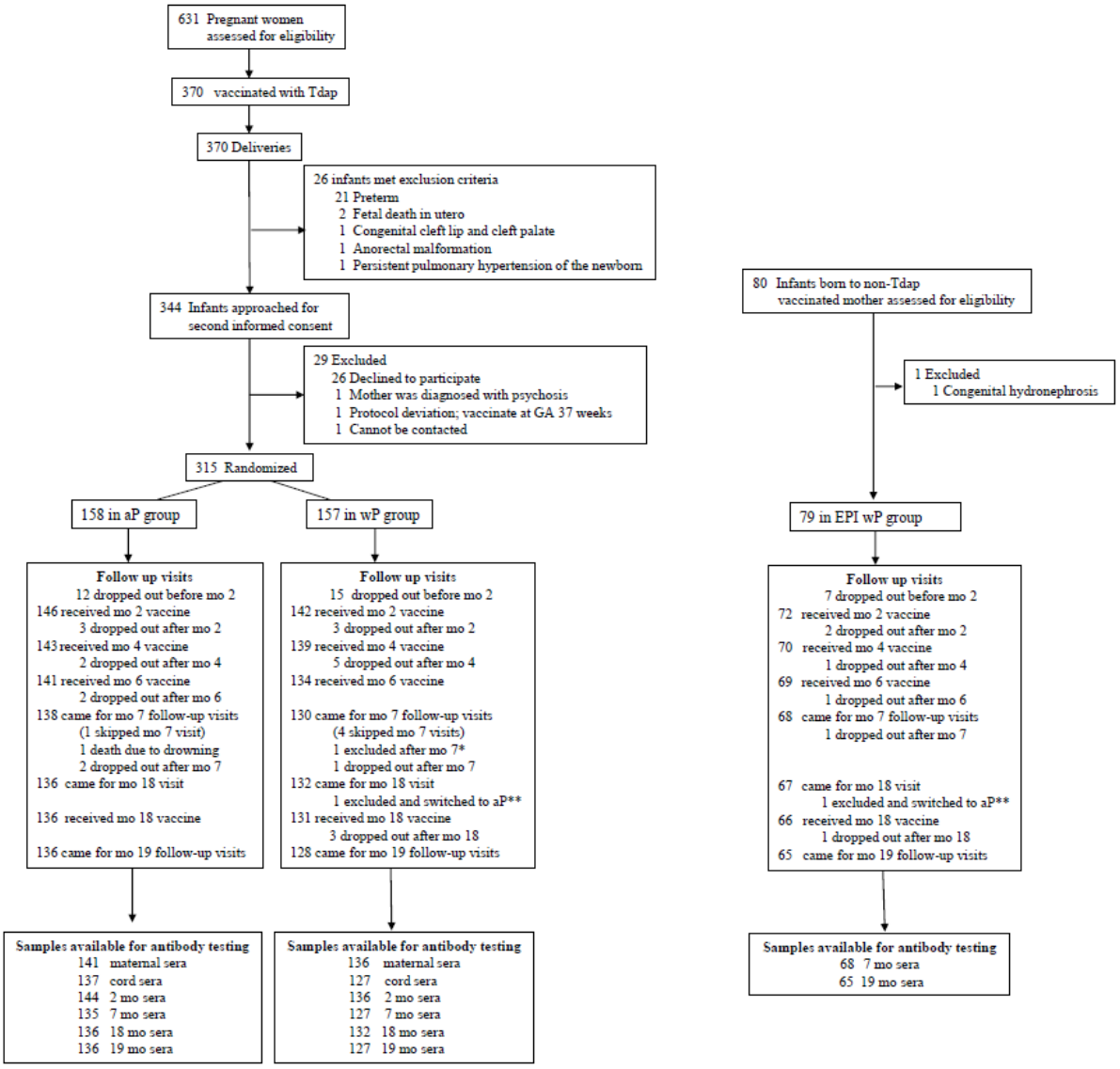


Table 6.4.1.1: Baseline characteristics of participants included in the study.

	aP (n=158)	wP (n=157)	EPI wP (n=79)
Mean GA at delivery (SD)	38.7 (1.1)	38.6 (1.1)	38.6 (1.2)
Mode of delivery			
- vaginal, n (%)	89 (56.3)	87 (55.4)	45 (56.3)
- cesarean, n (%)	69 (43.7)	70 (44.6)	35 (43.8)
Gender			
- male, n (%)	77 (48.7)	77 (49.0)	44 (55.0)
- female, n (%)	81 (51.3)	80 (51.0)	36 (45.0)
Mean weight at birth in grams (SD)	3127.6 (389.7)	3122.0 (320.6)	3237.4 (417.5)[a,b]
Mean length at birth in cm (SD)	49.6 (2.1)	49.7 (2.0)	N/D
Mean weight at mo 2 in kg (SD)	5.4 (0.6)	5.4 (0.6)	5.5 (0.6)
Mean length at mo 2 in cm (SD)	57.3 (2.3)	57.3 (2.6)	57.4 (2.3)
Mean weight at mo 4 in kg (SD)	6.7 (0.8)	6.8 (0.8)	6.9 (0.7)[c]
Mean length at mo 4 in cm (SD)	63.0 (2.5)	63.3 (2.5)	63.5 (2.3)
Mean weight at mo 6 in kg (SD)	7.5 (1.0)	7.6 (0.9)	7.8 (0.8)
Mean length at mo 6 in cm (SD)	67.2 (3.0)	67.3 (2.5)	67.5 (2.1)
Mean weight at mo 7 in kg (SD)	7.9 (1.0)	7.9 (0.9)	8.1 (0.8)
Mean length at mo 7 in cm (SD)	69.0 (2.6)	69.3 (2.9)	69.4 (2.2)
Mean weight at mo 18 in kg (SD)	10.9 (1.5)	10.9 (1.5)	10.9 (1.2)
Mean length at mo 18 in cm (SD)	81.7 (3.4)	81.6 (3.4)	82.1 (2.9)
Mean weight at mo 19 in kg (SD)	11.2 (1.5)	11.2 (1.5)	11.2 (1.3)
Mean length at mo 19 in cm (SD)	83.2 (3.2)	83.0 (3.3)	83.0 (4.5)
Mean duration from birth to mo 2 visit in days (SD)	63.0 (4.6)	62.6 (4.3)	61.6 (5.5)
Mean duration from mo 2 to mo 4 visit in days (SD)	59.9 (5.1)	60.0 (5.2)	61.8 (5.4)
Mean duration from mo 4 to mo 6 visit in days (SD)	60.5 (5.3)	61.6 (4.7)	61.5 (4.7)
Mean duration from mo 6 to mo 7 visit in days (SD)	30.8 (4.3)	31.1 (4.8)	31.7 (5.3)
Mean duration from mo 18 to mo 19 visit in days (SD)	31.8 (6.5)	32.1 (5.7)	31.6 (6.4)

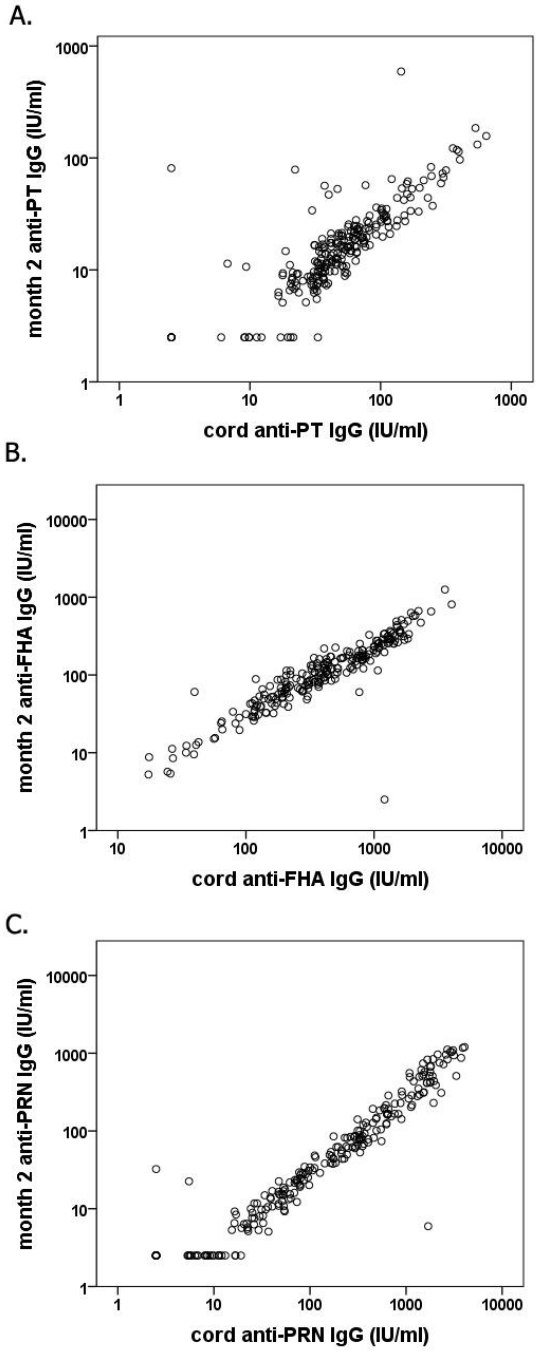
[a]$p = 0.046$ *vs.* aP, [b]$p = 0.019$ *vs.* wP, [c]$p = 0.035$ *vs.* aP (Independent *t*-test)

GA, Gestational age; SD, Standard Deviation; mo, month; cm, centimetre; kg, kilogram.

6.4.2 Correlations between the antibody levels in the cord and infant sera

There was a significant correlation between *B. pertussis*-specific antibody levels in the cord sera and in infants at two months of age before they received the first pertussis vaccine ($p < 0.001$) (Figure 6.4.2.1). The higher the anti-PT, anti-FHA and anti-PRN IgG levels in the cord sera, the higher these antibodies were in infants at 2 months. Using a multiple linear regression model, no associations between antibodies to *B. pertussis* antigens at month 2 and the gestational age of pregnant women at Tdap vaccination or the duration between maternal vaccination and delivery were found.

Figure 6.4.2.1: Correlations between (A) anti-PT, (B) anti-FHA and (C) anti-PRN IgG in cord and two-month-old infant sera. Kendall's tau coefficient for anti-PT = 0.70 ($p < 0.001$), anti-FHA= 0.81 ($p < 0.001$) and anti-PRN = 0.88 ($p < 0.001$).



6.4.3 Antibody response to *B. pertussis* antigens in aP and wP-vaccinated infants born to mothers who received Tdap during pregnancy

The GMC of antibodies to PT, FHA, and PRN in cord blood and infant sera at 2, 7, 18 and 19 months in aP and wP groups were compared (Figure 6.4.3.1). At one month post primary series vaccination (month 7), infants who had received aP-containing vaccine demonstrated significantly higher anti-PT and anti-PRN IgG antibodies when compared to the pre-priming levels (month 2), but their anti-FHA IgG remained at similar levels. Although infants who received wP also had significantly higher anti-PT IgG, their anti-FHA and anti-PRN IgG levels decreased significantly after primary series vaccination. GMCs of all three types of *B. pertussis*-specific antibodies were higher in aP-vaccinated infants compared to wP-vaccinated infants following primary series vaccination (Figure 6.4.3.2).

At 18 months of age just before children received the first pertussis booster, IgG to PT, FHA and PRN had waned rapidly and the remaining level was lower than the level at month 2 in both groups. One month after the administration of the 4th pertussis-containing vaccine (first booster), GMCs to anti-PT was comparable between aP and wP group, however, aP group possessed significantly higher anti-FHA and anti-PRN titers.

Figure 6.4.3.1: Kinetics of (A) anti-PT, (B) anti-FHA and (C) anti-PRN IgG in sera from the aP and wP groups at birth (cord), pre-vaccination (month 2), post-three-dose vaccination (month 7), pre-booster (month 18) and post-first booster vaccination (month 19). Bars indicate the 95% Confidence Interval (CI).

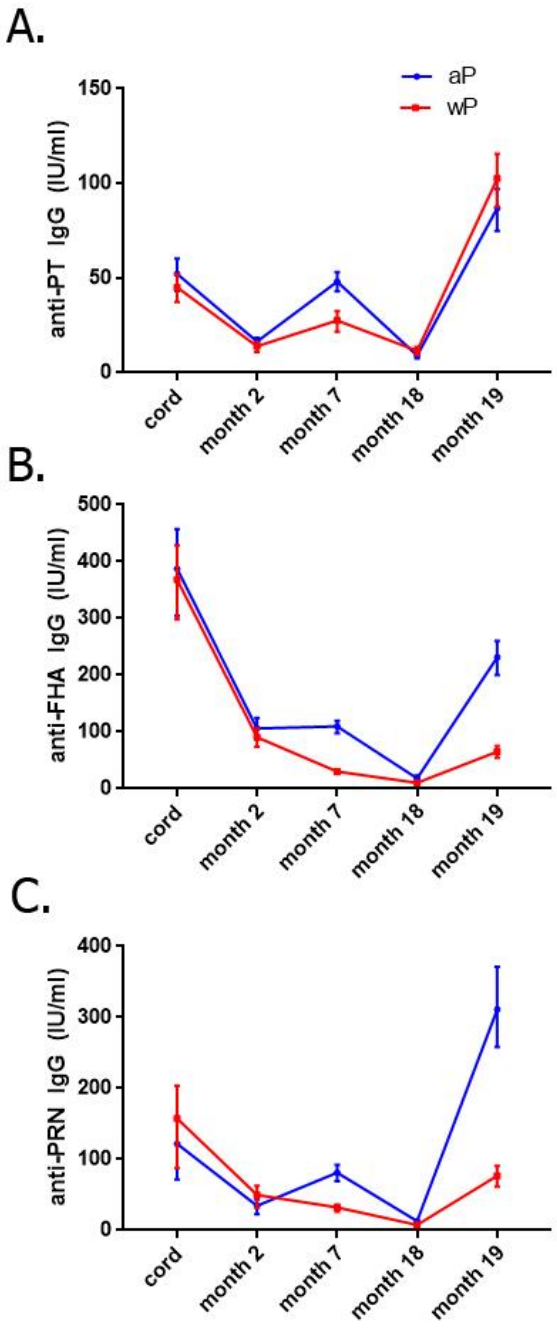
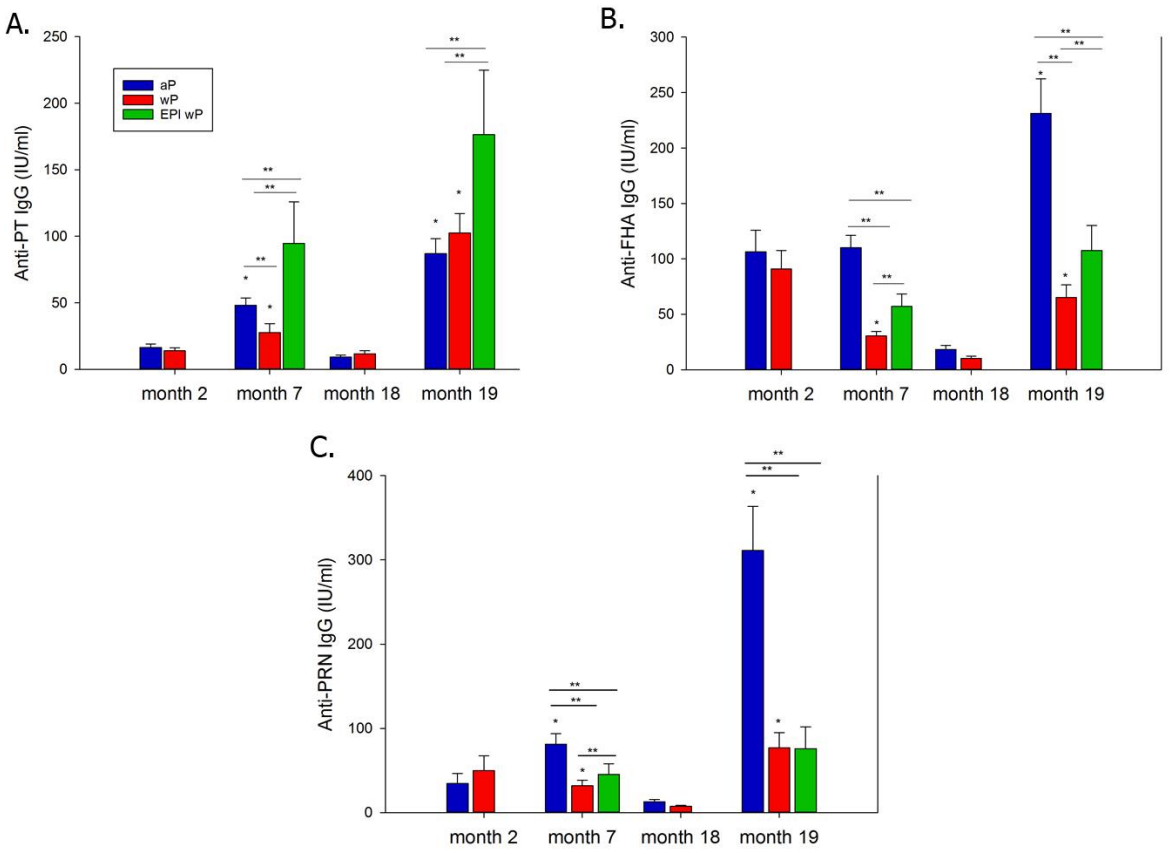


Figure 6.4.3.2: Geometric mean concentrations of (A) anti-PT, (B) anti-FHA and (C) anti-PRN IgG in the aP, wP and EPI wP groups at month 2, 7, 18 and 19. Error bars indicate the upper bound of 95% Confidence Interval (CI). *statistically significant difference compared to pre-priming or pre-booster, **statistically significant difference compared to other groups at month 7 and 19.



6.4.4 Interference of antibody response to *B. pertussis* antigens among wP-vaccinated infants

At one month after the third wP dose, infants born to Tdap-vaccinated mothers (wP group) had significantly lower anti-PT ($p < 0.001$), anti-FHA ($p < 0.001$), and anti-PRN ($p = 0.026$) titers compared to the EPI wP group, suggesting that maternal antibodies interfered with the infant immune response induced by wP (Figure 6.4.3.2) at post-third dose of vaccine. However, at one month after the administration of the fourth dose, maternal

antibodies interference with the wP-induced antibody response still persisted for anti-PT and anti-FHA, but not anti-PRN.

6.4.5 Effects of pre-existing maternal antibodies on aP and wP vaccine-induced antibody response

The correlations between antibody level at pre-priming (month 2) and post-priming (month 7) were evaluated. The results showed that higher titers of anti-PT IgG at month 2 resulted in lower titers at month 7, with a higher magnitude in the wP-vaccinated infants (Figure 6.4.5.1). This result suggested that pre-existing anti-PT IgG level at pre-vaccination had a negative effect on post-vaccination level. In contrast, a positive effect for anti-FHA IgG response in the wP group was found. For anti-PRN IgG, a small negative effect was found only in the aP group, not the wP group. When the correlations were re-evaluated at post booster (month 19), it was found that pre-existing maternal-derived antibody resulted in a significantly higher anti-PRN IgG after the first booster in the aP group but for other antibodies, the effect disappeared (Figure 6.4.5.2).

Figure 6.4.5.1: Correlations between anti-PT, anti-FHA and anti-PRN IgG between two and seven-month-old infant sera. Kendall's tau coefficient for anti-PT (aP) = -0.16 (p < 0.05), anti-PT (wP) = -0.26 (p < 0.05), anti-FHA (aP) = 0.02 (p: NS), anti-FHA (wP) = 0.17 (p < 0.05), anti-PRN (aP) = -0.12 (p < 0.05) and anti-PRN (wP) = 0.11 (p: NS). NS denotes not significant.

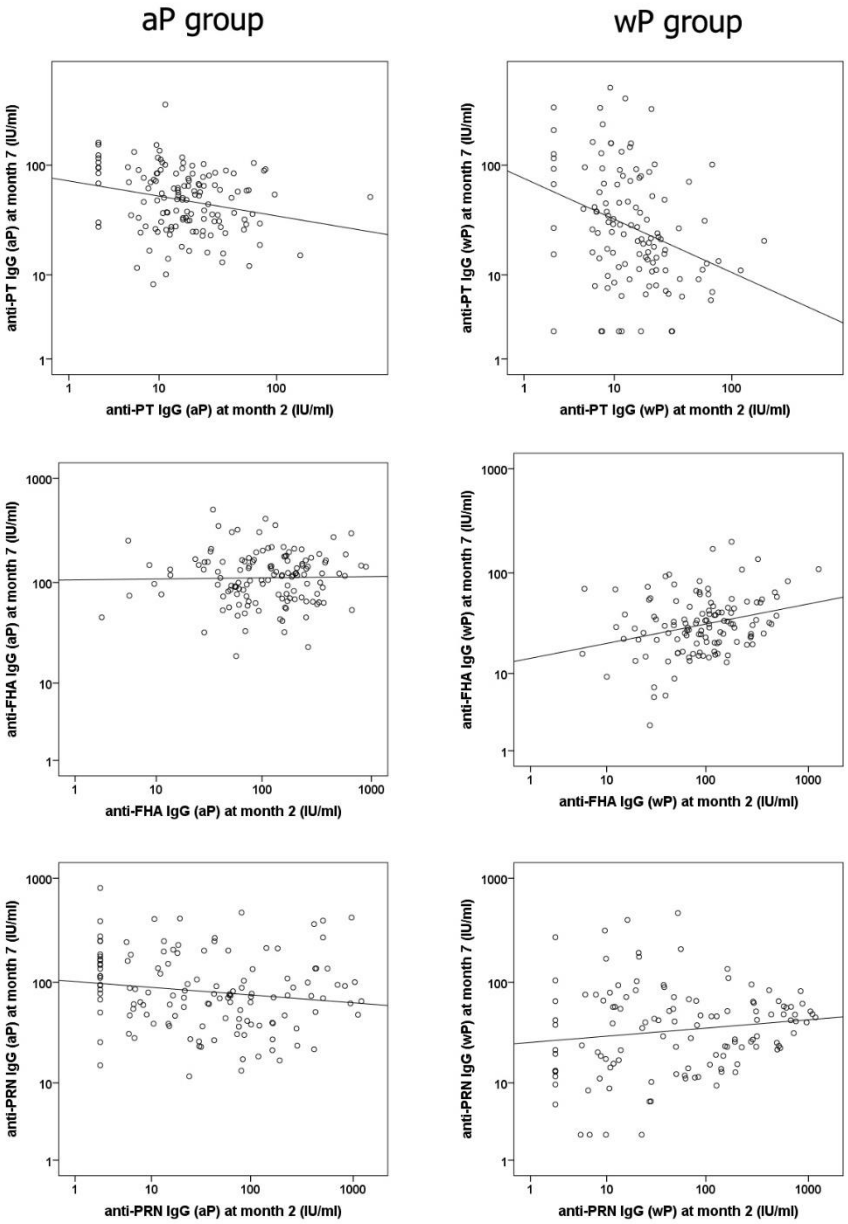
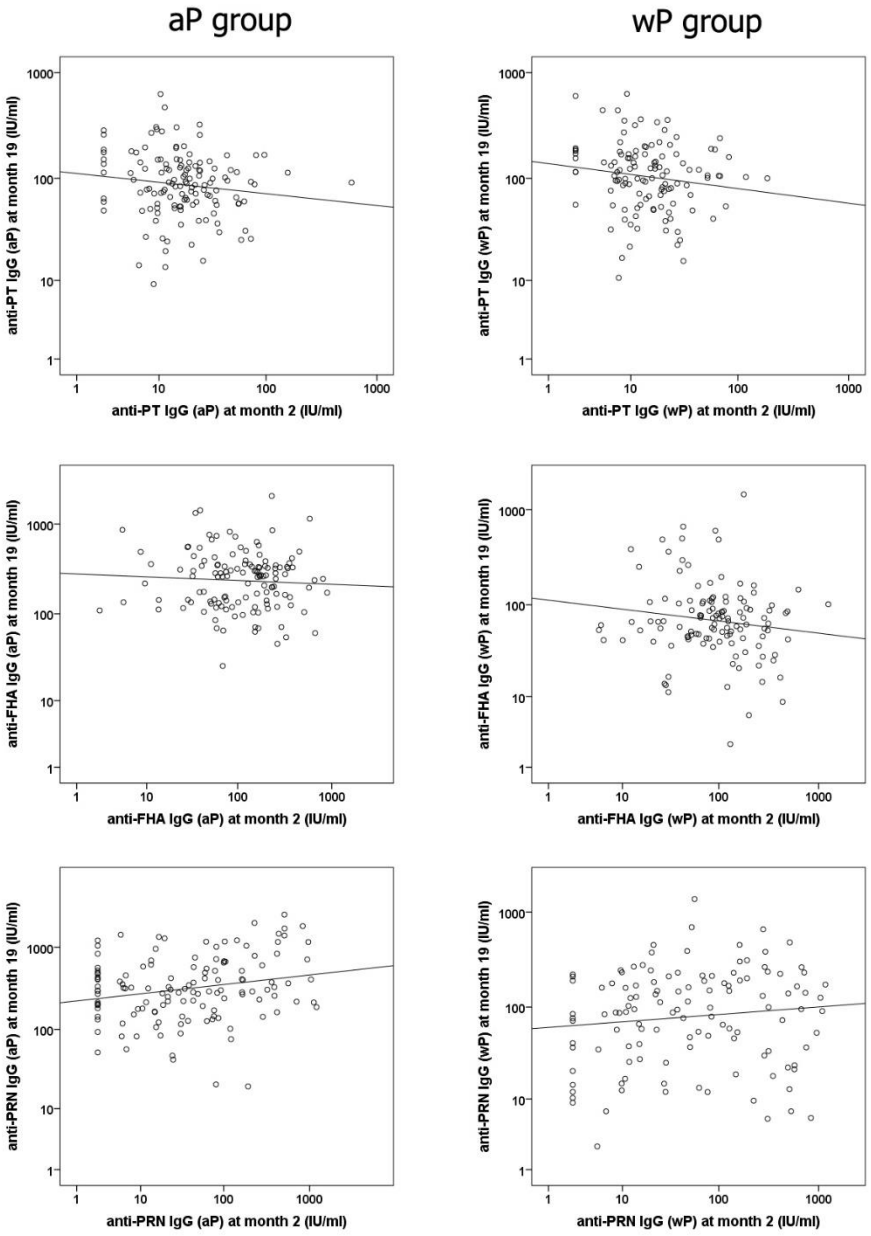


Figure 6.4.5.2: Correlations between anti-PT, anti-FHA and anti-PRN IgG between two and nineteen-month-old infant sera. Kendall's tau coefficient for anti-PT (aP) = -0.099 (*p*: NS), anti-PT (wP) = -0.113 (*p*: NS), anti-FHA (aP) = -0.048 (*p*: NS), anti-FHA (wP) = -0.087 (*p*: NS), anti-PRN (aP) = 0.145 ($p < 0.05$) and anti-PRN (wP) = 0.093 (*p*: NS). NS denotes not significant.



6.5 Discussion

In this study, the infant antibody responses to aP and wP vaccines used in the primary vaccination series and first booster following maternal Tdap vaccination during pregnancy were evaluated. In the presence of circulating *B. pertussis*-specific maternal antibodies, infants who received aP had significantly higher levels of anti-PT, anti-FHA and anti-PRN IgG at month 7 than infants who received wP. However, at month 19, anti-PT level were similar but anti-FHA and anti-PRN were still higher in the aP group. Previous comparative studies of infant antibody response after aP or wP vaccine without maternal pertussis immunization also showed that aP induced higher levels of antibodies to these three *B. pertussis* antigens than wP, which could be attributable to the relatively greater proportion of these antigens in aP compared to wP [9, 41, 49].

Similar to the blunting effect found in aP-vaccinated infants [14, 15, 88], the presence of *B. pertussis*-specific maternal antibodies induced by Tdap vaccination during pregnancy also influenced the infant immune response to wP-containing vaccine at month 7, with significantly higher antibody levels in the EPI wP group (no maternal immunization) compared to the wP group (with maternal immunization) for all three antigens studied. Although this study did not randomize infants to the EPI wP and wP groups and the baseline antibody levels to *B. pertussis* antigens at month 2 among the EPI wP infants were not available, it was expected that the antibody levels at month 2 in the EPI wP group were low, since the previous study demonstrated that antibody levels in cord sera of Tdap-unvaccinated pregnant women were low [117]. As previous reports on the persistence of the blunting effect to aP-induced antibody response in infants concluded that the blunting resolved after the first pertussis booster was given, except for one study in Belgium which still demonstrated the minor blunting effect for anti-PT [15, 114, 115], this infant cohort was followed into childhood when a booster dose was administered in the second year of life. The blunting effect for anti-

PT and anti-FHA, but not anti-PRN, still persisted after the first booster. However, a good antibody response to all measured antigens was observed in all groups.

A previous report showed positive correlations between the number of days between maternal vaccination and delivery and the remaining anti-PT and anti-FHA titers at month 2 [(118)]. The previous study stated that the interval between maternal vaccination and delivery correlated with the cord titers [(116)], but in this study no correlation between interval of vaccination-delivery and the remaining antibody titers at month 2 was observed. It is possible that the antibodies between birth to month 2 waned relatively quickly in this infant cohort, which lessens the significance of the effect of timing of maternal vaccination.

As expected, maternally-derived *B. pertussis* specific antibodies remained at month 2. After aP immunization, antibody levels to PT and PRN rose significantly at month 7, but aP did not appear to induce anti-FHA IgG following primary immunization. Ladhani et al. reported similar findings for anti-PT and anti-FHA in a cohort of UK aP-vaccinated children [(119)]. In contrast to aP, it was found in this study that following the primary immunization series with wP, anti-FHA and anti-PRN significantly decreased compared to the pre-priming level. Only anti-PT IgG levels appeared to increase at month 7 compared to month 2 after wP administration. Nevertheless, at one month post first booster, children in all groups showed an increase in antibody titers to all antigens. The clinical significance of the different antibody levels is yet to be defined.

Regarding the effect of pre-existing maternal antibodies on the antibody responses in infants who received aP or wP vaccine, Englund et al. showed that anti-PT response to DTaP was not affected by pre-existing maternal anti-PT. In contrast, anti-PT was negatively affected when DTwP was given [(95)]. Previous clinical studies have demonstrated that Tdap-induced maternal antibodies can blunt the antibody response in DTaP-vaccinated infants [(14,

[15, 88]. This present study compared the effect between aP and wP-vaccinated infants and found that for anti-PT, higher pre-existing maternal antibodies appeared to negatively affect the vaccine response at 7 months, and this effect was more apparent in DTwP-vaccinated group. Nevertheless, this effect was not statistically significant anymore after the first booster dose was given.

Although the clinical significance of the lower antibody level in wP- compared to aP-vaccinated infants is yet unknown, it is worth to note that if countries that use wP in infants EPI consider introducing maternal Tdap immunization, they should closely monitor the vaccine-induced immune protection and strengthen pertussis surveillance, as a negative effect on anti-PT and the interference with infant antibody response to *B. pertussis* antigens following primary immunization has been shown.

Chapter 7

Cell-mediated immune responses in acellular *versus* whole cell pertussis vaccinated infants born to mothers who received Tdap vaccine during pregnancy

7.1 Introduction

Pertussis is an infectious disease affecting people of all ages, however, severe complications occur mainly in young infants. Current efforts to protect young infants are through immunizing pregnant mothers [78]. Maternal immunization induces *B. pertussis*-specific antibodies detectable in cord sera of the newborn which are considered protective for young infants [62-64]. Since 2012, the Advisory Committee on Immunization Practices (ACIP) recommended to vaccinate pregnant women with Tetanus-Diphtheria-acellular pertussis (Tdap) vaccine during the third trimester of pregnancy and this has been implemented in an increasing number of countries worldwide [18, 94, 120].

Cellular immunity also plays a crucial role in the protection against pertussis. Several studies suggest a role for CD4+ T cells and interferon-gamma (IFN-γ) in protecting experimentally challenged mice against pertussis [39, 40]. Recent studies have demonstrated that Th17 cells are important in mediating bacterial clearance [43]. Immunization with wP results in faster bacterial clearance than aP vaccination in murine and baboon models due to the ability of wP to induce a strong Th1/Th17 response, while aP induced mostly Th2 but weak Th1/Th17 response [9, 40, 41, 43].

The previous study has shown that maternal immunization could blunt the antibody response to all three *B. pertussis* antigens studied in wP-vaccinated infants following primary immunization and this effect persisted until after the first booster for anti-PT and anti-FHA IgG. However, it is not known whether maternal immunization affects CD4+ T cell responses

in infants following vaccination. It is generally considered that B cell antibody production (e.g. following vaccination) is critically dependent on CD4+ T helper cells.

Detection of antigen-specific T cell proliferation has been used as a common method to evaluate T cell responses to vaccines or antigens *in vitro*. A study by Gans et al. demonstrated that the presence of passive antibody to measles in infants had no effect on the specific T cell proliferation or IFN-gamma production after measles stimulation [(121)]. Another study in rhesus macaques found that when rhesus macaques were immunized with live attenuated measles vaccine in the presence of passively acquired antibodies, proliferative T cell responses can be detected [(122)]. At present, data are limited regarding the effect of maternal-derived *B. pertussis*-specific antibodies on the proliferative T cell response in infants following primary series and first booster immunization.

In addition, it is currently unknown whether there is any interference by maternal immunization in the subsequent development of infant's T cell-related cytokine responses to vaccination. Data regarding the correlation between T cell and antibody response in aP *versus* wP-vaccinated infants are still limited. It will be important to understand whether and how infants' cellular immunity to vaccination could be affected in the presence of pre-existing maternal antibodies.

7.2 Aims of study

1. To assess the influence of maternal vaccination/maternal antibodies on T cell-mediated immune response in wP-vaccinated infants one month after primary series, before and after the first booster vaccination

2. To compare T cell-mediated immune response after the administration of different pertussis vaccines (aP *vs.* wP) to the infants born in the presence of high level of maternal-derived antibodies in their circulation.

7.3 Material and methods

7.3.1 Study design

This study was conducted according to the Declaration of Helsinki and Good Clinical Practice Guidelines (ICH-GCP). Written informed consent was obtained from the parents prior to infant enrollment. The Institutional Review Board of the Faculty of Medicine at Chulalongkorn University (IRB no. 604/57) and the ethical committee of the University of Antwerp (IRB no. 14/49/511) approved this study.

This study is a subset of the randomized controlled clinical trial that aims to characterize the kinetics and interference of humoral infant immune response to aP and wP vaccines after TdaP vaccination of their mothers during pregnancy. The detail of enrolled participants is provided in chapter 6. Briefly, there were 370 pregnant Thai women enrolled at King Chulalongkorn Memorial Hospital and consented to Tdap vaccination (Boostrix®, GSK, Rixensart, Belgium) during their third trimester of pregnancy (ClinicalTrial.gov NCT02408926). Healthy full-term and late preterm infants born at gestational age of 36 weeks with birth weight greater than 2500 grams were randomized to receive either the aP vaccine in the form of DTaP-Hib-HB-IPV (Infanrix hexa®, GlaxoSmithKline Biologicals, Rixensart, Belgium) or wP vaccine in the form of DTwP-HB-Hib (Quinvaxem®, Crucell-Janssen, Incheon, South Korea). Eighty full-term infants born to non-Tdap-vaccinated women were also recruited and this group received Quinvaxem (EPI wP group).

7.3.2 Study vaccines

All infants received pertussis-containing vaccines at 2, 4, 6 and 18 months of age according to the current recommendation in Thailand. Infanrix hexa is administered to infants in the aP group. Quinvaxem is administered to infants in the wP and EPI wP groups. The components of the vaccine are described in Chapter 6.

7.3.3 Sample collection

Addition 4 ml of heparinized blood in a subset of infant from each group at 7 (one month after primary series), 18 and 19 months (before and one month after a booster) were obtained in order to assess T cell-mediated immune response. These samples were taken after the written informed consent was obtained from the parents. Heparinized blood was processed within four hours after collection and freshly-isolated infant peripheral blood mononuclear cells (PBMC) were cultured for subsequent experiments.

7.3.4 Measurements of cytokine production in cultured PBMC supernatants

To characterize cytokine responses, PBMC from infants were stimulated with heat-inactivated PT and assessed for cytokine production. PBMC were isolated using Ficoll density gradient centrifugation (GE Healthcare) and washed twice in RPMI-1640 medium supplemented with 10% fetal bovine serum, 1× non-essential amino acids, 100 U/ml of penicillin, 100 µg/ml of streptomycin, 4 mM of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 1.6 mM of L-glutamine and 50 µM of β-mercaptoethanol. Cells were resuspended at 2 x 10^6 cells/ml and seeded into 96-well flat-bottom plate. PT (Calbiochem, Merck, Germany) was heat-inactivated at 80 ^oC for 15 minutes and used at a final concentration of 5 µg/ml as previously described [51]. Cells were incubated at 37 ^oC with 5% CO_2 for 88 hours. Culture supernatants from PT-treated and

untreated (medium control) cells were then harvested and stored at -80 ^{o}C. Th1 (IFN-γ, IL-12p70, TNF-α), Th2 (IL-4, IL-5, IL-6) and Th17 (IL-17A) cytokines were analyzed by a commercial multiplex bead-based ELISA kit (ProcartaPlex®, ThermoFisher Scientific, Vienna, Austria) according to the manufacturer's instructions. Data were acquired using Bio-plex instruments with Luminex xMap Technology (Bio Rad).Values below the lower limit of quantification (LLOQ) were quantified as half of the LLOQ. For IL-6 values that exceeded the upper limit of quantification in some samples, 50-fold dilution was performed and IL-6 levels were evaluated using a human IL-6 simplex kit (ProcartaPlex®, ThermoFisher Scientific, Vienna, Austria). There were 21 samples with limited amount of cell culture supernatant (Table 9.2.7), therefore, the IL-6 levels were estimated using the upper limit of quantification (31,684.49 pg/ml).

7.3.5 Intracellular cytokine staining assay

After cell culture supernatants were harvested, Brefeldin A solution (eBioscience, ThermoFisher Scientific) was added to cells at the final concentration of 3 µg/ml for 4 hours prior to cell staining. Cells were then fixed using fixation buffer (eBioscience) and stained with surface markers including anti-CD3-PE/Cy7 (Biolegend, San Diego, California, USA; clone UCHT1) and anti-CD4-APC/Cy7 or anti-CD4-APC/Fire750 (Biolegend, San Diego, California, USA; clone SK3). Afterwards, stained cells were permeabilized using permeabilization buffer (eBioscience) and stained with anti- IFN-γ-APC (eBioscience; clone 4S.B3) and anti-IL-17A-PE (BD Bioscience; clone SCPL1362). Data acquisition was performed on BD LSRII analyzer (BD Biosciences). Gating strategies are demonstrated in Figure 2.5.1. Positive control was phorbol 12-myristate 13-acetate (PMA) (Sigma) at a concentration of 10 ng/ml and ionomycin (Sigma) at a concentration of 1 µg/ml. BD FACSDIVA software (BD Biosciences) was used for data acquisition and Flowjo software (Tree Star, Oregon, USA) was used for data analysis. Percentages of cytokine producing

CD4+ T cells were recorded. Only samples showing cytokine producing CD4+ T cell after positive control stimulation were included in the data analysis.

7.3.6 T cell proliferation assay

Infant PBMC were isolated and resuspended at the concentration of 2 x 10^6 cells/ml in a 15 ml conical tube at a total volume of 1 ml. Carboxyfluorescein diacetate succinimidyl ester (CFSE) (Biolegend, Oregon, USA) at a volume of 1.1 µl was added on top of 110 µl sterile phosphate-buffered saline (PBS) on the side of the tube. Cell suspensions were mixed thoroughly by Vortexing at high speed to make the final working concentration of 5 µM CFSE. Cells were then incubated at 37°C for 4 minutes in the dark. Then, the reaction was stopped by adding 10 ml of complete RPMI-1640 culture medium (cRPMI). Thereafter, cells were centrifuged at 300 × *g* for 5 minutes, and the pellet was resuspended in cRPMI at the concentration of 1 x 10^6 cells/ml. Cells were seeded into 96-well flat bottom plates at the concentration of 200,000 cells/well and incubated with or without stimulation reagents at 37°C in 5% CO_2 incubator for 6 days. Under stimulated condition, pertussis toxin (Calbiochem, Merck, Germany) at the final concentration of 5 µg/ml was heat-inactivated at 80 ^{0}C for 15 minutes prior to use. Positive control for this experiment was anti-CD3 (Biolegend, Oregon, USA) at the concentration of 1 µg/ml. After six days of incubation, cells were harvested and stained with anti-CD3-PE/Cy7 (Biolegend, Oregon, USA; clone UCHT1) and anti-CD4-APC/Cy7 (Biolegend, Oregon, USA; clone RPA-T4). Data acquisition was performed on BD LSRII analyzer (BD Biosciences). Gating strategies were demonstrated in Figure 2.6.1. BD FACSDIVA software (BD Biosciences) was used for data acquisition and Flowjo software (Tree Star, Oregon, USA) was used for data analysis. CD4+ and CD3+ T cell proliferations were recorded. Proliferating CD4+ T cell was calculated from the percentages of CD4+CFSEdim cells among CD4+ T cell. Proliferating CD3+ T cell was calculated from the percentages of CD3+CFSEdim cells among CD3+ T cells. Only samples

showing proliferating CD4+ and CD3+ T cells after positive control stimulation were included in the data analysis.

7.3.7 Statistical analysis

A Wilcoxon signed ranks test was used to compare the differences in cytokine levels, percentages of cytokine producing or proliferating T cells before and after stimulation. Kruskal-Wallis and Mann-Whitney U tests were used to compare the difference in cytokine levels, proliferating cells and cytokine producing cells between infant groups. Kendall's tau coefficient was used to evaluate the correlations between cytokine responses and antibody levels to *B. pertussis* antigens. A p-value < 0.05 was considered statistically significant.

7.4 Results

7.4.1 Baseline characteristics of infants enrolled in the cell-mediated immune response (CMIR) study

Participants enrolled for measurement of cytokines, intracellular cytokine staining assay and T cell proliferation assay are presented in Table 7.4.1.1-4. In the measurement of cytokine levels, a subset of 7-month-old children cohort were selected. Also, a subset of 18-month-old children was longitudinally followed up until 19 months of age to see the changes in response between pre and post booster. For samples selected for the intracellular cytokine staining and T cell proliferation assays, a subset of children at each time point were selected in a cross-sectional design. There are some children whose PBMCs were tested at pre and post booster but the sample size of this group was insufficient to analyse the results separately. Codes of individual infants participated in the study are demonstrated in Table 9.2.8.

Table 7.4.1.1: Participants enrolled at 7 months of age for measurement of cytokine levels.

	aP (n=13)	wP (n=15)	EPI wP (n=9)
Mean GA at delivery (SD)	38.5 (1.4)	38.3 (1.4)	38.6 (1.2)
Mode of delivery			
- vaginal, n (%)	5 (38.5)	10 (66.7)	4 (44.4)
- cesarean, n (%)	8 (61.5)	5 (33.3)	5 (55.6)
Gender			
-male, n (%)	6 (46.2)	7 (46.7)	5 (55.6)
-female, n (%)	7 (53.8)	8 (53.3)	4 (44.4)
Mean weight at birth in grams (SD)	3001.5 (428.0)	3075.7 (321.6)	3139.4 (426.0)
Mean length at birth in cm (SD)	48.5 (2.3)	49.9 (1.7)	49.6 (2.0)
Mean weight at mo 2 in kg (SD)	5.2 (0.6)	5.2 (0.5)	5.3 (0.7)
Mean length at mo 2 in cm (SD)	56.1 (2.3)	57.0 (2.4)	57.0 (1.5)
Mean weight at mo 4 in kg (SD)	6.5 (0.7)	6.8 (0.7)	7.0 (0.8)
Mean length at mo 4 in cm (SD)	62.0 (2.5)	63.3 (2.2)	63.4 (1.8)
Mean weight at mo 6 in kg (SD)	7.3 (0.7)	7.8 (0.9)	7.9 (0.8)
Mean length at mo 6 in cm (SD)	65.6 (2.5)	67.4 (2.1)	66.8 (1.8)
Mean duration from birth to mo 2 visit in days (SD)	64.6 (5.9)	62.3 (1.9)	57.7 (5.1)
Mean duration from mo 2 to mo 4 visit in days (SD)	62.2 (5.7)	62.7 (3.9)	64.2 (7.8)
Mean duration from mo 4 to mo 6 visit in days (SD)	60.7 (5.4)	60.0 (4.3)	59.9 (5.1)
Mean duration from mo 6 to mo 7 visit in days (SD)	31.9 (5.0)	32.9 (5.6)	31.9 (3.7)

GA, Gestational age; SD, Standard Deviation; mo, month; cm, centimetres; kg, kilograms.

Table 7.4.1.2: Participants enrolled at 18 and 19 months of age for measurement of cytokine levels.

	aP (n=13)	wP (n=12)	EPI wP (n=13)
Mean GA at delivery (SD)	39.0 (1.1)	39.1 (1.4)	38.6 (1.3)
Mode of delivery			
- vaginal, n (%)	10 (76.5)	6 (50.0)	7 (53.8)
- cesarean, n (%)	3 (23.1)	6 (50.0)	6 (46.2)
Gender			
-male, n (%)	6 (46.2)	5 (41.7)	7 (53.8)
-female, n (%)	7 (53.8)	7 (58.3)	6 (46.2)
Mean weight at birth in grams (SD)	3305.4 (414.1)	3167.5 (210.3)	3238.5 (440.2)
Mean length at birth in cm (SD)	50.8 (1.1)	50.4 (1.2)	50.1 (1.3)
Mean weight at mo 2 in kg (SD)	5.6 (0.7)	5.4 (0.9)	5.3 (0.7)
Mean length at mo 2 in cm (SD)	57.8 (2.3)	58.0 (3.5)	57.5 (2.2)
Mean weight at mo 4 in kg (SD)	7.1 (0.6)	6.8 (1.0)	6.8 (0.8)
Mean length at mo 4 in cm (SD)	63.6 (2.1)	62.7 (2.5)	63.5 (2.2)
Mean weight at mo 6 in kg (SD)	7.9 (0.7)	7.6 (1.1)	7.7 (0.9)
Mean length at mo 6 in cm (SD)	68.3 (2.3)	67.5 (2.0)	67.5 (1.8)
Mean weight at mo 18 in kg (SD)	11.4 (1.5)	11.1 (1.8)	11.0 (0.9)
Mean length at mo 18 in cm (SD)	83.1 (2.3)	81.8 (3.6)	83.2 (5.4)
Mean duration from birth to mo 2 visit in days (SD)	63.1 (7.8)	65.4 (8.4)	61.5 (10.6)
Mean duration from mo 2 to mo 4 visit in days (SD)	62.2 (9.0)	62.6 (8.1)	64.9 (7.6)
Mean duration from mo 4 to mo 6 visit in days (SD)	60.6 (12.2)	58.7 (8.7)	60.2 (6.8)
Mean duration from mo 6 to mo 7 visit in days (SD)	31.8 (8.1)	30.9 (4.7)	30.1 (3.4)
Mean duration from mo 18 to mo 19 visit in days (SD)	30.5 (6.1)	30.3 (3.4)	31.2 (6.1)

GA, Gestational age; SD, Standard Deviation; mo, month; cm, centimetres; kg, kilograms.

Table 7.4.1.3: Participants enrolled for the intracellular cytokine staining assay.

	aP pre-boost (n=23)	aP post-boost (n=35)	wP pre-boost (n=22)	wP post-boost (n=36)	EPI wP pre-boost (n=12)	EPI wP post-boost (n=26)
Mean GA at delivery (SD)	39.1 (1.1)	38.9 (1.0)	39.1 (1.0)	38.7 (1.1)	38.4 (1.2)	38.3 (1.2)
Mode of delivery						
- vaginal, n (%)	13 (56.5)	21 (60.0)	15 (68.2)	19 (52.8)	8 (66.7)	15 (57.7)
- cesarean, n (%)	10 (43.5)	14 (40.0)	7 (31.8)	17 (47.2)	4 (33.3)	11 (42.3)
Gender						
-male, n (%)	12 (52.2)	21 (60.0)	11 (50.0)	16 (44.4)	8 (66.7)	15 (57.7)
-female, n (%)	11 (47.8)	14 (40.0)	11 (50.0)	20 (55.6)	4 (33.3)	11 (42.3)
Mean weight at birth in grams (SD)	3256.1 (339.6)	3174.1 (404.1)	3208.6 (289.2)	3103.2 (311.3)	3237.1 (460.2)	3217.5 (369.5)
Mean length at birth in cm (SD)	50.3 (1.4)	49.8 (2.0)	50.3 (1.9)	49.7 (1.8)	49.8 (1.3)	49.8 (1.6)
Mean weight at mo 2 in kg (SD)	5.5 (0.7)	5.4 (0.6)	5.5 (0.7)	5.4 (0.6)	5.3 (0.6)	5.5 (0.7)
Mean length at mo 2 in cm (SD)	57.1 (2.1)	57.9 (2.1)	58.1 (2.8)	57.4 (2.4)	58.4 (2.7)	57.5 (1.9)
Mean weight at mo 4 in kg (SD)	6.9 (0.6)	6.8 (0.7)	6.9 (0.8)	6.8 (0.8)	6.8 (0.7)	7.0 (0.8)
Mean length at mo 4 in cm (SD)	62.9 (1.9)	63.2 (2.0)	63.6 (2.6)	63.2 (2.5)	63.4 (1.5)	63.7 (1.7)
Mean weight at mo 6 in kg (SD)	7.8 (0.8)	7.6 (0.9)	7.7 (1.0)	7.7 (1.0)	7.5 (0.7)	7.9 (0.8)
Mean length at mo 6 in cm (SD)	67.2 (3.0)	67.2 (3.3)	67.9 (2.5)	67.7 (2.4)	66.9 (1.3)	67.5 (2.1)
Mean weight at mo 18 in kg (SD)	10.8 (1.4)	10.8 (1.2)	11.0 (1.5)	10.9 (1.6)	11.3 (1.1)	10.9 (1.2)
Mean length at mo 18 in cm (SD)	81.7 (2.8)	82.3 (3.1)	82.2 (3.4)	82.1 (3.9)	83.0 (3.2)	81.7 (2.5)
Mean duration from birth to mo 2 visit in days (SD)	63.5 (5.2)	63.8 (5.9)	64.6 (6.3)	63.6 (6.1)	60.9 (3.9)	60.3 (4.9)
Mean duration from mo 2 to mo 4 visit in days (SD)	61.8 (5.9)	60.5 (5.1)	59.5 (3.6)	59.7 (5.0)	61.4 (4.3)	62.3 (6.3)
Mean duration from mo 4 to mo 6 visit in days (SD)	62.1 (6.1)	62.2 (5.7)	62.4 (5.0)	62.8 (5.9)	62.7 (5.3)	61.3 (5.1)
Mean duration from mo 6 to mo 7 visit in days (SD)	31.9 (6.8)	29.9 (3.6)	29.6 (3.7)	31.4 (5.2)	31.1 (3.5)	32.0 (6.8)
Mean duration from mo 18 to mo 19 visit in days (SD)	30.3 (4.9)	32.4 (8.3)	32.0 (5.5)	32.5 (5.8)	35.5 (10.7)	30.1 (3.7)

GA, Gestational age; SD, Standard Deviation; mo, month; cm, centimetres; kg, kilograms.

Table 7.4.1.4: Participants enrolled for the T cell proliferation assay.

	aP pre-boost (n=15)	aP post-boost (n=25)	wP pre-boost (n=17)	wP post-boost (n=26)	EPI wP pre-boost (n=10)	EPI wP post-boost (n=21)
Mean GA at delivery (SD)	38.5 (1.2)	38.8 (1.1)	38.6 (1.2)	38.4 (1.0)	38.5 (1.3)	38.3 (1.3)
Mode of delivery						
- vaginal, n (%)	9 (60.0)	14 (56.0)	11 (64.7)	13 (50.0)	7 (70.0)	13 (61.9)
- cesarean, n (%)	6 (40.0)	11 (44.0)	6 (35.3)	13 (50.0)	3 (30.0)	8 (38.1)
Gender						
-male, n (%)	6 (40.0)	15 (60.0)	10 (58.8)	12 (46.2)	5 (50.0)	11 (52.4)
-female, n (%)	9 (60.0)	10 (40.0)	7 (41.2)	14 (53.8)	5 (50.0)	10 (47.6)
Mean weight at birth in grams (SD)	3250.0 (381.9)	3197.4 (306.4)	3227.6 (195.0)	3085.4 (324.8)	3249.0 (498.3)	3217.4 (400.1)
Mean length at birth in cm (SD)	50.1 (1.4)	49.8 (1.5)	50.4 (1.6)	49.5 (1.8)	50.1 (1.6)	49.8 (1.8)
Mean weight at mo 2 in kg (SD)	5.5 (0.5)	5.5 (0.5)	5.5 (0.6)	5.5 (0.6)	5.4 (0.5)	5.5 (0.7)
Mean length at mo 2 in cm (SD)	56.8 (2.1)	57.9 (2.1)	57.7 (2.2)	57.7 (2.4)	58.7 (2.9)	57.6 (1.9)
Mean weight at mo 4 in kg (SD)	6.9 (0.5)	6.7 (0.5)	6.9 (0.7)	6.9 (0.8)	6.9 (0.7)	7.0 (0.8)
Mean length at mo 4 in cm (SD)	63.1 (1.6)	63.3 (1.9)	63.9 (2.3)	63.8 (2.2)	63.9 (1.1)	63.7 (1.7)
Mean weight at mo 6 in kg (SD)	7.9 (0.7)	7.5 (0.6)	7.9 (0.9)	7.8 (1.0)	7.6 (0.6)	7.9 (08)
Mean length at mo 6 in cm (SD)	67.8 (2.5)	67.0 (3.4)	68.0 (1.9)	68.0 (2.3)	66.8 (1.5)	67.9 (1.9)
Mean weight at mo 18 in kg (SD)	11.0 (1.5)	10.6 (1.0)	11.0 (1.3)	11.2 (1.5)	11.3 (1.0)	10.8 (1.1)
Mean length at mo 18 in cm (SD)	81.7 (2.8)	81.9 (3.3)	82.8 (3.0)	82.5 (3.3)	84.1 (2.6)	81.7 (2.6)
Mean duration from birth to mo 2 visit in days (SD)	63.0 (6.5)	64.1 (5.8)	61.2 (13.7)	63.2 (3.7)	60.8 (4.0)	59.5 (4.7)
Mean duration from mo 2 to mo 4 visit in days (SD)	62.1 (6.7)	61.8 (5.6)	61.8 (6.6)	60.2 (5.6)	59.6 (3.1)	63.2 (6.4)
Mean duration from mo 4 to mo 6 visit in days (SD)	62.6 (7.2)	60.9 (4.5)	63.6 (6.1)	62.8 (5.7)	63.3 (5.4)	61.6 (5.4)
Mean duration from mo 6 to mo 7 visit in days (SD)	30.1 (4.1)	29.3 (2.4)	30.2 (4.1)	29.8 (3.0)	31.7 (3.5)	31.7 (5.2)
Mean duration from mo 18 to mo 19 visit in days (SD)	30.3 (5.1)	31.8 (7.6)	32.7 (6.6)	32.8 (6.1)	32.0 (4.8)	29.7 (3.8)

GA, Gestational age; SD, Standard Deviation; mo, month; cm, centimetres; kg, kilograms.

7.4.2 Cytokine levels in cell culture supernatants after primary series vaccination

The concentrations of different cytokines released into the cell culture supernatants from medium-treated and PT-stimulated PBMC derived from infants in the aP (n=13), wP (n=15) and EPI wP (n=9) groups at seven months of age were evaluated (Figure 7.4.2.1). Among the Th1 cytokines, there was a statistically significant increase in IFN-γ, IL-12p70 and TNF-α levels detected in PT-stimulated PBMC compared to the medium control in all three groups, except for TNF-α in the wP and EPI wP groups. PT-stimulated PBMC also resulted in significantly higher levels of Th2 cytokines (IL-4, IL-5, and IL-6) than the medium control in all three groups. PT stimulation resulted in statistically significant increase in IL-17A production compared to the medium control only in the wP group. There appeared to be an increase in IL-17A production after PT stimulation in the EPI wP group as well, although it did not reach statistical significance. When the PT-stimulated cytokine levels were substracted with the levels in medium control to compare the magnitude of responses between each infant group (Figure 7.4.2.2). It was found that the magnitude of cytokine responses were similar among all groups except that aP-vaccinated infant PBMC secreted higher level of IL-4 in response to PT compared to EPI wP group (p = 0.03, Mann-Whitney U test).

Figure 7.4.2.1: Cytokine production in medium-treated and PT-stimulated PBMC from aP, wP and EPI wP infants at seven months of age. Box plots represent the medians and interquartile ranges of the values. Vertical lines represent values between the 10th to the 90th percentile, with red dots indicating outliers. *$p < 0.05$ *vs.* medium control

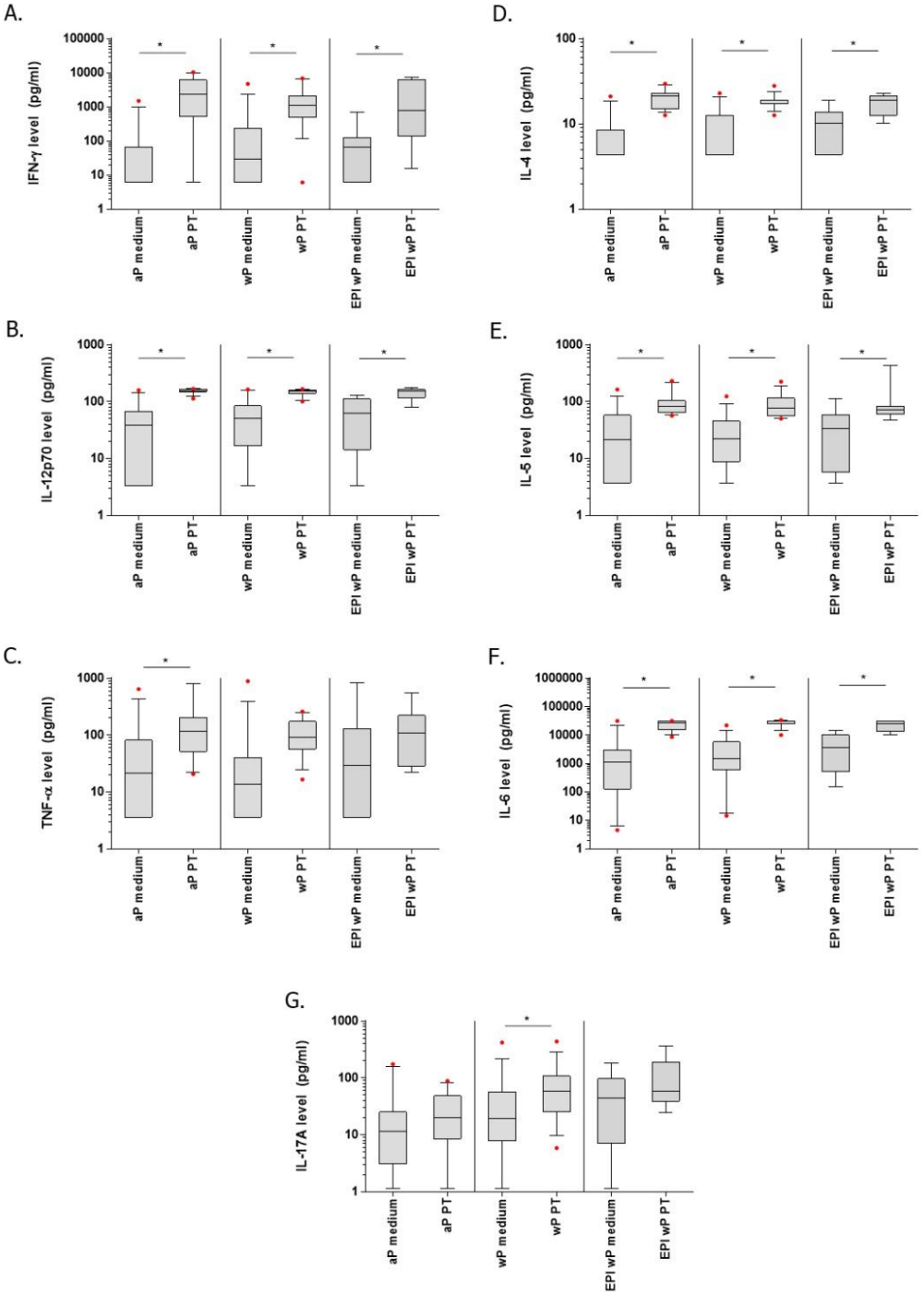
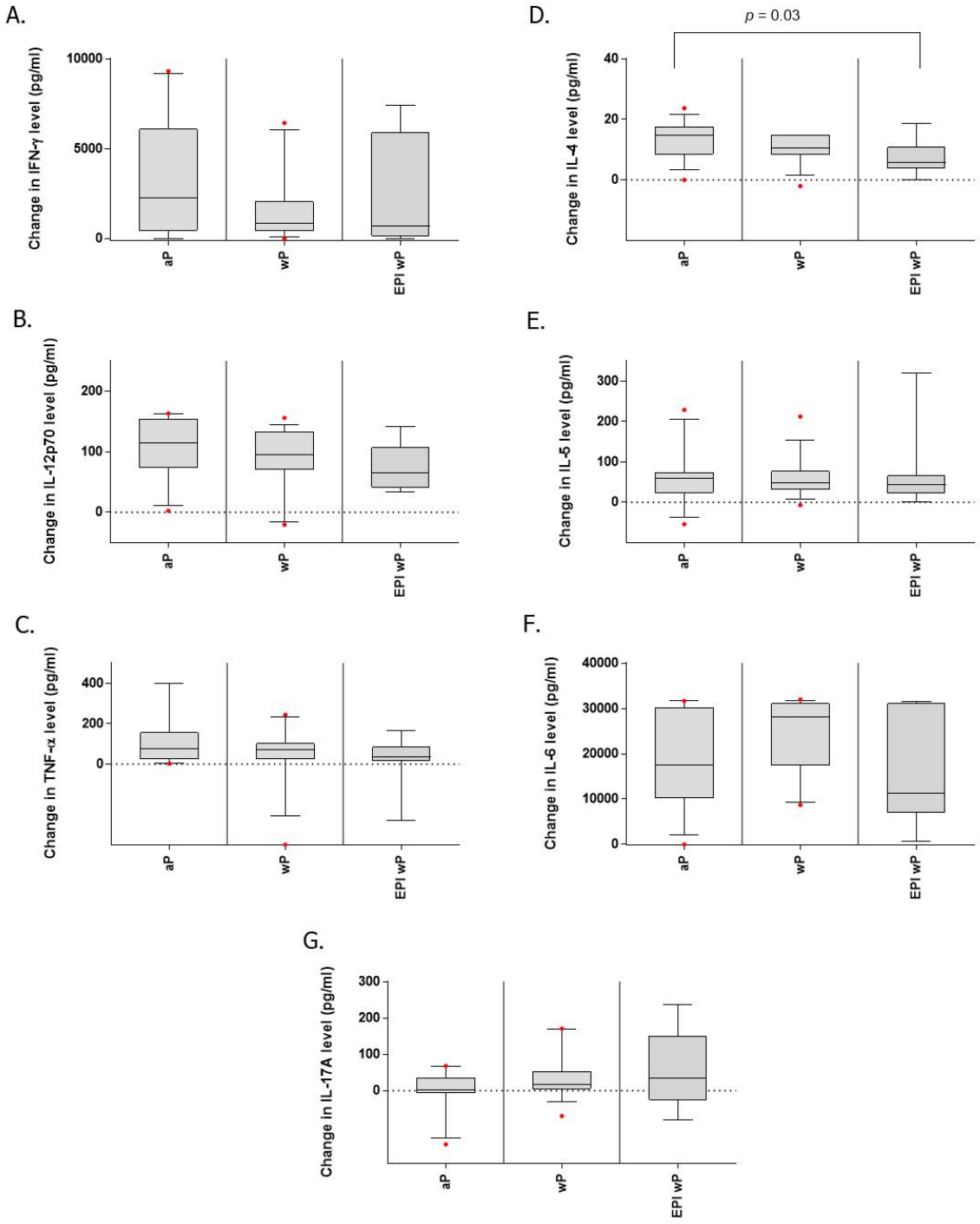


Figure 7.4.2.2: **Changes in cytokine levels in the supernatants of the cultured PBMC of seven-month-old infants.** Box plots represent the medians and interquartile ranges of the values. Vertical lines represent values between the 10th to the 90th percentile, with red dots indicating outliers.



7.4.3 Cytokine levels in cell culture supernatants at pre and post first booster

The concentrations of cytokines released into the cell culture supernatants from medium-treated and PT-stimulated PBMC derived from infants in the aP (n=13), wP (n=12) and EPI wP (n=13) groups at pre and post first booster were evaluated (Figure 7.4.3.1 and Figure 7.4.3.2, respectively). At 18 months of age (pre-booster), there was a statistically significant increase in IFN-γ, IL-12p70 and TNF-α levels detected in PT-stimulated PBMC compared to the medium control in all three groups. PT-stimulated PBMC also resulted in significantly higher levels of Th2 cytokines (IL-4, IL-5, and IL-6) than the medium control in all three groups, except for IL-4 in the wP group. In contrast to the findings at month 7, PT-stimulation did not result in a significant increase in IL-17A production compared to the medium control only in the wP group. At one month after the first booster, all cytokines tested increased after PT stimulation except for IL-4 in the aP group.

The PT-stimulated cytokine levels were subtracted with the levels in medium control to compare the magnitude of response between each infant group. It was found that for Th1 cytokines (Figure 7.4.3.3, A-C), aP-vaccinated infant PBMC secreted higher levels of IFN-γ and IL-12p70 compared to EPI wP at pre booster. The higher response was also observed for IL-12p70 when compared to the wP group. Overall, the Th1 cytokine response between pre and post booster was similar except for IFN-γ in EPI wP group and TNF-α in the aP group that showed significantly increased response after the booster.

Regarding the Th2 cytokines (Figure 7.4.3.3, D-F), there was no difference in magnitude of response between infant groups at pre ad post booster, but the findings demonstrated a higher response after booster for IL-5 in the wP group and IL-6 in the EPI wP group when compared to the pre-booster levels. Similar to the findings in Th2 cytokines, Th17 cytokine response (Figure 7.4.3.3, G) at pre and post booster was similar among all groups but an increase in magnitude of response after the first booster was detected only in the wP group.

Figure 7.4.3.1: **Cytokine production in medium-treated and PT-stimulated PBMC from aP, wP and EPI wP infants at 18 months of age.** Box plots represent the medians and interquartile ranges of the values. Vertical lines represent values between the 10th to the 90th percentile, with red dots indicating outliers. *$p < 0.05$ vs. medium control

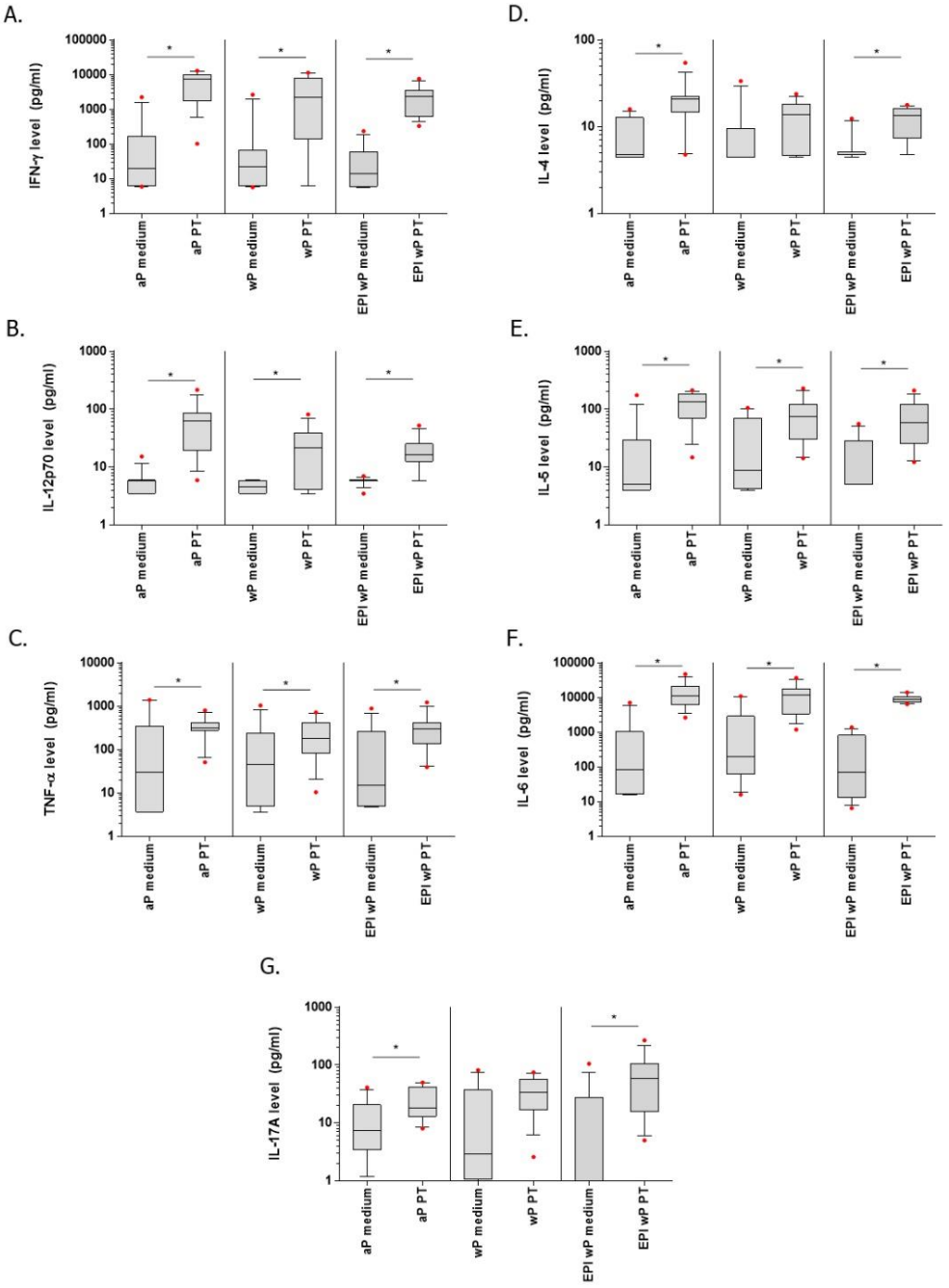


Figure 7.4.3.2: Cytokine production in medium-treated and PT-stimulated PBMC from aP, wP and EPI wP infants at 19 months of age. Box plots represent the medians and interquartile ranges of the values. Vertical lines represent values between the 10th to the 90th percentile, with red dots indicating outliers. *$p < 0.05$ vs. medium control

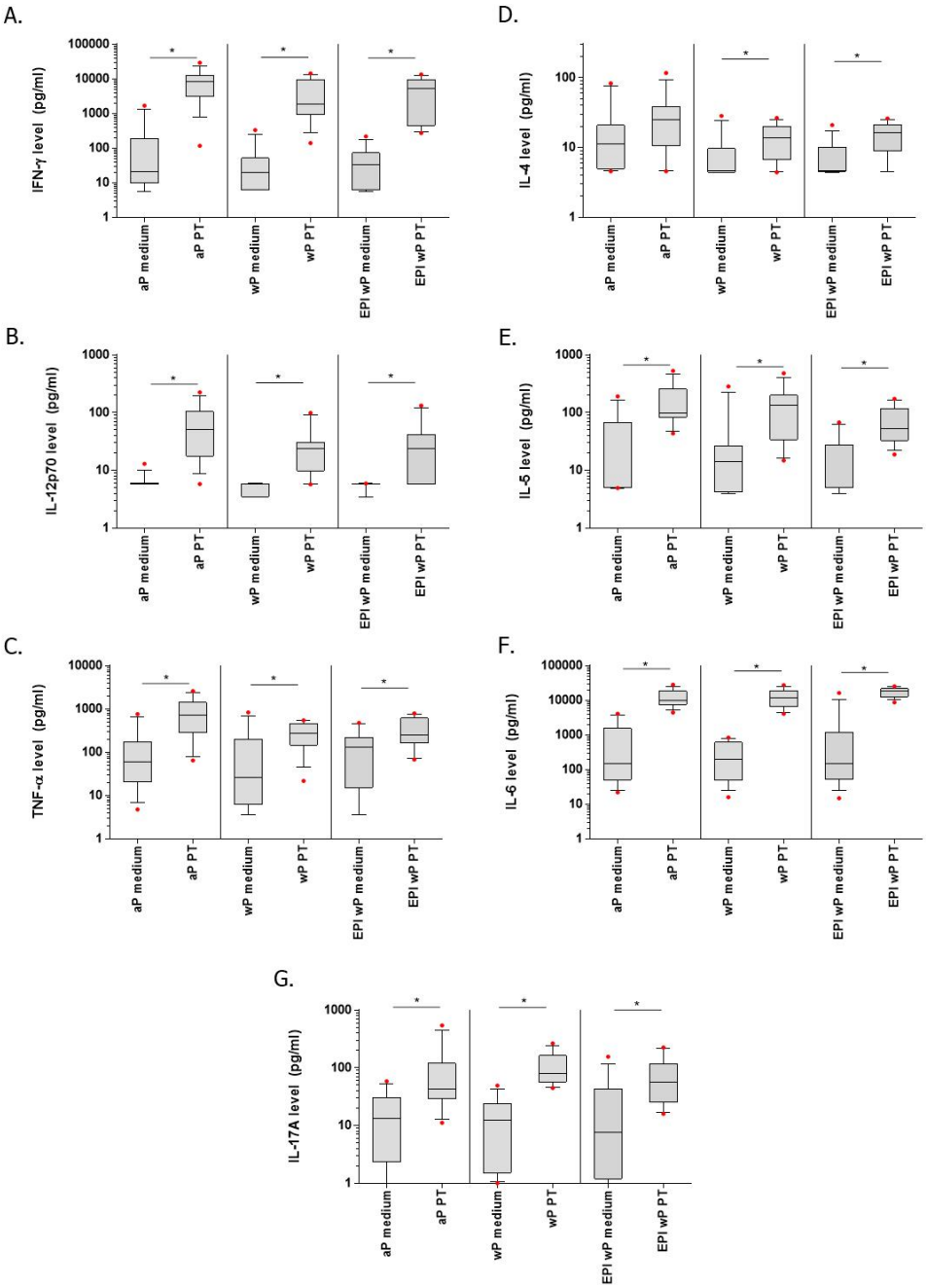
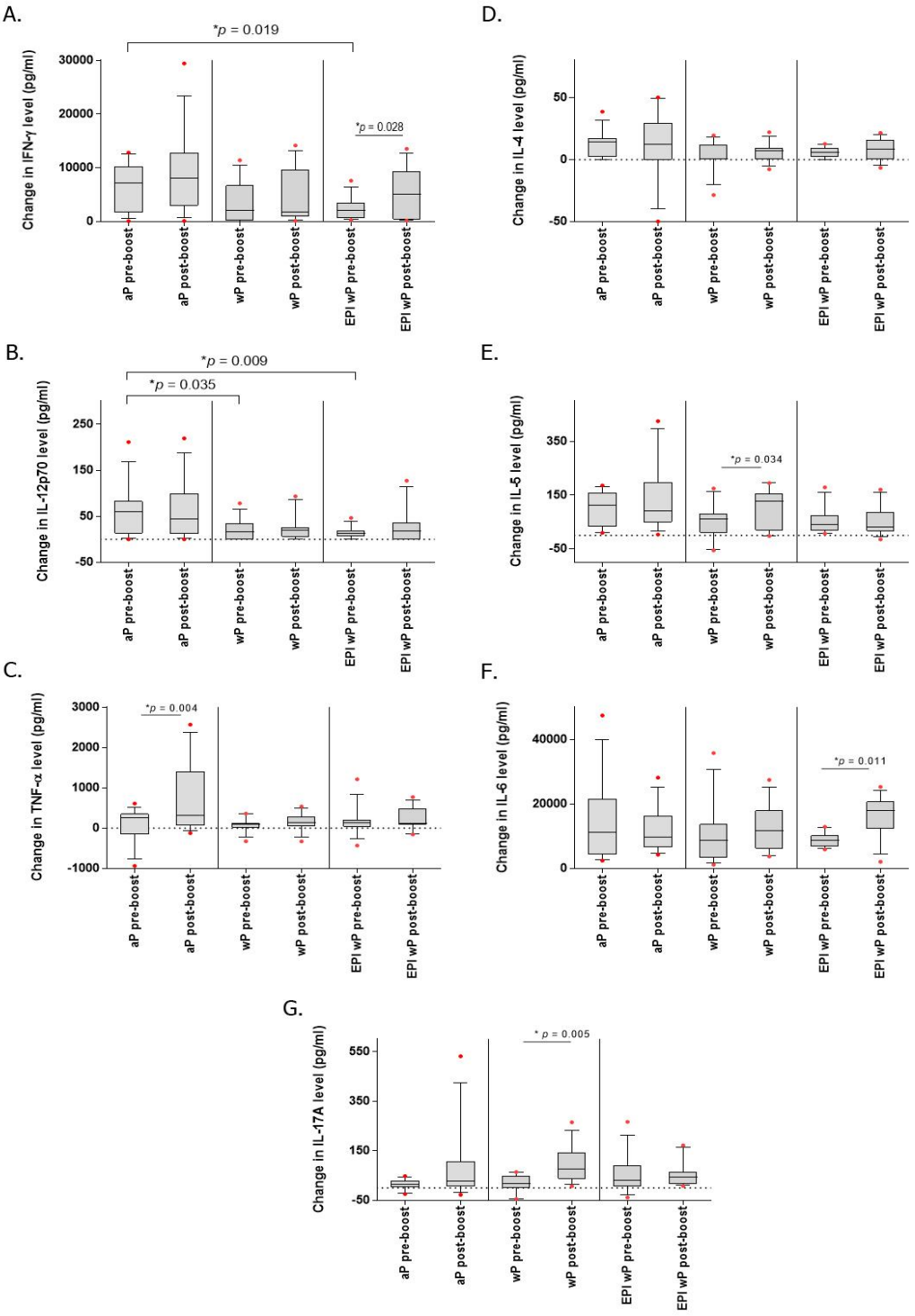


Figure 7.4.3.3: Changes in cytokine levels at pre-and post-booster. Box plots represent the medians and interquartile ranges of the values. Vertical lines represent values between the 10th to the 90th percentile, with red dots indicating outliers.

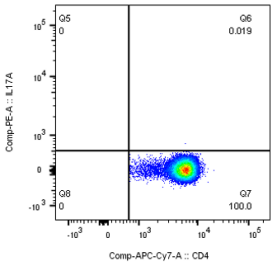
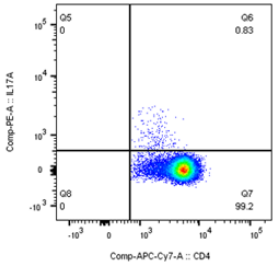
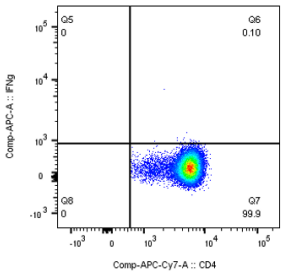
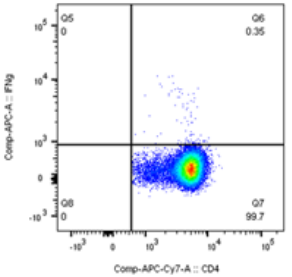


7.4.4 Cytokine secreting CD4+ T cells in response to PT at pre- and post-first booster

Comparing aP- to wP-primed children at pre-booster vaccination, the proportion of PT-specific IL-17A- and IFN-γ-secreting CD4+ T cells were similar in all infant groups (Table 7.4.4.1). After the first booster, the percentages of IL-17A-secreting CD4+ T cells did not significantly increase compared to pre booster level in all infant groups and no differences among all groups can be detected.

Regarding the IFN-γ-secreting CD4+ T cell, only aP-vaccinated children PBMC displayed higher percentages of IFN-γ-secreting CD4+ T cells after the booster. In addition, aP-vaccinated children had higher percentages of IFN-γ-producing CD4+ T cells compared to infants from other groups at one month post-booster.

Table 7.4.4.1: Percentages of cytokine producing CD4+ T cells in children at pre- and post- booster vaccination (Per sample, the un-stimulated (control) cytokine producing CD4+ T cell data were subtracted).

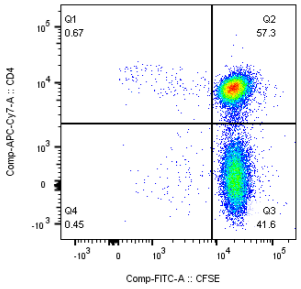
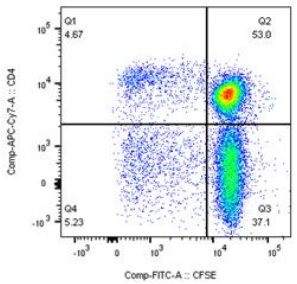
	Pre-booster				One month post-booster				
	% of IL-17A-producing CD4+ T cells in response to PT				% of IL-17A-producing CD4+ T cells in response to PT				
	aP	wP	EPI wP	*p*-value	aP	wP	EPI wP	*p*-value	*p*-value (pre- *vs.* post-boost)
	0.53	0.73	0.94	NS	0.68	0.82	0.55	NS	NS
95% CI	0.27, 0.80	0.42, 1.04	0.39, 1.49		0.48, 0.89	0.44, 1.21	0.33, 0.78		
No. of children	23	22	11		20	25	26		
	% of IFN-γ-producing CD4+ T cells in response to PT				% of IFN-γ-producing CD4+ T cells in response to PT				
	aP	wP	EPI wP	*p*-value	aP	wP	EPI wP	*p*-value	*p*-value (pre- *vs.* post-boost)
	0.08	-0.16	0.15	NS	0.44	0.13	0.10	$p = 0.008$ (aP *vs.* wP) $p = 0.004$ (aP *vs.* EPI wP)	$p = 0.01$ for aP
95% CI	0.00, 0.17	-0.45, 0.13	-0.13, 0.34		0.23, 0.64	0.03, 0.22	0.01, 0.19		
No. of children	14	9	9		29	26	24		
Representative flow cytometry plots	IL-17A-producing CD4+ T cells (unstimulated) 		IL-17A-producing CD4+ T cells (PT-stimulated) 		IFN-γ-producing CD4+ T cells (unstimulated) 		IFN-γ-producing CD4+ T cells (PT-stimulated) 		

NS; not significance.

7.4.5 T cell proliferation in response to PT at pre- and post first- booster

In general, the frequencies of proliferated CD4+ and CD3+ T cells after stimulation with PT were higher in the wP group than in the aP and EPI wP groups at pre-booster, although it did not reach statistical significance (Table 7.4.5.1). The total frequencies of PT-specific proliferating CD4+ T cells of aP-primed children were significantly increased post-booster. In contrast, the PT stimulation of wP-primed children PBMC resulted in comparable frequencies of proliferated CD4+ and CD3+ T cells before and after wP booster vaccination. Infants in the wP group (with maternal immunization) had higher percentages of proliferating CD4+ and CD3+ T cells at pre- and post-booster than infants in the EPI wP group (without maternal immunization), suggesting that maternal vaccination might enhance the infant T cell proliferative response to wP-containing vaccine. However, the sample size may be too small to reach statistical significance. At one month post-booster, infants in the aP and wP group had similar percentages of proliferating CD4+ and CD3+ T cells.

Table 7.4.5.1: Proliferation of CD4+ and CD3+ T cells determined by CFSE staining (Per sample, the non-stimulated T cell proliferation data were subtracted).

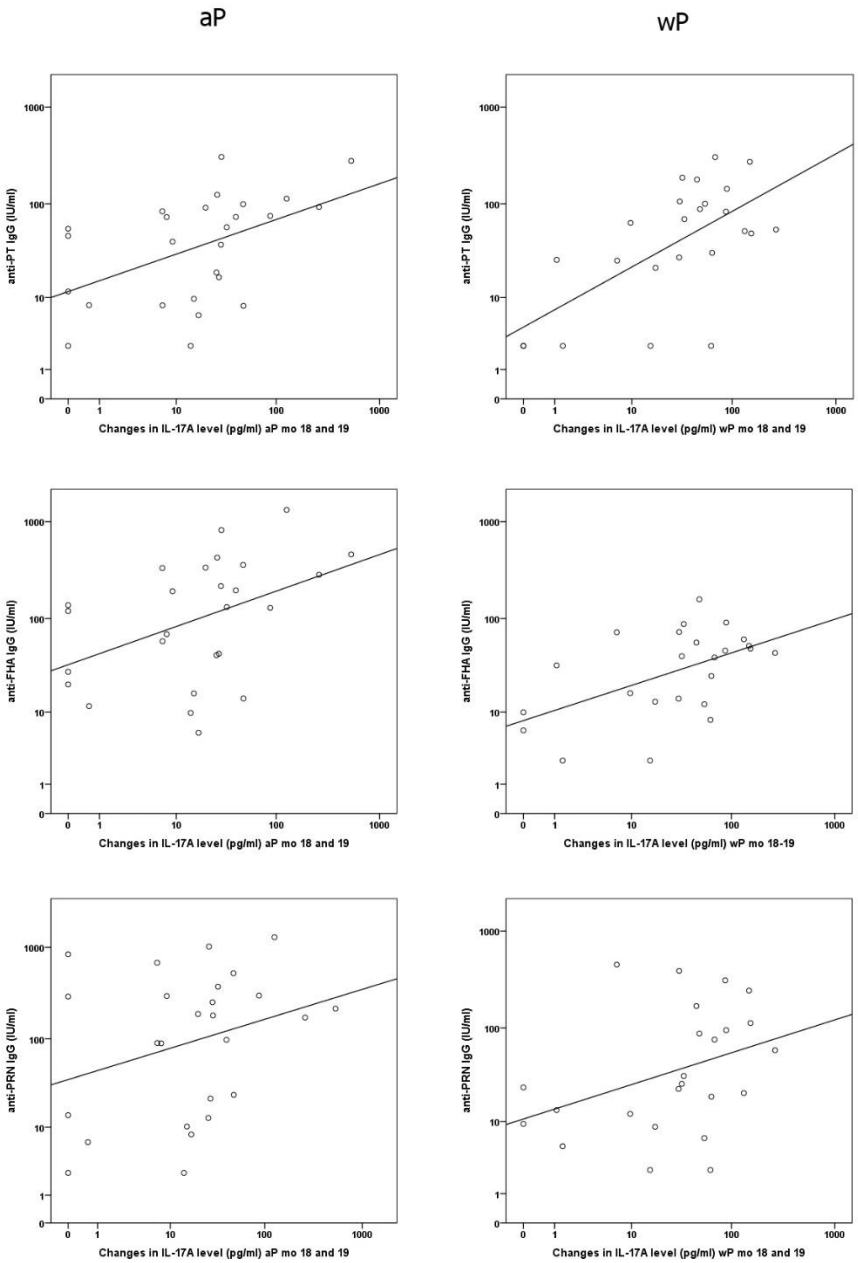
	Pre-booster			One month post-booster		
	% of proliferated CD4+ T cells	95% CI	No. of children	% of proliferated CD4+ T cells	95% CI	No. of children
aP	5.52	0.68, 10.36	15	14.23*	7.45, 21.01	25
wP	13.27	3.52, 23.01	17	13.80	6.08, 21.52	26
EPI wP	6.07	0.92, 11.22	10	6.27	1.89, 10.66	21
	% of proliferated CD3+ T cells	95% CI	No. of children	% of proliferated CD3+ T cells	95% CI	No. of children
aP	5.85	1.42, 10.28	15	14.35	7.24, 21.47	25
wP	13.13	3.58, 22.69	17	13.87	6.05, 21.70	26
EPI wP	6.58	0.59, 12.57	10	6.67	1.81, 11.53	21
Representative flow cytometry plots	Proliferated T cells (unstimulated) 	Proliferated T cells (PT-stimulated) 				

* *p*-value = 0.049 *vs.* pre-booster (Mann-Whitney U test); CI, Confidence Interval.

7.4.6 Correlations between cytokine responses and antibody level to *B. pertussis* antigens

The correlation between changes in cytokine levels after PT stimulation (IL-17A: Th17 cytokine, IFN-γ: Th1 cytokine and IL-5: Th2 cytokine) and anti-PT, anti-FHA and anti-PRN IgG level at month 18-19 was evaluated. It was found that the magnitude of IL-17A responses significantly correlated with anti-PT and anti-FHA IgG level (Figure 7.4.6.1). However, no significant correlation was observed between IFN-γ or IL-5 levels and antibody titers (Data not shown). These results supported the evidence of T cell and B cell correlation in the adaptive immune responses.

Figure 7.4.6.1: Correlations between IL-17A responses after PT stimulation and anti-PT, anti-FHA and anti-PRN IgG titers in 18- and 19-month-old infant sera. Kendall's tau coefficient for anti-PT (aP) = 0.379 (p = 0.007), anti-PT (wP) = 0.399 (p = 0.007), anti-FHA (aP) = 0.326 (p = 0.020), anti-FHA (wP) = 0.327 (p = 0.025), anti-PRN (aP) = 0.224 (p: NS) and anti-PRN (wP) = 0.255 (p: NS). NS denotes not significant.



7.5. Discussion

Recent epidemiological data suggest that wP-vaccinated children are better protected from pertussis than aP-vaccinated children [8, 10, 66]. Although the immunological basis for this is yet to be defined, evidence from animal studies suggests that this might be due to differences in the cellular immunity induced by these two types of vaccines [40, 41]. There is limited data in children on the cellular immunity induced by pertussis vaccination. Mascart et al. found that both aP and wP vaccine induced Th1 cytokines in infants, but aP vaccine recipients also developed a Th2-biased response which can be detected in children up to 13 months of age [51, 52]. Recent studies also demonstrated that in baboons, wP induced strong Th1 and Th17 responses, while aP induced high Th2, but weak Th1 and very low Th17 response [9, 123]. In contrast, studies in mice showed that both aP and wP vaccine can induce IL-17A production [43].

In this study, the cellular cytokine responses using cytokine array in PBMC samples collected from infants who received aP or wP vaccines were analyzed. After primary series vaccination, both Th1 (IFN-γ, IL-12p70, TNF-α) and Th2 (IL-4, IL-5) cytokine responses were detected from PBMC culture following stimulation by PT in both aP and wP vaccinated infants. No significant differences in the Th1 and Th2 responses between the two groups were shown apart from a small difference in IL-4, for which the levels were generally low. For IL-17A response, which is known to be generally low in young infants, no response was detected in the aP group. However, there appeared to be a significant IL-17A response in the wP and EPI wP group at the age of 7 months. This early onset of Th17 response in the wP vaccinated infants provides the first evidence supporting the hypothesis that wP promotes a Th17 response in children.

The cytokine responses measured at 18 months (pre-booster) and 19 month (post-booster) were analyzed. The results showed that almost all the cytokines including IL-17A

increased markedly after PT stimulation at 18 and 19 months, and the levels were significantly higher than at 7 months. The aP-vaccinated infants showed higher IFN-γ production in response to PT compared to EPI wP infants, and higher IL-12p70 production compared to wP and EPI wP groups. This supports a stronger Th1 cytokine response at pre-booster in aP vaccinated group than wP group, which, to our knowledge provides the first evidence that aP vaccination activated a superior Th1 response in young children. Previous studies reported equal responses among aP and wP-vaccinated infants [52, 124]. Moreover, in contrast to the previous study described by Dirix et al. [52], this present study found that the Th2-biased response was no longer detected anymore in the aP group during the second year of life. The reason for these contradictory findings may be differences between the populations of children and the laboratory techniques. However, all studies agreed that most aP- and wP-vaccinated children PBMC displayed *B. pertussis*-specific Th1 and Th2 cytokine responses before the first booster dose is given.

This study provides additional information that Th17 cytokine responses are detected equally among all groups at pre-booster. At one month post-booster, the IL-17A responses were also similar among all groups. It is possible that as children become older, memory Th17 responses become stronger and both aP and wP vaccines can activate an effective Th17 cytokine response which contributes to the protection against pertussis.

The intracellular cytokine staining (ICS) assay was conducted in a subset of samples to further examine the Th1 and Th17 cytokine producing CD4+T cells in response to PT. Overall, the results were consistent with the conclusion drawn from the above cytokine results. The geometric mean percentages of Th17 response at pre-booster for wP (0.73%) and EPI wP (0.94%) were higher than in aP group (0.53%), supporting a trend of a stronger Th17 response in wP-vaccinated children, although it did not reach statistically significant difference likely due to the small sample size. Moreover, at post-booster, PBMC of aP group

showed a significant increase in IFN-γ-producing CD4+ T cell (Th1) and the frequencies were markedly higher compared to wP and EPI wP groups. This finding is also consistent with the cytokine results presented earlier that support a stronger Th1 response in aP-vaccinated children. The post-booster Th17 responses were similar among all groups, again consistent with results as demonstrated in the measurement of cytokines.

Previous clinical studies demonstrated that, in the ^{3}H-thymidine incorporation lymphoproliferative assay, aP-vaccinated children responded to PT better than wP-vaccinated children at the second year of life before the booster dose is given [125]. However, there was no significant difference in lymphoproliferative responses between pre- and post-booster among aP-vaccinated infants [52]. In contrast, this present study found that at pre-booster, there was a trend that the frequencies of proliferating CD3+ and CD4+ T cells were highest in wP-vaccinated infants born to Tdap-vaccinated mothers (wP group). At post-booster, the frequencies of the CD3+ and CD4+ proliferating T cells significantly increased in the aP group and became equal to the wP group. The cell proliferation assay mimicked the response following subsequent antigen exposure. This present study found that at pre-booster, the majority of infants still responded to PT and the booster effect was found only in the aP group. The mechanism of the booster effect in this group needs to be further investigated.

When taking antibody response into account, the anti-PT IgG level was very low at pre-booster but increased significantly at post-booster in both aP and wP groups. The level was similar among aP and wP groups, but lower than the EPI group probably due to the blunting effect caused by maternal Tdap immunization. In contrast, T cell responses were detected at similar level at pre-booster among all groups and maintained the same after the booster. These findings suggest that despite the rapid waning of antibody, memory T cell response exists and could contribute to the protection against pertussis. It was observed the significant correlations between changes in IL-17A levels and anti-PT and anti-FHA IgG

levels in both aP and wP groups, suggesting that T and B cell responses are correlated. The underlying mechanism of B and T cell collaboration needs further investigations.

Recent findings on the effect of maternal immunization on the T cell responses shows that maternally-derived antibodies do not interfere with the cellular immune responses after primary vaccination, despite the complete inhibition of serological response [122, 126]. Nevertheless, Rowe et al. demonstrated that T-cell responses were even boosted as evidenced by increased IL-4, IL-5 and IL-13 responses in the presence of tetanus toxoid maternal antibody, even though the humoral immune response was suppressed [127]. Bertley et al. reported that at five years of age, the pre-existing maternal-derived antibody level was strongly correlated with the lymphoproliferative response against measles vaccine [128]. Our results were in line with the previous findings, as most of the cytokine responses to *B. pertussis* antigens were similar in children born to Tdap or non-Tdap vaccinated mothers. However, there was higher frequencies of proliferating CD3+ and CD4+ T cells in the wP group (with maternal immunization) compared to EPI wP group (without maternal immunization) at pre- and post-booster, suggesting maternal immunization might enhance the cell proliferative responses to wP-containing vaccine.

There are some limitations in this study. The cellular responses were assessed only in a small convenience-sample subset of children in the cohort and understandably may not be generalized for all vaccinated children. Moreover, the number of children in each group was low, and this reduced the power of any statistical comparison between different vaccines and infant groups. Nevertheless, the majority of measured cytokine levels increased after antigen stimulation, and there is a trend supporting a stronger Th1 response in aP-vaccinated and a higher Th17 response in wP-vaccinated infants. There are some un-treated samples (medium control) with high level of cytokines which may be caused by cells that secreted cytokines in response to common childhood infections or allergic diseases. The lack of unvaccinated

infants made it difficult to confirm that these responses are induced by adaptive immunity induced by aP and wP vaccine, not innate immune cells [129]. Further experiments that sort naïve and memory T cells before antigen stimulation will help better clarify that these responses are generated by the *B. pertussis*-specific memory CD4+ T cells.

In conclusion, this study found that aP-vaccinated infants had higher Th1 cytokine responses at pre- and post-pertussis booster than wP-vaccinated children. For Th17 response following primary vaccination, the wP-vaccinated infants appeared to prime a better Th17 response than aP-vaccinated infants. Nevertheless, these differences tend to disappear following the first booster. These cellular immune responses after the primary immunization series, before and after the first booster did not appear to be significantly affected by maternal Tdap vaccination, but the study may not have been powered enough to see subtle differences. The result of this study shed light on kinetics of aP and wP-induced immune response in infants born to Tdap-vaccinated mothers. A long-term follow-up before and after the second booster is ongoing. Longitudinal data will help determine the proper time of pertussis booster in this infant cohort.

Chapter 8

General Discussion and Conclusions

8.1 General discussion

Even though maternal immunization has proven to be safe and effective, some adverse effects have come to light. Recent studies suggest that the presence of maternal antibody at the time of primary vaccination could have a blunting effect on infant specific immune response which may result in decreased disease protection. This may raise concerns regarding the increased susceptibility to disease with no known protective antibody level such as pertussis. However, vaccine protection does not only rely on humoral immunity, as the cellular immune response also plays an important role in providing disease protection. Therefore, a lowered antibody titer caused by the blunting effect may not necessarily imply reduced protection.

Conducting the cell-mediated immune response (CMIR) studies is challenging as it required a large volume of blood samples. It is also costly and labour-intensive. In addition, there is still no accepted standardized CMIR assay, which makes comparison between different studies difficult. This study aims to evaluate and compare the antibody titers and CMIR induced by currently available aP and wP-containing vaccine in infants born to mothers who received Tdap during pregnancy. It was demonstrated here that in the presence of circulating *B. pertussis*-specific maternal antibodies induced by Tdap vaccination, infants who received aP had significantly higher levels of antibodies against all three *B. pertussis* antigens tested at month 7 compared to infants who had received wP. However, at one month post-booster dose (month 19), anti-PT level was similar but anti-FHA and anti-PRN were still higher in the aP group.

The interference of maternal antibodies on the infant immune responses to wP was demonstrated by significantly higher antibody level in the EPI wP group (no maternal immunization) compared to the wP group (with maternal immunization) for all three antigens studied. However, at one month post-booster dose (month 19), it was found that the blunting effect persisted for anti-PT and anti-FHA but not for anti-PRN. The clinical significance of these blunting effects is still unknown. However, monitoring the immunity of this cohort of children should be done in order to understand the long-term impact of these immunological findings.

Unlike the rapid waning of humoral immune response found at pre-booster (month 18), cell-mediated immune response to PT was still detectable and there appeared to be a trend showing aP vaccination activated a stronger Th1, and wP induced a higher Th17 response. These cellular immune responses did not appear to be significantly affected by maternal Tdap vaccination.

Apart from the quantitative analysis of the humoral and cellular responses, the quality or functionality of the vaccine-induced antibodies is also of importance to understand the protective effect induced by different types of vaccines and the clinical implication of the blunting effect. Functionality testing of the antibodies in maternal, cord and infant sera (PT neutralization assay and serum bacterial killing assay) will be conducted and results will be analysed with the antibody concentrations. In addition, further studies regarding the peripheral T follicular helper cells and their roles in helping B cells with antibody production are ongoing.

Further evaluation on the *B. pertussis*-specific IgG subclass distribution and its correlation with T helper cell polarization will reveal changes in each subclass and their correspondence to the T cell response following booster immunization. In addition,

evaluation of breast milk immunoglobulin A (IgA) to PT, FHA and PRN in Tdap-vaccinated women will provide additional information on the extent of maternal-derived IgA transfer which serves as an extra benefit for protection against pertussis during the early months of life.

8.2 Conclusions

Maternal Tdap immunization can potentially reduce the antibody responses in infants vaccinated with the wP vaccine. Long-term monitoring of pertussis vaccine-induced immunity coupled with improved disease surveillance is warranted especially in countries using wP vaccines in their infant immunization program. However, cellular immune responses after the primary immunization and first booster did not appear to be significantly affected by maternal Tdap vaccination.

Chapter 9

Appendices

9.1 Inclusion and exclusion criteria of pregnant women and infants in the study

Inclusion criteria for pregnant women

1. Women aged 18-40 years

2. Willing to be immunized with a pertussis containing vaccine during pregnancy at GA 27-36 weeks gestation

3. Intend to be available for follow-up visits and phone call through 19 months postpartum

4. Willing to have infant immunized with a pertussis containing vaccine at 2, 4, 6 months and 18 months of age

5. At low risk for pregnancy related complications as determined by the investigator and a second trimester ultrasound with no significant abnormalities

Exclusion criteria for pregnant women

Pregnant women who met any exclusion criteria at baseline were excluded from the study.

1. Multiple pregnancies

2. Serious obstetrical risk:

- incompetent cervix

- known placenta previa

- preeclampsia not responding to state of the art treatment

- thyroid diseases not responding to state of the art treatment

- gestational hypertension not responding to state of the art treatment

- diabetes gravidarum

- grand multiparae (>5 pregnancies and deliveries)

- history of PPROM or preterm deliveries <37 weeks

- history of early onset preeclampsia (less than 34 weeks) in previous pregnancies

- repeated (more than three) lost pregnancies

- serious congenital defects in previous children or pregnancies

- any medical condition that shows a risk according to the medical doctors in charge

3. Serious underlying medical condition (e.g., immunosuppressive disease or therapy, human immunodeficiency virus (HIV) infection, collagen vascular disease, diabetes mellitus, chronic hypertension, moderate to severe asthma, lung/heart disease, liver/kidney disease, chronic or recurrent infections, primary immune deficiencies)

4. Significant mental illness (e.g. schizophrenia, psychosis, major depression)

5. History of a febrile illness (greater than or equal to 38° Celsius) within the past 72 hours before injection. Vaccination can be postponed to a later moment if this is the only exclusion criterion

6. Previous severe reaction to any vaccine

7. Receipt of tetanus-diphtheria toxoid immunization within the past 1 month (except for the control group)

8. Receipt of a pertussis containing vaccine (Tdap) immunization in the last 5 years

9. Receipt of a vaccine, blood product (excluding Rhogam) within the 4 weeks prior to injection through 4 weeks following injection and IVIG within 12 weeks period. One month interval should be respected with another vaccine (except influenza) in order to evaluate eventual adverse events following one of both vaccines.

10. Receipt of an experimental drug during pregnancy

11. Anything in the opinion of the investigator that would prevent women from completing the study or put the woman at risk

9.2 Supplementary tables

Table 9.2.1: Duration of redness after Tdap vaccination during pregnancy

ID	D0	D1	D2	D3	D4	D5	D6	D7	Duration (days)
M062	mild redness	NO	NO	NO	NO	NO	NO	NO	1
M078	mild redness	mild redness	mild redness	mild redness	mild redness	mild redness	NO	NO	6
M259	mild redness	mild redness	mild redness	NO	NO	NO	NO	NO	3
M266	mild redness	NO	NO	NO	NO	NO	NO	NO	1
M283	mild redness	mild redness	mild redness	mild redness	NO	NO	NO	NO	4

*n=5, mean duration = 3 days (SD 2.1), median duration = 3 days (25^{th} to 75^{th} percentile 1,5)

Table 9.2.2: Duration of fever after Tdap vaccination during pregnancy

ID	D0	D1	D2	D3	D4	D5	D6	D7	Duration (days)
M009	Low grade fever	NO	NO	NO	NO	NO	NO	NO	1
M022	Low grade fever	Low grade fever	NO	NO	NO	NO	NO	NO	2
M035	Low grade fever	Low grade fever	NO	NO	NO	NO	NO	NO	2
M043	NO	Low grade fever	NO	NO	NO	NO	NO	NO	1
M079	NO	Low grade fever	Low grade fever	Low grade fever	Low grade fever	NO	NO	NO	4
M084	Low grade fever	Low grade fever	NO	NO	NO	NO	NO	NO	2

M137	Low grade fever	Low grade fever	Low grade fever	NO	NO	NO	NO	NO	3
M163	Low grade fever	Low grade fever	NO	NO	NO	NO	NO	NO	2
M167	Low grade fever	Low grade fever	NO	NO	NO	NO	NO	NO	2
M179	Low grade fever	Low grade fever	Low grade fever	Low grade fever	Low grade fever	Low grade fever	NO	NO	6
M212	Low grade fever	Low grade fever	Low grade fever	Low grade fever	NO	NO	NO	NO	4
M224	Low grade fever	NO	NO	NO	NO	NO	NO	NO	1
M257	Low grade fever	Low grade fever	Low grade fever	NO	NO	NO	NO	NO	3
M259	Low grade fever	Low grade fever	Low grade fever	NO	NO	NO	NO	NO	3
M261	NO	Low grade fever	Low grade fever	Low grade fever	Low grade fever	Low grade fever	Low grade fever	NO	6
M278	Low grade fever	Low grade fever	NO	NO	NO	NO	NO	NO	2
M287	Low grade fever	NO	NO	NO	NO	NO	NO	NO	1
M303	Low grade fever	NO	NO	NO	NO	NO	NO	NO	1
M365	Low grade fever	Low grade fever	Low grade fever	NO	NO	NO	NO	NO	3

N=19 mean duration of fever = 2.6 (SD 1.5) median duration = 2 (25^{th} to 75^{th} percentile 1,3)

Table 9.2.3: Duration of swelling after Tdap vaccination during pregnancy

ID	D0	D1	D2	D3	D4	D5	D6	D7	Duration (days)
M062	mild swelling	mild swelling	no	no	no	no	no	no	2
M078	mild swelling	mild swelling	no	no	no	no	no	no	2
M113	mild swelling	mild swelling	mild swelling	no	no	no	no	no	3
M159	mild swelling	mild swelling	no	no	no	no	no	no	2
M176	mild swelling	no	no	no	no	no	no	no	1
M186	mild swelling	mild swelling	no	no	no	no	no	no	2
M221	mild swelling	mild swelling	mild swelling	no	no	no	no	no	3
M249	mild swelling	mild swelling	no	no	no	no	no	no	2
M260	mild swelling	no	no	no	no	no	no	no	1
M261	mild swelling	mild swelling	mild swelling	no	no	no	no	no	3
M262	mild swelling	no	no	no	no	no	no	no	1
M266	mild swelling	mild swelling	no	no	no	no	no	no	2
M299	no	mild swelling	no	no	no	no	no	no	1
M303	mild swelling	mild swelling	no	no	no	no	no	no	2
M339	mild swelling	mild swelling	no	no	no	no	no	no	2

N=15 mean duration of swelling = 1.9 (SD 0.7) median duration = 2 (25^{th} to 75^{th} percentile 1,2)

Table 9.2.4: Duration of pain after Tdap-vaccination during pregnancy

ID	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	duration (days)
M003	mild pain	mild pain	NO	NO	NO	NO	NO	NO	2
M004	moderate pain	NO	NO	NO	NO	NO	NO	NO	1
M005	mild pain	mild pain	mild pain	NO	NO	NO	NO	NO	3
M006	mild pain	mild pain	mild pain	NO	NO	NO	NO	NO	3
M007	mild pain	mild pain	mild pain	NO	NO	NO	NO	NO	3
M008	mild pain	mild pain	mild pain	NO	NO	NO	NO	NO	3
M009	moderate pain	mild pain	mild pain	NO	NO	NO	NO	NO	3
M010	moderate pain	mild pain	NO	NO	NO	NO	NO	NO	2
M011	mild pain	mild pain	mild pain	NO	NO	NO	NO	NO	3
M012	mild pain	mild pain	mild pain	NO	NO	NO	NO	NO	3
M014	mild pain	mild pain	mild pain	NO	NO	NO	NO	NO	3
M015	mild pain	mild pain	mild pain	NO	NO	NO	NO	NO	3
M016	mild pain	mild pain	NO	NO	NO	NO	NO	NO	2
M018	mild pain	mild pain	NO	NO	NO	NO	NO	NO	2
M019	mild pain	mild pain	mild pain	NO	NO	NO	NO	NO	3
M021	mild pain	mild pain	NO	NO	NO	NO	NO	NO	2
M022	mild pain	mild pain	NO	NO	NO	NO	NO	NO	2
M023	NO	mild pain	mild pain	NO	NO	NO	NO	NO	2
M027	mild pain	mild pain	NO	NO	NO	NO	NO	NO	2
M029	mild pain	mild pain	mild pain	mild pain	NO	NO	NO	NO	4
M030	mild pain	mild pain	mild pain	NO	NO	NO	NO	NO	3
M031	mild pain	mild pain	mild pain	mild pain	NO	NO	NO	NO	4
M032	mild pain	mild pain	mild pain	mild pain	NO	NO	NO	NO	4
M033	mild pain	mild pain	mild pain	mild pain	mild pain	NO	NO	NO	5
M034	mild pain	mild pain	NO	NO	NO	NO	NO	NO	2

M036	mild pain	mild pain	NO	NO	NO	NO	NO	NO	2
M037	NO	mild pain	mild pain	NO	NO	NO	NO	NO	2
M039	mild pain	mild pain	mild pain	NO	NO	NO	NO	NO	3
M040	mild pain	mild pain	mild pain	mild pain	mild pain	mild pain	NO	NO	6
M041	NO	mild pain	mild pain	NO	NO	NO	NO	NO	2
M042	mild pain	mild pain	mild pain	mild pain	NO	NO	NO	NO	4
M043	NO	mild pain	NO	NO	NO	NO	NO	NO	1
M045	NO	mild pain	mild pain	NO	NO	NO	NO	NO	2
M046	moderate pain	mild pain	NO	NO	NO	NO	NO	NO	2
M047	NO	mild pain	NO	NO	NO	NO	NO	NO	1
M048	mild pain	mild pain	NO	NO	NO	NO	NO	NO	2
M049	mild pain	mild pain	NO	NO	NO	NO	NO	NO	2
M050	mild pain	mild pain	mild pain	mild pain	mild pain	NO	NO	NO	5
M051	NO	mild pain	NO	NO	NO	NO	NO	NO	1
M052	mild pain	mild pain	mild pain	NO	NO	NO	NO	NO	3
M054	mild pain	NO	NO	NO	NO	NO	NO	NO	1
M055	mild pain	mild pain	NO	NO	NO	NO	NO	NO	2
M056	moderate pain	mild pain	mild pain	mild pain	mild pain	mild pain	NO	NO	6
M057	NO	mild pain	NO	NO	NO	NO	NO	NO	1
M059	mild pain	mild pain	mild pain	mild pain	NO	NO	NO	NO	4
M060	mild pain	mild pain	mild pain	NO	NO	NO	NO	NO	3
M061	mild pain	mild pain	mild pain	NO	NO	NO	NO	NO	3
M062	moderate pain	moderate pain	mild pain	NO	NO	NO	NO	NO	3
M063	mild pain	NO	NO	NO	NO	NO	NO	NO	1
M064	mild pain	mild pain	NO	NO	NO	NO	NO	NO	2
M065	mild pain	mild pain	NO	NO	NO	NO	NO	NO	2
M067	mild pain	mild pain	mild pain	NO	NO	NO	NO	NO	3
M068	mild pain	mild pain	NO	NO	NO	NO	NO	NO	2

M070	mild pain	mild pain	mild pain	NO	NO	NO	NO	NO	3
M071	NO	mild pain	mild pain	NO	NO	NO	NO	NO	2
M072	mild pain	mild pain	NO	NO	NO	NO	NO	NO	2
M073	mild pain	NO	NO	NO	NO	NO	NO	NO	1
M075	NO	mild pain	mild pain	mild pain	mild pain	mild pain	NO	NO	5
M076	moderate pain	moderate pain	moderate pain	mild pain	NO	NO	NO	NO	4
M077	mild pain	mild pain	NO	NO	NO	NO	NO	NO	2
M078	moderate pain	moderate pain	mild pain	NO	NO	NO	NO	NO	3
M079	mild pain	NO	NO	NO	NO	NO	NO	NO	1
M081	mild pain	mild pain	mild pain	NO	NO	NO	NO	NO	3
M082	moderate pain	moderate pain	mild pain	NO	NO	NO	NO	NO	3
M083	mild pain	mild pain	NO	NO	NO	NO	NO	NO	2
M084	moderate pain	mild pain	mild pain	NO	NO	NO	NO	NO	3
M085	mild pain	mild pain	mild pain	NO	NO	NO	NO	NO	3
M088	mild pain	mild pain	mild pain	mild pain	NO	NO	NO	NO	4
M089	NO	mild pain	mild pain	NO	NO	NO	NO	NO	2
M090	mild pain	mild pain	mild pain	NO	NO	NO	NO	NO	3
M091	NO	mild pain	mild pain	NO	NO	NO	NO	NO	2
M092	mild pain	mild pain	mild pain	NO	NO	NO	NO	NO	3
M093	mild pain	mild pain	mild pain	NO	NO	NO	NO	NO	3
M094	moderate pain	moderate pain	mild pain	mild pain	NO	NO	NO	NO	4
M096	mild pain	mild pain	NO	NO	NO	NO	NO	NO	2
M097	mild pain	mild pain	mild pain	NO	NO	NO	NO	NO	3
M100	moderate pain	moderate pain	moderate pain	mild pain	mild pain	mild pain	NO	NO	6

M101	mild pain	mild pain	NO	NO	NO	NO	NO	NO	2
M102	moderate pain	mild pain	NO	NO	NO	NO	NO	NO	2
M103	mild pain	mild pain	NO	NO	NO	NO	NO	NO	2
M104	mild pain	mild pain	NO	NO	NO	NO	NO	NO	2
M105	NO	mild pain	NO	NO	NO	NO	NO	NO	1
M106	moderate pain	mild pain	NO	NO	NO	NO	NO	NO	2
M107	NO	mild pain	mild pain	NO	NO	NO	NO	NO	2
M108	mild pain	mild pain	NO	NO	NO	NO	NO	NO	2
M110	NO	moderate pain	mild pain	NO	NO	NO	NO	NO	2
M111	mild pain	mild pain	NO	NO	NO	NO	NO	NO	2
M112	mild pain	mild pain	NO	NO	NO	NO	NO	NO	2
M113	mild pain	mild pain	mild pain	mild pain	mild pain	mild pain	NO	NO	6
M114	mild pain	mild pain	mild pain	NO	NO	NO	NO	NO	3
M115	mild pain	mild pain	mild pain	NO	NO	NO	NO	NO	3
M116	mild pain	NO	NO	NO	NO	NO	NO	NO	1
M117	mild pain	mild pain	NO	NO	NO	NO	NO	NO	2
M118	mild pain	mild pain	NO	NO	NO	NO	NO	NO	2
M119	mild pain	NO	NO	NO	NO	NO	NO	NO	1
M120	mild pain	mild pain	NO	NO	NO	NO	NO	NO	2
M121	mild pain	mild pain	NO	NO	NO	NO	NO	NO	2
M122	mild pain	mild pain	mild pain	NO	NO	NO	NO	NO	3
M124	mild pain	NO	NO	NO	NO	NO	NO	NO	1
M125	mild pain	mild pain	mild pain	NO	NO	NO	NO	NO	3
M126	mild pain	NO	NO	NO	NO	NO	NO	NO	1
M127	mild pain	mild pain	mild pain	NO	NO	NO	NO	NO	3
M130	mild pain	NO	NO	NO	NO	NO	NO	NO	1
M132	mild pain	mild pain	mild pain	NO	NO	NO	NO	NO	3
M133	mild pain	mild pain	mild pain	NO	NO	NO	NO	NO	3

M134	mild pain	mild pain	mild pain	NO	NO	NO	NO	NO	3
M136	mild pain	NO	NO	NO	NO	NO	NO	NO	1
M137	moderate pain	mild pain	mild pain	NO	NO	NO	NO	NO	3
M138	moderate pain	mild pain	NO	NO	NO	NO	NO	NO	2
M139	mild pain	mild pain	mild pain	NO	NO	NO	NO	NO	3
M141	mild pain	mild pain	NO	NO	NO	NO	NO	NO	2
M142	mild pain	mild pain	mild pain	NO	NO	NO	NO	NO	3
M147	mild pain	mild pain	NO	NO	NO	NO	NO	NO	2
M148	mild pain	mild pain	mild pain	NO	NO	NO	NO	NO	3
M150	moderate pain	mild pain	mild pain	NO	NO	NO	NO	NO	3
M151	mild pain	mild pain	mild pain	NO	NO	NO	NO	NO	3
M152	mild pain	mild pain	mild pain	NO	NO	NO	NO	NO	3
M155	mild pain	mild pain	mild pain	mild pain	NO	NO	NO	NO	4
M157	mild pain	mild pain	NO	NO	NO	NO	NO	NO	2
M159	moderate pain	mild pain	mild pain	NO	NO	NO	NO	NO	3
M160	mild pain	mild pain	mild pain	NO	NO	NO	NO	NO	3
M161	mild pain	mild pain	mild pain	NO	NO	NO	NO	NO	3
M163	moderate pain	mild pain	mild pain	NO	NO	NO	NO	NO	3
M165	mild pain	mild pain	NO	NO	NO	NO	NO	NO	2
M166	mild pain	mild pain	mild pain	mild pain	mild pain	NO	NO	NO	5
M168	mild pain	mild pain	NO	NO	NO	NO	NO	NO	2
M169	mild pain	mild pain	mild pain	NO	NO	NO	NO	NO	3
M171	mild pain	NO	NO	NO	NO	NO	NO	NO	1
M172	mild pain	mild pain	mild pain	NO	NO	NO	NO	NO	3
M174	mild pain	mild pain	mild pain	NO	NO	NO	NO	NO	3
M175	mild pain	mild pain	mild pain	mild pain	NO	NO	NO	NO	4
M176	mild pain	NO	NO	NO	NO	NO	NO	NO	1
M177	mild pain	NO	NO	NO	NO	NO	NO	NO	1
M178	mild pain	mild pain	NO	NO	NO	NO	NO	NO	2

M180	mild pain	mild pain	NO	NO	NO	NO	NO	NO	2
M181	mild pain	mild pain	NO	NO	NO	NO	NO	NO	2
M184	mild pain	mild pain	NO	NO	NO	NO	NO	NO	2
M186	mild pain	mild pain	mild pain	NO	NO	NO	NO	NO	3
M187	mild pain	mild pain	mild pain	NO	NO	NO	NO	NO	3
M188	mild pain	mild pain	mild pain	NO	NO	NO	NO	NO	3
M189	mild pain	mild pain	mild pain	NO	NO	NO	NO	NO	3
M191	mild pain	mild pain	NO	NO	NO	NO	NO	NO	2
M192	mild pain	mild pain	NO	NO	NO	NO	NO	NO	2
M193	mild pain	mild pain	NO	NO	NO	NO	NO	NO	2
M194	mild pain	mild pain	NO	NO	NO	NO	NO	NO	2
M195	moderate pain	moderate pain	moderate pain	mild pain	NO	NO	NO	NO	4
M196	mild pain	mild pain	mild pain	NO	NO	NO	NO	NO	3
M198	mild pain	mild pain	NO	NO	NO	NO	NO	NO	2
M199	mild pain	mild pain	mild pain	NO	NO	NO	NO	NO	3
M200	mild pain	NO	NO	NO	NO	NO	NO	NO	1
M201	mild pain	mild pain	NO	NO	NO	NO	NO	NO	2
M202	mild pain	NO	NO	NO	NO	NO	NO	NO	1
M204	moderate pain	moderate pain	moderate pain	mild pain	NO	NO	NO	NO	4
M205	mild pain	NO	NO	NO	NO	NO	NO	NO	1
M208	mild pain	mild pain	mild pain	NO	NO	NO	NO	NO	3
M209	mild pain	mild pain	NO	NO	NO	NO	NO	NO	2
M210	mild pain	mild pain	NO	NO	NO	NO	NO	NO	2
M211	mild pain	mild pain	mild pain	NO	NO	NO	NO	NO	3
M212	moderate pain	mild pain	mild pain	NO	NO	NO	NO	NO	3
M213	mild pain	mild pain	NO	NO	NO	NO	NO	NO	2
M214	mild pain	mild pain	mild pain	NO	NO	NO	NO	NO	3

M216	mild pain	mild pain	NO	NO	NO	NO	NO	NO	2
M217	mild pain	mild pain	NO	NO	NO	NO	NO	NO	2
M219	moderate pain	mild pain	mild pain	NO	NO	NO	NO	NO	3
M220	mild pain	mild pain	NO	NO	NO	NO	NO	NO	2
M221	mild pain	mild pain	mild pain	NO	NO	NO	NO	NO	3
M222	mild pain	mild pain	mild pain	NO	NO	NO	NO	NO	3
M223	mild pain	mild pain	NO	NO	NO	NO	NO	NO	2
M224	moderate pain	mild pain	NO	NO	NO	NO	NO	NO	2
M226	mild pain	mild pain	mild pain	mild pain	NO	NO	NO	NO	4
M228	mild pain	mild pain	NO	NO	NO	NO	NO	NO	2
M229	mild pain	mild pain	mild pain	NO	NO	NO	NO	NO	3
M231	mild pain	mild pain	mild pain	NO	NO	NO	NO	NO	3
M232	mild pain	mild pain	NO	NO	NO	NO	NO	NO	2
M233	mild pain	mild pain	NO	NO	NO	NO	NO	NO	2
M234	mild pain	NO	NO	NO	NO	NO	NO	NO	1
M235	mild pain	mild pain	NO	NO	NO	NO	NO	NO	2
M238	mild pain	mild pain	mild pain	NO	NO	NO	NO	NO	3
M239	mild pain	mild pain	NO	NO	NO	NO	NO	NO	2
M240	mild pain	NO	NO	NO	NO	NO	NO	NO	1
M241	mild pain	mild pain	NO	NO	NO	NO	NO	NO	2
M242	mild pain	mild pain	NO	NO	NO	NO	NO	NO	2
M243	moderate pain	mild pain	mild pain	NO	NO	NO	NO	NO	3
M245	mild pain	NO	NO	NO	NO	NO	NO	NO	1
M246	mild pain	mild pain	mild pain	NO	NO	NO	NO	NO	3
M247	mild pain	mild pain	NO	NO	NO	NO	NO	NO	2
M249	mild pain	mild pain	mild pain	NO	NO	NO	NO	NO	3
M250	mild pain	mild pain	mild pain	mild pain	NO	NO	NO	NO	4
M252	mild pain	NO	NO	NO	NO	NO	NO	NO	1
M256	mild pain	mild pain	NO	NO	NO	NO	NO	NO	2

M257	mild pain	mild pain	mild pain	NO	NO	NO	NO	NO	3
M258	moderate pain	mild pain	NO	NO	NO	NO	NO	NO	2
M259	mild pain	mild pain	mild pain	NO	NO	NO	NO	NO	3
M260	moderate pain	mild pain	NO	NO	NO	NO	NO	NO	2
M261	mild pain	mild pain	mild pain	NO	NO	NO	NO	NO	3
M262	moderate pain	moderate pain	mild pain	NO	NO	NO	NO	NO	3
M263	mild pain	mild pain	NO	NO	NO	NO	NO	NO	2
M264	mild pain	mild pain	NO	NO	NO	NO	NO	NO	2
M265	mild pain	mild pain	NO	NO	NO	NO	NO	NO	2
M266	moderate pain	mild pain	mild pain	NO	NO	NO	NO	NO	3
M267	mild pain	mild pain	mild pain	mild pain	mild pain	mild pain	NO	NO	6
M268	mild pain	mild pain	NO	NO	NO	NO	NO	NO	2
M270	moderate pain	moderate pain	mild pain	NO	NO	NO	NO	NO	3
M271	mild pain	mild pain	NO	NO	NO	NO	NO	NO	2
M272	mild pain	mild pain	mild pain	NO	NO	NO	NO	NO	3
M273	mild pain	NO	NO	NO	NO	NO	NO	NO	1
M274	moderate pain	mild pain	mild pain	NO	NO	NO	NO	NO	3
M276	moderate pain	moderate pain	mild pain	NO	NO	NO	NO	NO	3
M277	mild pain	NO	NO	NO	NO	NO	NO	NO	1
M278	moderate pain	mild pain	mild pain	mild pain	NO	NO	NO	NO	4
M279	mild pain	NO	NO	NO	NO	NO	NO	NO	1
M281	mild pain	mild pain	NO	NO	NO	NO	NO	NO	2
M282	moderate pain	mild pain	NO	NO	NO	NO	NO	NO	2
M283	mild pain	mild pain	mild pain	mild pain	NO	NO	NO	NO	4
M284	mild pain	mild pain	NO	NO	NO	NO	NO	NO	2
M286	moderate pain	mild pain	mild pain	mild pain	NO	NO	NO	NO	4

M287	mild pain	mild pain	NO	NO	NO	NO	NO	NO	2
M288	mild pain	NO	NO	NO	NO	NO	NO	NO	1
M289	mild pain	NO	NO	NO	NO	NO	NO	NO	1
M290	moderate pain	moderate pain	mild pain	mild pain	NO	NO	NO	NO	4
M291	mild pain	mild pain	mild pain	NO	NO	NO	NO	NO	3
M292	moderate pain	mild pain	NO	NO	NO	NO	NO	NO	2
M293	moderate pain	moderate pain	mild pain	mild pain	NO	NO	NO	NO	4
M294	moderate pain	mild pain	mild pain	NO	NO	NO	NO	NO	3
M295	mild pain	NO	NO	NO	NO	NO	NO	NO	1
M297	mild pain	mild pain	NO	NO	NO	NO	NO	NO	2
M298	mild pain	mild pain	NO	NO	NO	NO	NO	NO	2
M299	mild pain	mild pain	NO	NO	NO	NO	NO	NO	2
M300	moderate pain	mild pain	mild pain	NO	NO	NO	NO	NO	3
M301	mild pain	NO	NO	NO	NO	NO	NO	NO	1
M302	mild pain	NO	NO	NO	NO	NO	NO	NO	1
M303	moderate pain	mild pain	mild pain	NO	NO	NO	NO	NO	3
M305	mild pain	mild pain	mild pain	NO	NO	NO	NO	NO	3
M306	mild pain	mild pain	mild pain	NO	NO	NO	NO	NO	3
M307	mild pain	NO	NO	NO	NO	NO	NO	NO	1
M308	mild pain	NO	NO	NO	NO	NO	NO	NO	1
M310	moderate pain	moderate pain	mild pain	NO	NO	NO	NO	NO	3
M312	mild pain	NO	NO	NO	NO	NO	NO	NO	1
M313	mild pain	NO	NO	NO	NO	NO	NO	NO	1
M314	mild pain	NO	NO	NO	NO	NO	NO	NO	1
M315	mild pain	mild pain	NO	NO	NO	NO	NO	NO	2
M317	mild pain	mild pain	mild pain	NO	NO	NO	NO	NO	3

M318	mild pain	mild pain	NO	NO	NO	NO	NO	NO	2
M319	mild pain	mild pain	mild pain	mild pain	NO	NO	NO	NO	4
M320	moderate pain	moderate pain	mild pain	mild pain	NO	NO	NO	NO	4
M321	mild pain	mild pain	mild pain	NO	NO	NO	NO	NO	3
M323	mild pain	NO	NO	NO	NO	NO	NO	NO	1
M324	mild pain	NO	NO	NO	NO	NO	NO	NO	1
M325	mild pain	mild pain	mild pain	mild pain	NO	NO	NO	NO	4
M326	mild pain	NO	NO	NO	NO	NO	NO	NO	1
M328	mild pain	NO	NO	NO	NO	NO	NO	NO	1
M329	moderate pain	moderate pain	mild pain	NO	NO	NO	NO	NO	3
M330	mild pain	NO	NO	NO	NO	NO	NO	NO	1
M332	moderate pain	mild pain	NO	NO	NO	NO	NO	NO	2
M333	mild pain	mild pain	NO	NO	NO	NO	NO	NO	2
M334	mild pain	NO	NO	NO	NO	NO	NO	NO	1
M335	mild pain	mild pain	NO	NO	NO	NO	NO	NO	2
M336	mild pain	mild pain	NO	NO	NO	NO	NO	NO	2
M337	mild pain	mild pain	NO	NO	NO	NO	NO	NO	2
M338	mild pain	NO	NO	NO	NO	NO	NO	NO	1
M339	moderate pain	mild pain	mild pain	NO	NO	NO	NO	NO	3
M340	mild pain	mild pain	mild pain	NO	NO	NO	NO	NO	3
M342	mild pain	mild pain	NO	NO	NO	NO	NO	NO	2
M343	mild pain	mild pain	mild pain	NO	NO	NO	NO	NO	3
M345	moderate pain	mild pain	mild pain	NO	NO	NO	NO	NO	3
M346	mild pain	mild pain	NO	NO	NO	NO	NO	NO	2
M347	mild pain	mild pain	NO	NO	NO	NO	NO	NO	2
M348	moderate pain	moderate pain	mild pain	NO	NO	NO	NO	NO	3

M351	moderate pain	moderate pain	mild pain	NO	NO	NO	NO	NO	3
M353	mild pain	mild pain	mild pain	NO	NO	NO	NO	NO	3
M354	mild pain	mild pain	NO	NO	NO	NO	NO	NO	2
M356	mild pain	mild pain	NO	NO	NO	NO	NO	NO	2
M357	mild pain	mild pain	mild pain	NO	NO	NO	NO	NO	3
M358	mild pain	mild pain	NO	NO	NO	NO	NO	NO	2
M360	mild pain	mild pain	NO	NO	NO	NO	NO	NO	2
M362	mild pain	mild pain	NO	NO	NO	NO	NO	NO	2
M363	mild pain	NO	NO	NO	NO	NO	NO	NO	1
M364	mild pain	mild pain	mild pain	NO	NO	NO	NO	NO	3
M367	mild pain	NO	NO	NO	NO	NO	NO	NO	1
M368	moderate pain	moderate pain	mild pain	mild pain	NO	NO	NO	NO	4
M369	mild pain	mild pain	NO	NO	NO	NO	NO	NO	2
M370	mild pain	mild pain	mild pain	NO	NO	NO	NO	NO	3

N=282, mean duration of pain = 2.5 days (SE 0.06), median = 2 (range 1-6)

Table 9.2.5: List of outliers removed from the final multiple linear regression analysis to satisfy the assumptions of normality and homoscedasticity (equal variances) of the residuals.

Antibodies to *B. pertussis* antigen tested	Code	Level in cord serum (IU/ml)	Level in serum at month 2 (IU/ml)
Anti-PT IgG	024	18.7	14.7
	031	22.1	78.6
	034	37.3	56.5
	047	2.5	81.0
	057	29.9	34.0
	187	40.1	46.9
	207	9.4	10.7
	221	46.8	52.9
	234	6.728	11.4
	249	21.5	2.5
	269	19.5	2.5
	325	33.1	2.5
	366	20.5	2.5
Anti-FHA IgG	067	767.3	60.1
	232	39.7	2.5
	366	1206.5	2.5
Anti-PRN IgG	057	5.5	22.5
	168	1696.1	5.9
	232	2.5	32.4

Table 9.2.6: Deviations in the study visits

a) Between birth and month 2

No.	Group	Code	Interval (days)	Number of deviated days	Reason
1	aP	C067	78	8	Illness
2	aP	C084	71	1	Delayed visit
3	aP	C100	73	2	Delayed visit
4	aP	C234	79	9	Delayed visit
5	aP	C289	73	3	Illness
6	aP	C333	80	10	Delayed visit
7	wP	C022	71	1	Illness
8	wP	C127	77	7	Delayed visit
9	wP	C153	79	9	Illness
10	wP	C173	71	1	Delayed visit
11	wP	C208	72	2	Delayed visit
12	wP	C215	73	3	Delayed visit
13	EPI wP	C501	88	18	Delayed visit
14	EPI wP	C504	74	4	Delayed visit

b) Between month 2 and month 4

No.	Group	Code	Interval (days)	Number of deviated days	Reason
1	aP	C062	80	10	Delayed visit
2	aP	C316	72	2	Delayed visit
3	aP	C333	77	7	Delayed visit
4	wP	C030	77	7	Illness
5	wP	C184	77	7	Illness
6	wP	C290	77	7	Delayed visit
7	EPI wP	C501	73	3	Delayed visit
8	EPI wP	C511	77	7	Illness
9	EPI wP	C558	73	3	Delayed visit

10	EPI wP	C577	84	14	Illness

c) Between month 4 and month 6

No.	Group	Code	Interval (days)	Number of deviated days	Reason
1	aP	C274	75	5	Illness
2	aP	C299	87	17	Delayed visit
3	aP	C316	84	14	Delayed visit
4	wP	C184	84	14	Delayed visit
5	wP	C230	73	3	Illness
6	wP	C264	77	7	Delayed visit
7	EPI wP	C518	73	3	Illness
8	EPI wP	C521	77	7	Illness
9	EPI wP	C529	77	7	Delayed visit

d) Between month 6 and month 7

No.	Group	Code	Interval (days)	Number of deviated days	Reason
1	aP	C032	42	7	Illness
2	aP	C034	36	1	Delayed visit
3	aP	C052	51	16	Delayed visit
4	aP	C062	27	-1	Limited availability
5	aP	C076	42	7	Illness
6	aP	C090	42	7	Illness
7	aP	C138	42	7	Illness
8	aP	C196	40	5	Illness
9	aP	C207	42	7	Delayed visit
10	aP	C326	27	-1	Limited availability
11	aP	C338	skip	-	Relocation
12	aP	C343	44	9	Delayed visit
13	wP	C041	42	7	Delayed visit
14	wP	C043	39	4	Illness
15	wP	C057	skip	-	Relocation

16	wP	C099	skip	-	Relocation
17	wP	C114	27	-1	Limited availability
18	wP	C119	42	7	Illness
19	wP	C132	49	14	Illness
20	wP	C157	42	7	Delayed visit
21	wP	C209	42	7	Illness
22	wP	C221	skip	-	Relocation
23	wP	C222	37	2	Delayed visit
24	wP	C229	skip	-	Relocation
25	wP	C260	37	2	Delayed visit
26	wP	C302	42	7	Delayed visit
27	wP	C337	42	7	Delayed visit
28	wP	C350	42	7	Illness
29	wP	C351	42	7	Delayed visit
30	wP	C364	47	12	Delayed visit
31	EPI wP	C518	53	18	Delayed visit
32	EPI wP	C519	42	7	Illness
33	EPI wP	C531	49	14	Delayed visit
34	EPI wP	C536	42	7	Delayed visit
35	EPI wP	C542	39	4	Delayed visit
36	EPI wP	C567	41	6	Delayed visit

e) Between month 18 and month 19

No.	Group	Code	Interval (days)	Number of deviated days	Reason
1	aP	C026	49	14	Delayed visit
2	aP	C033	42	7	Delayed visit
3	aP	C047	43	8	Illness
4	aP	C062	44	9	Illness
5	aP	C063	42	7	Delayed visit
6	aP	C088	25	-3	Limited availability
7	aP	C100	49	14	Delayed visit

8	aP	C122	42	7	Illness
9	aP	C150	21	-7	Limited availability
10	aP	C155	42	7	Delayed visit
11	aP	C196	42	7	Illness
12	aP	C206	56	21	Illness
13	aP	C207	42	7	Delayed visit
14	aP	C227	42	7	Illness
15	aP	C232	43	8	Delayed visit
16	aP	C243	36	1	Illness
17	aP	C268	36	1	Delayed visit
18	aP	C273	37	2	Delayed visit
19	aP	C291	42	7	Delayed visit
20	aP	C295	63	28	Delayed visit
21	aP	C320	42	7	Delayed visit
22	aP	C338	50	15	Illness
23	aP	C339	42	7	Delayed visit
24	aP	C342	42	7	Illness
25	wP	C036	59	24	Delayed visit
26	wP	C045	42	7	Delayed visit
27	wP	C115	37	2	Illness
28	wP	C119	42	7	Illness
29	wP	C127	42	7	Delayed visit
30	wP	C132	42	7	Delayed visit
31	wP	C160	49	14	Illness
32	wP	C161	42	7	Delayed visit
33	wP	C166	42	7	Illness
34	wP	C184	49	14	Illness

35	wP	C188	40	5	Illness
36	wP	C229	37	2	Delayed visit
37	wP	C260	42	7	Delayed visit
38	wP	C266	42	7	Delayed visit
39	wP	C359	42	7	Delayed visit
40	wP	C369	45	10	Illness
41	EPI wP	C547	61	26	Delayed visit
42	EPI wP	C550	49	14	Delayed visit
43	EPI wP	C565	25	-3	Limited availability
44	EPI wP	C568	40	5	Illness
45	EPI wP	C575	42	7	Illness

Table 9.2.7: Samples with high IL-6 values that were replaced with the upper limit of quantification.

No.	Time points	Groups	Code	Description
1	7 months	aP	333	PT stimulated
2	7 months	aP	343	PT stimulated
3	7 months	wP	350	PT stimulated
4	7 months	aP	352	PT stimulated
5	7 months	wP	353	PT stimulated
6	7 months	wP	359	PT stimulated
7	7 months	aP	330	Medium control
8	7 months	aP	330	PT stimulated
9	7 months	wP	337	PT stimulated
10	7 months	aP	339	PT stimulated
11	7 months	wP	346	PT stimulated
12	7 months	wP	349	PT stimulated
13	7 months	wP	351	PT stimulated
14	7 months	wP	355	PT stimulated
15	7 months	EPI wP	577	PT stimulated
16	7 months	EPI wP	580	PT stimulated
17	7 months	EPI wP	575	PT stimulated
18	7 months	aP	329	PT stimulated
19	7 months	wP	347	PT stimulated
20	7 months	EPI wP	566	PT stimulated
21	7 months	wP	313	PT stimulated

Table 9.2.8: Codes of individual infants participated in the Cell-Mediated Immune Response study. C denoted children.

	aP	wP	EPI wP
Cytokine levels at month 7	C329 C330 C333 C339 C341 C342 C343 C344 C352 C356 C360 C362 C365	C313 C336 C337 C345 C346 C347 C348 C349 C350 C351 C353 C355 C359 C368 C369	C559 C566 C570 C571 C572 C575 C577 C578 C580
Cytokine levels at month 18 and 19	C207 C220 C228 C242 C253 C316 C317 C318 C321 C324 C328 C330 C334	C183 C197 C200 C208 C209 C217 C286 C298 C312 C336 C348 C349	C524 C529 C540 C550 C553 C557 C558 C562 C564 C566 C577 C578 C579
Intracellular cytokine staining assay	Month 18 C192 C207 C213 C220 C223 C228 C231 C242 C253 C300 C316 C317 C318 C321 C324	Month 18 C183 C193 C197 C208 C209 C216 C217 C224 C236 C239 C246 C266 C269 C286 C298	Month 18 C524 C527 C529 C540 C549 C550 C553 C561 C562 C564 C574 C575

	C328 C333 C339 C343 C352 C356 C358 C359	C304 C310 C312 C313 C325 C336 C348	
	Month 19 C123 C139 C176 C185 C196 C206 C207 C213 C220 C227 C228 C234 C241 C242 C250 C253 C254 C255 C256 C258 C273 C279 C281 C289 C295 C305 C306 C316 C323 C329 C330 C333 C334 C339 C344	Month 19 C148 C160 C161 C173 C183 C184 C193 C197 C200 C208 C209 C210 C215 C217 C229 C230 C237 C252 C257 C264 C266 C277 C286 C297 C298 C308 C311 C331 C332 C336 C348 C349 C350 C351 C364 C368	Month 19 C524 C529 C531 C533 C534 C537 C538 C540 C541 C543 C544 C548 C553 C554 C557 C558 C566 C570 C571 C572 C573 C575 C577 C578 C579 C580
Proliferation assay	Month 18 C174 C192 C207 C213	Month 18 C183 C197 C200 C208	Month 18 C524 C527 C529 C535

	C228 C300 C316 C318 C321 C328 C333 C339 C352 C356 C358	C217 C224 C266 C269 C286 C290 C298 C302 C304 C310 C312 C348 C359	C540 C549 C561 C562 C574 C575
	Month 19 C123 C139 C168 C176 C185 C213 C234 C241 C250 C256 C271 C273 C279 C281 C289 C295 C305 C306 C323 C329 C330 C333 C334 C339 C344	Month 19 C146 C148 C160 C161 C173 C183 C184 C197 C200 C203 C204 C210 C215 C237 C252 C257 C275 C278 C280 C286 C311 C331 C332 C336 C346 C349	Month 19 C524 C529 C531 C533 C534 C537 C540 C544 C546 C553 C554 C557 C558 C566 C570 C572 C575 C577 C578 C579 C580

9.3 List of publications.

No.	Authors	Title	Journal
1.	Wanlapakorn N, Ngaovithunvong V, Thongmee T, Vichaiwattana P, Vongpunsawad S, Poovorawan Y.	Seroprevalence of Antibodies to Pertussis Toxin among Different Age Groups in Thailand after 37 Years of Universal Whole-Cell Pertussis Vaccination.	PLoS One. 2016 Feb 2;11(2):e0148338
2.	Wanlapakorn N, Thongmee T, Vichaiwattana P, Leuridan E, Vongpunsawad S, Poovorawan Y.	Antibodies to *Bordetella pertussis* antigens in maternal and cord blood pairs: a Thai cohort study.	PeerJ. 2017 Nov 23;5:e4043.
3.	Wanlapakorn N, Maertens K, Chaithongwongwatthana S, Srimuan D, Suratannon N, Vongpunsawad S, Mai Phuong Tran T, Hens N , Van Damme P, Locht C, Pooworavan Y, Leuridan E.	Assessing the reactogenicity of Tdap vaccine administered during pregnancy and antibodies to *Bordetella pertussis* antigens in maternal and cord sera of Thai women.	Vaccine. 2018 Mar 7;36(11):1453-1459.

9.4 International presentation

Participating the Fourth International Neonatal and Maternal Immunization Symposium (10-12 September 2017)

1. Oral presentation: “Anti-pertussis titers in maternal and cord sera after Tdap vaccination during pregnancy in Thailand”

2. Poster presentation: “Evaluating the safety of Tdap vaccination during pregnancy in Thailand”

3. Poster presentation: “Seroprevalence of anti-pertussis antibodies in maternal and cord sera of Thai women without pertussis vaccination during pregnancy”

Chapter 10

References

1. Octavia S, Sintchenko V, Gilbert GL, Lawrence A, Keil AD, Hogg G, et al. Newly emerging clones of *Bordetella pertussis* carrying *prn2* and *ptxP3* alleles implicated in Australian pertussis epidemic in 2008-2010. J Infect Dis. 2012;205(8):1220-4.
2. van der Maas NA, Mooi FR, de Greeff SC, Berbers GA, Spaendonck MA, de Melker HE. Pertussis in the Netherlands, is the current vaccination strategy sufficient to reduce disease burden in young infants? Vaccine. 2013;31(41):4541-7.
3. Winter K, Harriman K, Zipprich J, Schechter R, Talarico J, Watt J, et al. California pertussis epidemic, 2010. J Pediatr. 2012;161(6):1091-6.
4. Koh MT, Liu CS, Chiu CH, Boonsawat W, Watanaveeradej V, Abdullah N, et al. Under-recognized pertussis in adults from Asian countries: a cross-sectional seroprevalence study in Malaysia, Taiwan and Thailand. Epidemiol Infect. 2016;144(6):1192-200.
5. Snapp B, Fischetti D. *Bordetella pertussis* infection in infants: a reemerging disease. Adv Neonatal Care. 2013;13(2):103-7.
6. Mooi FR, Van Der Maas NA, De Melker HE. Pertussis resurgence: waning immunity and pathogen adaptation - two sides of the same coin. Epidemiol Infect. 2014;142(4):685-94.
7. Tartof SY, Lewis M, Kenyon C, White K, Osborn A, Liko J, et al. Waning immunity to pertussis following 5 doses of DTaP. Pediatrics. 2013;131(4):e1047-52.
8. Klein NP, Bartlett J, Fireman B, Rowhani-Rahbar A, Baxter R. Comparative effectiveness of acellular *versus* whole-cell pertussis vaccines in teenagers. Pediatrics. 2013;131(6):e1716-22.
9. Warfel JM, Zimmerman LI, Merkel TJ. Acellular pertussis vaccines protect against disease but fail to prevent infection and transmission in a nonhuman primate model. Proc Natl Acad Sci U S A. 2014;111(2):787-92.
10. Liko J, Robison SG, Cieslak PR. Priming with whole-cell versus acellular pertussis vaccine. N Engl J Med. 2013;368(6):581-2.
11. Broder KR, Cortese MM, Iskander JK, Kretsinger K, Slade BA, Brown KH, et al. Preventing tetanus, diphtheria, and pertussis among adolescents: use of tetanus toxoid, reduced diphtheria toxoid and acellular pertussis vaccines recommendations of the Advisory Committee on Immunization Practices (ACIP). MMWR Recomm Rep. 2006;55(RR-3):1-34.
12. Murphy TV, Slade BA, Broder KR, Kretsinger K, Tiwari T, Joyce PM, et al. Prevention of pertussis, tetanus, and diphtheria among pregnant and postpartum women and

their infants recommendations of the Advisory Committee on Immunization Practices (ACIP). MMWR Recomm Rep. 2008;57(RR-4):1-51.

13. Berenson AB, Hirth JM, Rahman M, Laz TH, Rupp RE, Sarpong KO. Maternal and infant outcomes among women vaccinated against pertussis during pregnancy. Hum Vaccin Immunother. 2016;12(8):1965-71.

14. Maertens K, Caboré RN, Huygen K, Hens N, Van Damme P, Leuridan E. Pertussis vaccination during pregnancy in Belgium: Results of a prospective controlled cohort study. Vaccine. 2016;34(1):142-50.

15. Munoz FM, Bond NH, Maccato M, Pinell P, Hammill HA, Swamy GK, et al. Safety and immunogenicity of tetanus diphtheria and acellular pertussis (Tdap) immunization during pregnancy in mothers and infants: a randomized clinical trial. JAMA. 2014;311(17):1760-9.

16. Gall SA, Myers J, Pichichero M. Maternal immunization with tetanus-diphtheria-pertussis vaccine: effect on maternal and neonatal serum antibody levels. Am J Obstet Gynecol. 2011;204(4):334.e1-5.

17. Updated recommendations for use of tetanus toxoid, reduced diphtheria toxoid and acellular pertussis vaccine (Tdap) in pregnant women and persons who have or anticipate having close contact with an infant aged <12 months --- Advisory Committee on Immunization Practices (ACIP), 2011. MMWR Recomm Rep. 2011;60(41):1424-6.

18. Moreno-Perez D, Alvarez Garcia FJ, Aristegui Fernandez J, Cilleruelo Ortega MJ, Corretger Rauet JM, Garcia Sanchez N, et al. Immunisation schedule of the Spanish Association of Paediatrics: 2017 recommendations. An Pediatr (Barc). 2017;86(2):98.e1-98.e9.

19. Kilgore PE, Salim AM, Zervos MJ, Schmitt HJ. Pertussis: Microbiology, Disease, Treatment, and Prevention. Clin Microbiol Rev. 2016;29(3):449-86.

20. Locht C, Antoine R, Jacob-Dubuisson F. *Bordetella pertussis*, molecular pathogenesis under multiple aspects. Curr Opin Microbiol. 2001;4(1):82-9.

21. Hinds PW, 2nd, Yin C, Salvato MS, Pauza CD. Pertussis toxin induces lymphocytosis in rhesus macaques. J Med Primatol. 1996;25(6):375-81.

22. Sim YB, Park SH, Kim SS, Lim SM, Jung JS, Lee JK, et al. Pertussis toxin administered spinally induces a hypoglycemic effect on normal and diabetic mice. Pharmacology. 2014;94(1-2):29-40.

23. Paddock CD, Sanden GN, Cherry JD, Gal AA, Langston C, Tatti KM, et al. Pathology and pathogenesis of fatal *Bordetella pertussis* infection in infants. Clin Infect Dis. 2008;47(3):328-38.

24. Melvin JA, Scheller EV, Miller JF, Cotter PA. *Bordetella pertussis* pathogenesis: current and future challenges. Nat Rev Microbiol. 2014;12(4):274-88.

25. Lapidot R, Gill CJ. The Pertussis resurgence: putting together the pieces of the puzzle. Trop Dis Travel Med Vaccines. 2016;2:26.

26. Baraff LJ, Shields WD, Beckwith L, Strome G, Marcy SM, Cherry JD, et al. Infants and children with convulsions and hypotonic-hyporesponsive episodes following diphtheria-tetanus-pertussis immunization: follow-up evaluation. Pediatrics. 1988;81(6):789-94.

27. Cody CL, Baraff LJ, Cherry JD, Marcy SM, Manclark CR. Nature and rates of adverse reactions associated with DTP and DT immunizations in infants and children. Pediatrics. 1981;68(5):650-60.

28. Pichichero ME, Deloria MA, Rennels MB, Anderson EL, Edwards KM, Decker MD, et al. A safety and immunogenicity comparison of 12 acellular pertussis vaccines and one whole-cell pertussis vaccine given as a fourth dose in 15- to 20-month-old children. Pediatrics. 1997;100(5):772-88.

29. Gustafsson L, Hallander HO, Olin P, Reizenstein E, Storsaeter J. A controlled trial of a two-component acellular, a five-component acellular, and a whole-cell pertussis vaccine. N Engl J Med. 1996;334(6):349-55.

30. The World Health Organization. Immunization, vaccines and biologicals. Data, statistics and graphs. Topic 6. Immunization schedule [Internet]. [cited 2018 Jun 16]. Available from: http://www.who.int/immunization/monitoring_surveillance/data/en/

31. Stein PE, Boodhoo A, Armstrong GD, Cockle SA, Klein MH, Read RJ. The crystal structure of pertussis toxin. Structure. 1994;2(1):45-57.

32. Mazar J, Cotter PA. Topology and maturation of filamentous haemagglutinin suggest a new model for two-partner secretion. Mol Microbiol. 2006;62(3):641-54.

33. Scheller EV, Cotter PA. *Bordetella* filamentous hemagglutinin and fimbriae: critical adhesins with unrealized vaccine potential. Pathog Dis. 2015;73(8):ftv079.

34. Mobberley-Schuman PS, Connelly B, Weiss AA. Phagocytosis of *Bordetella pertussis* incubated with convalescent serum. J Infect Dis. 2003;187(10):1646-53.

35. Phongsisay V, Iizasa E, Hara H, Yoshida H. Pertussis toxin targets the innate immunity through DAP12, FcRgamma, and MyD88 adaptor proteins. Immunobiology. 2017;222(4):664-71.

36. Inatsuka CS, Julio SM, Cotter PA. *Bordetella* filamentous hemagglutinin plays a critical role in immunomodulation, suggesting a mechanism for host specificity. Proc Natl Acad Sci U S A. 2005;102(51):18578-83.

37. Emsley P, Charles IG, Fairweather NF, Isaacs NW. Structure of *Bordetella pertussis* virulence factor P.69 pertactin. Nature. 1996;381(6577):90-2.

38. Henderson IR, Nataro JP. Virulence functions of autotransporter proteins. Infect Immun. 2001;69(3):1231-43.

39. Mills KH, Barnard A, Watkins J, Redhead K. Cell-mediated immunity to *Bordetella pertussis*: role of Th1 cells in bacterial clearance in a murine respiratory infection model. Infect Immun. 1993;61(2):399-410.

40. Redhead K, Watkins J, Barnard A, Mills KH. Effective immunization against *Bordetella pertussis* respiratory infection in mice is dependent on induction of cell-mediated immunity. Infect Immun. 1993;61(8):3190-8.

41. Mills KH, Ryan M, Ryan E, Mahon BP. A murine model in which protection correlates with pertussis vaccine efficacy in children reveals complementary roles for humoral and cell-mediated immunity in protection against *Bordetella pertussis*. Infect Immun. 1998;66(2):594-602.

42. Louten J, Boniface K, de Waal Malefyt R. Development and function of TH17 cells in health and disease. J Allergy Clin Immunol. 2009;123(5):1004-11.

43. Ross PJ, Sutton CE, Higgins S, Allen AC, Walsh K, Misiak A, et al. Relative contribution of Th1 and Th17 cells in adaptive immunity to *Bordetella pertussis*: towards the rational design of an improved acellular pertussis vaccine. PLoS Pathog. 2013;9(4):e1003264.

44. Zhang L, Prietsch SO, Axelsson I, Halperin SA. Acellular vaccines for preventing whooping cough in children. Cochrane Database Syst Rev. 2014(9):Cd001478.

45. Jefferson T, Rudin M, DiPietrantonj C. Systematic review of the effects of pertussis vaccines in children. Vaccine. 2003;21(17-18):2003-14.

46. Diaz-Mitoma F, Halperin SA, Tapiero B, Hoffenbach A, Zappacosta PS, Radley D, et al. Safety and immunogenicity of three different formulations of a liquid hexavalent diphtheria-tetanus-acellular pertussis-inactivated poliovirus-*Haemophilus influenzae* b conjugate-hepatitis B vaccine at 2, 4, 6 and 12-14 months of age. Vaccine. 2011;29(6):1324-31.

47. Edwards KM, Berbers GA. Immune responses to pertussis vaccines and disease. J Infect Dis. 2014;209 Suppl 1:S10-5.

48. Halperin SA, Eastwood BJ, Barreto L, Friesen B, Medd L, Meekison W, et al. Adverse reactions and antibody response to four doses of acellular or whole cell pertussis

vaccine combined with diphtheria and tetanus toxoids in the first 19 months of life. Vaccine. 1996;14(8):767-72.

49. Vermeulen F, Verscheure V, Damis E, Vermeylen D, Leloux G, Dirix V, et al. Cellular immune responses of preterm infants after vaccination with whole-cell or acellular pertussis vaccines. Clin Vaccine Immunol. 2010;17(2):258-62.

50. Mascart F, Verscheure V, Malfroot A, Hainaut M, Pierard D, Temerman S, et al. *Bordetella pertussis* infection in 2-month-old infants promotes type 1 T cell responses. J Immunol. 2003;170(3):1504-9.

51. Mascart F, Hainaut M, Peltier A, Verscheure V, Levy J, Locht C. Modulation of the infant immune responses by the first pertussis vaccine administrations. Vaccine. 2007;25(2):391-8.

52. Dirix V, Verscheure V, Goetghebuer T, Hainaut M, Debrie AS, Locht C, et al. Cytokine and antibody profiles in 1-year-old children vaccinated with either acellular or whole-cell pertussis vaccine during infancy. Vaccine. 2009;27(43):6042-7.

53. The World Health Organization. Immunization, Vaccines and Biologicals. Data, statistics and graphics. Topic 3. Disease Incidence [Internet]. [cited 2018 Jan 26]. Available from: http://www.who.int/immunization/monitoring_surveillance/data/en/

54. The Centers for Disease Control and Prevention. Final Pertussis Surveillance report 2014 [Internet]. [cited 2016 May 8]. Available from: https://www.cdc.gov/pertussis/downloads/pertuss-surv-report-2014.pdf

55. Black RE, Cousens S, Johnson HL, Lawn JE, Rudan I, Bassani DG, et al. Global, regional, and national causes of child mortality in 2008: a systematic analysis. Lancet. 2010;375(9730):1969-87.

56. The Bureau of Epidemiology, The Ministry of Public Health, Thailand. National Disease Surveillance (Report 506): Annual report of pertussis [Internet]. [cited 2017 Aug 12]. Available from: http://www.boe.moph.go.th/Annual/AESR2013/annual/Pertussis.pdf

57. The Bureau of Epidemiology, The Ministry of Public Health, Thailand. National Disease Surveillance (Report 506): Incidence of communicable diseases (Pertussis) in 2015-2016 [Internet]. [cited 2017 May 24]. Available from: http://www.boe.moph.go.th/boedb/surdata/index.php

58. Siriyakorn N, Leethong P, Tantawichien T, Sripakdee S, Kerdsin A, Dejsirilert S, et al. Adult pertussis is unrecognized public health problem in Thailand. BMC Infect Dis. 2016;16:25.

59. Suntarattiwong P. Revisit of pertussis in Thai children. Annual Pediatric Infectious Disease Society of Thailand (PIDST) meeting 12 May 2013. Regent Cha-am Hotel, Petchburee, Thailand. 2013.

60. Vizzotti C, Neyro S, Katz N, Juarez MV, Perez Carrega ME, Aquino A, et al. Maternal immunization in Argentina: A storyline from the prospective of a middle income country. Vaccine. 2015;33(47):6413-9.

61. The Australian Immunization Program for Pregnant women 2015 [Internet]. [cited 2017 Dec 16]. Available from: http://www.immunise.health.gov.au/

62. Amirthalingam G, Andrews N, Campbell H, Ribeiro S, Kara E, Donegan K, et al. Effectiveness of maternal pertussis vaccination in England: an observational study. Lancet. 2014;384(9953):1521-8.

63. Dabrera G, Amirthalingam G, Andrews N, Campbell H, Ribeiro S, Kara E, et al. A case-control study to estimate the effectiveness of maternal pertussis vaccination in protecting newborn infants in England and Wales, 2012-2013. Clin Infect Dis. 2015;60(3):333-7.

64. Vizzotti C, Juarez MV, Bergel E, Romanin V, Califano G, Sagradini S, et al. Impact of a maternal immunization program against pertussis in a developing country. Vaccine. 2016;34(50):6223-8.

65. Wanlapakorn N, Ngaovithunvong V, Thongmee T, Vichaiwattana P, Vongpunsawad S, Poovorawan Y. Seroprevalence of Antibodies to Pertussis Toxin among Different Age Groups in Thailand after 37 Years of Universal Whole-Cell Pertussis Vaccination. PloS One. 2016;11(2):e0148338.

66. Sheridan SL, Ware RS, Grimwood K, Lambert SB. Number and order of whole cell pertussis vaccines in infancy and disease protection. JAMA. 2012;308(5):454-6.

67. Smits K, Pottier G, Smet J, Dirix V, Vermeulen F, De Schutter I, et al. Different T cell memory in preadolescents after whole-cell or acellular pertussis vaccination. Vaccine. 2013;32(1):111-8.

68. Cherry JD. Historical review of pertussis and the classical vaccine. J Infect Dis. 1996;174 Suppl 3:S259-63.

69. van de Waterbeemd B, Streefland M, Pennings J, van der Pol L, Beuvery C, Tramper J, et al. Gene-expression-based quality scores indicate optimal harvest point in *Bordetella pertussis* cultivation for vaccine production. Biotechnol Bioeng. 2009;103(5):900-8.

70. Shamsizadeh A, Nikfar R, Yusefi H, Abbasi-Montazeri E, Cheraghian B. Seroprevalence of pertussis antibodies in 6-17-year-old students in Ahvaz, south-west Islamic Republic of Iran. East Mediterr Health J. 2014;20(10):623-6.

71. Syed MA, Ahmed W, Naeem A, Bokhari H. Seroprevalence of *Bordetella pertussis* antibodies to pertussis toxin among healthy children. J Coll Physicians Surg Pak. 2012;22(10):677-8.

72. Lugauer S, Heininger U, Cherry JD, Stehr K. Long-term clinical effectiveness of an acellular pertussis component vaccine and a whole cell pertussis component vaccine. Eur J Pediatr. 2002;161(3):142-6.

73. Grimprel E, Begue P, Anjak I, Njamkepo E, Francois P, Guiso N. Long-term human serum antibody responses after immunization with whole-cell pertussis vaccine in France. Clin Diagn Lab Immunol. 1996;3(1):93-7.

74. He Q, Viljanen MK, Nikkari S, Lyytikainen R, Mertsola J. Outcomes of *Bordetella pertussis* infection in different age groups of an immunized population. J Infect Dis. 1994;170(4):873-7.

75. Jenkinson D. Duration of effectiveness of pertussis vaccine: evidence from a 10 year community study. Br Med J (Clin Res Ed). 1988;296(6622):612-4.

76. Salmaso S, Mastrantonio P, Tozzi AE, Stefanelli P, Anemona A, Ciofi degli Atti ML, et al. Sustained efficacy during the first 6 years of life of 3-component acellular pertussis vaccines administered in infancy: the Italian experience. Pediatrics. 2001;108(5):E81.

77. Gustafsson L, Hessel L, Storsaeter J, Olin P. Long-term follow-up of Swedish children vaccinated with acellular pertussis vaccines at 3, 5, and 12 months of age indicates the need for a booster dose at 5 to 7 years of age. Pediatrics. 2006;118(3):978-84.

78. The Centers for Disease Control and Prevention. Updated recommendations for use of tetanus toxoid, reduced diphtheria toxoid, and acellular pertussis vaccine (Tdap) in pregnant women--Advisory Committee on Immunization Practices (ACIP), 2012. MMWR Morb Mortal Wkly Rep. 2013;62(7):131-5.

79. de Voer RM, van der Klis FR, Nooitgedagt JE, Versteegh FG, van Huisseling JC, van Rooijen DM, et al. Seroprevalence and placental transportation of maternal antibodies specific for Neisseria meningitidis serogroup C, *Haemophilus influenzae* type B, diphtheria, tetanus, and pertussis. Clin Infect Dis. 2009;49(1):58-64.

80. Ercan TE, Sonmez C, Vural M, Erginoz E, Torunoglu MA, Perk Y. Seroprevalance of pertussis antibodies in maternal and cord blood of preterm and term infants. Vaccine. 2013;31(38):4172-6.

81. Gonik B, Puder KS, Gonik N, Kruger M. Seroprevalence of *Bordetella pertussis* antibodies in mothers and their newborn infants. Infect Dis Obstet Gynecol. 2005;13(2):59-61.

82. Healy CM, Munoz FM, Rench MA, Halasa NB, Edwards KM, Baker CJ. Prevalence of pertussis antibodies in maternal delivery, cord, and infant serum. J Infect Dis. 2004;190(2):335-40.

83. Healy CM, Rench MA, Edwards KM, Baker CJ. Pertussis serostatus among neonates born to Hispanic women. Clin Infect Dis. 2006;42(10):1439-42.

84. Heininger U, Riffelmann M, Leineweber B, Wirsing von Koenig CH. Maternally derived antibodies against *Bordetella pertussis* antigens pertussis toxin and filamentous hemagglutinin in preterm and full term newborns. Pediatr Infect Dis J. 2009;28(5):443-5.

85. Shakib JH, Ralston S, Raissy HH, Stoddard GJ, Edwards KM, Byington CL. Pertussis antibodies in postpartum women and their newborns. J Perinatol. 2010;30(2):93-7.

86. van den Berg JP, Westerbeek EA, Berbers GA, van Gageldonk PG, van der Klis FR, van Elburg RM. Transplacental transport of IgG antibodies specific for pertussis, diphtheria, tetanus, *haemophilus influenzae* type b, and *Neisseria meningitidis* serogroup C is lower in preterm compared with term infants. Pediatr Infect Dis J. 2010;29(9):801-5.

87. Villarreal Perez JZ, Ramirez Aranda JM, de la OCM, Zamudio Osuna MJ, Perales Davila J, Ballesteros Elizondo MR, et al. Randomized clinical trial of the safety and immunogenicity of the Tdap vaccine in pregnant Mexican women. Hum Vaccin Immunother. 2017;13(1):128-35.

88. Hoang HT, Leuridan E, Maertens K, Nguyen TD, Hens N, Vu NH, et al. Pertussis vaccination during pregnancy in Vietnam: Results of a randomized controlled trial pertussis vaccination during pregnancy. Vaccine. 2016;34(1):151-9.

89. Hellwig SM, Rodriguez ME, Berbers GA, van de Winkel JG, Mooi FR. Crucial role of antibodies to pertactin in *Bordetella pertussis* immunity. J Infect Dis. 2003;188(5):738-42.

90. Nooitgedagt JE, de Greeff SC, Elvers BH, de Melker HE, Notermans DW, van Huisseling H, et al. Seroprevalence of *Bordetella pertussis* infection during pregnancy measured by IgG antibodies against pertussis toxin. Clin Infect Dis. 2009;49(7):1086-9.

91. Plans P, Alvarez E, de Ory F, Campins M, Paya T, Balfagon P, et al. Prevalence of antibody to *Bordetella pertussis* in neonates and prevalence of recent pertussis infection in pregnant women in Catalonia (Spain) in 2003 and 2013. Pediatr Infect Dis J. 2014;33(11):1114-8.

92. Plans P, Jansa J, Doshi N, Harrison TG, Plasencia A. Prevalence of pertussis antibodies in umbilical cord blood samples in Catalonia, Spain. Pediatr Infect Dis J. 2008;27(11):1023-5.
93. Hardy-Fairbanks AJ, Pan SJ, Decker MD, Johnson DR, Greenberg DP, Kirkland KB, et al. Immune responses in infants whose mothers received Tdap vaccine during pregnancy. Pediatr Infect Dis J. 2013;32(11):1257-60.
94. Kharbanda EO, Vazquez-Benitez G, Lipkind HS, Klein NP, Cheetham TC, Naleway AL, et al. Maternal Tdap vaccination: Coverage and acute safety outcomes in the vaccine safety datalink, 2007-2013. Vaccine. 2016;34(7):968-73.
95. Englund JA, Anderson EL, Reed GF, Decker MD, Edwards KM, Pichichero ME, et al. The effect of maternal antibody on the serologic response and the incidence of adverse reactions after primary immunization with acellular and whole-cell pertussis vaccines combined with diphtheria and tetanus toxoids. Pediatrics. 1995;96(3 Pt 2):580-4.
96. Agresti A. Categorical data analysis. 2nd ed. New York, NY: Wiley; 2002. 710 p.
97. Eberhardt CS, Blanchard-Rohner G, Lemaitre B, Boukrid M, Combescure C, Othenin-Girard V, et al. Maternal immunization earlier in pregnancy maximizes antibody transfer and expected infant seropositivity against pertussis. Clin Infect Dis. 2016;62(7):829-36.
98. Abu Raya B, Srugo I, Kessel A, Peterman M, Bader D, Gonen R, et al. The effect of timing of maternal tetanus, diphtheria, and acellular pertussis (Tdap) immunization during pregnancy on newborn pertussis antibody levels - a prospective study. Vaccine. 2014;32(44):5787-93.
99. Cabore RN, Maertens K, Dobly A, Leuridan E, Van Damme P, Huygen K. Influence of maternal vaccination against diphtheria, tetanus, and pertussis on the avidity of infant antibody responses to a pertussis containing vaccine in Belgium. Virulence. 2017:1-10.
100. Calvert A, Jones CE. Placental transfer of antibody and its relationship to vaccination in pregnancy. Curr Opin Infect Dis. 2017;30(3):268-73.
101. Morgan JL, Baggari SR, McIntire DD, Sheffield JS. Pregnancy outcomes after antepartum tetanus, diphtheria, and acellular pertussis vaccination. Obstet Gynecol. 2015;125(6):1433-8.
102. Sukumaran L, McCarthy NL, Kharbanda EO, McNeil MM, Naleway AL, Klein NP, et al. Association of Tdap Vaccination with acute events and adverse birth outcomes among pregnant women with prior tetanus-containing immunizations. JAMA. 2015;314(15):1581-7.

103. Donegan K, King B, Bryan P. Safety of pertussis vaccination in pregnant women in UK: observational study. BMJ. 2014;349:g4219.
104. Gkentzi D, Katsakiori P, Marangos M, Hsia Y, Amirthalingam G, Heath PT, et al. Maternal vaccination against pertussis: a systematic review of the recent literature. Arch Dis Child Fetal Neonatal Ed. 2017;102(5):F456-F63.
105. Shakib JH, Korgenski K, Sheng X, Varner MW, Pavia AT, Byington CL. Tetanus, diphtheria, acellular pertussis vaccine during pregnancy: pregnancy and infant health outcomes. J Pediatr. 2013;163(5):1422-6.e1-4.
106. Walls T, Graham P, Petousis-Harris H, Hill L, Austin N. Infant outcomes after exposure to Tdap vaccine in pregnancy: an observational study. BMJ Open. 2016;6(1):e009536.
107. Zheteyeva YA, Moro PL, Tepper NK, Rasmussen SA, Barash FE, Revzina NV, et al. Adverse event reports after tetanus toxoid, reduced diphtheria toxoid, and acellular pertussis vaccines in pregnant women. Am J Obstet Gynecol. 2012;207(1):59.e1-7.
108. Kharbanda EO, Vazquez-Benitez G, Lipkind HS, Klein NP, Cheetham TC, Naleway A, et al. Evaluation of the association of maternal pertussis vaccination with obstetric events and birth outcomes. JAMA. 2014;312(18):1897-904.
109. Layton JB, Butler AM, Li D, Boggess KA, Weber DJ, McGrath LJ, et al. Prenatal Tdap immunization and risk of maternal and newborn adverse events. Vaccine. 2017;35(33):4072-8.
110. Warfel JM, Papin JF, Wolf RF, Zimmerman LI, Merkel TJ. Maternal and neonatal vaccination protects newborn baboons from pertussis infection. J Infect Dis. 2014;210(4):604-10.
111. Feunou PF, Mielcarek N, Locht C. Reciprocal interference of maternal and infant immunization in protection against pertussis. Vaccine. 2016;34(8):1062-9.
112. Ahmad SM, Alam J, Afsar NA, Huda N, Kabir Y, Qadri F, et al. Comparisons of the effect of naturally acquired maternal pertussis antibodies and antenatal vaccination induced maternal tetanus antibodies on infant's antibody secreting lymphocyte responses and circulating plasma antibody levels. Hum Vaccin Immunother. 2016;12(4):886-93.
113. Booy R, Aitken SJ, Taylor S, Tudor-Williams G, Macfarlane JA, Moxon ER, et al. Immunogenicity of combined diphtheria, tetanus, and pertussis vaccine given at 2, 3, and 4 months versus 3, 5, and 9 months of age. Lancet. 1992;339(8792):507-10.

114. Maertens K, Cabore RN, Huygen K, Vermeiren S, Hens N, Van Damme P, et al. Pertussis vaccination during pregnancy in Belgium: Follow-up of infants until 1 month after the fourth infant pertussis vaccination at 15 months of age. Vaccine. 2016;34(31):3613-9.

115. Maertens K, Hoang TT, Nguyen TD, Cabore RN, Duong TH, Huygen K, et al. The effect of maternal pertussis immunization on infant vaccine responses to a booster pertussis-containing vaccine in Vietnam. Clin Infect Dis. 2016;63(suppl 4):S197-S204.

116. Wanlapakorn N, Maertens K, Chaithongwongwatthana S, Srimuan D, Suratannon N, Vongpunsawad S, et al. Assessing the reactogenicity of Tdap vaccine administered during pregnancy and antibodies to *Bordetella pertussis* antigens in maternal and cord sera of Thai women. Vaccine. 2018;36(11):1453-1459.

117. Wanlapakorn N, Thongmee T, Vichaiwattana P, Leuridan E, Vongpunsawad S, Poovorawan Y. Antibodies to *Bordetella pertussis* antigens in maternal and cord blood pairs: a Thai cohort study. PeerJ. 2017;5:e4043.

118. Kent A, Ladhani SN, Andrews NJ, Matheson M, England A, Miller E, et al. Pertussis antibody concentrations in infants born prematurely to mothers vaccinated in pregnancy. Pediatrics. 2016;138(1).

119. Ladhani SN, Andrews NJ, Southern J, Jones CE, Amirthalingam G, Waight PA, et al. Antibody responses after primary immunization in infants born to women receiving a pertussis-containing vaccine during pregnancy: single arm observational study with a historical comparator. Clin Infect Dis. 2015;61(11):1637-44.

120. The European Centre for Disease Prevention and Control. Vaccination in pregnancy (Ireland, portugual, UK,Beligum,Czech republic, Italy) 2017 [internet]. [cited 2017 Jun 15]. Available from: http://vaccine-schedule.ecdc.europa.eu/Pages/Scheduler.aspx

121. Gans HA, Maldonado Y, Yasukawa LL, Beeler J, Audet S, Rinki MM, et al. IL-12, IFN-gamma, and T cell proliferation to measles in immunized infants. J Immunol. 1999;162(9):5569-75.

122. van Binnendijk RS, Poelen MC, van Amerongen G, de Vries P, Osterhaus AD. Protective immunity in macaques vaccinated with live attenuated, recombinant, and subunit measles vaccines in the presence of passively acquired antibodies. J Infect Dis. 1997;175(3):524-32.

123. Warfel JM, Zimmerman LI, Merkel TJ. Comparison of three whole-cell pertussis vaccines in the baboon model of pertussis. Clin Vaccine Immunol. 2015;23(1):47-54.

124. Vermeulen F, Dirix V, Verscheure V, Damis E, Vermeylen D, Locht C, et al. Persistence at one year of age of antigen-induced cellular immune responses in preterm

infants vaccinated against whooping cough: comparison of three different vaccines and effect of a booster dose. Vaccine. 2013;31(15):1981-6.

125. Cassone A, Ausiello CM, Urbani F, Lande R, Giuliano M, La Sala A, et al. Cell-mediated and antibody responses to *Bordetella pertussis* antigens in children vaccinated with acellular or whole-cell pertussis vaccines. The Progetto Pertosse-CMI Working Group. Arch Pediatr Adolesc Med. 1997;151(3):283-9.

126. Siegrist CA, Cordova M, Brandt C, Barrios C, Berney M, Tougne C, et al. Determinants of infant responses to vaccines in presence of maternal antibodies. Vaccine. 1998;16(14-15):1409-14.

127. Rowe J, Poolman JT, Macaubas C, Sly PD, Loh R, Holt PG. Enhancement of vaccine-specific cellular immunity in infants by passively acquired maternal antibody. Vaccine. 2004;22(29-30):3986-92.

128. Bertley FM, Ibrahim SA, Libman M, Ward BJ. Measles vaccination in the presence of maternal antibodies primes for a balanced humoral and cellular response to revaccination. Vaccine. 2004;23(4):444-9.

129. Cua DJ, Tato CM. Innate IL-17-producing cells: the sentinels of the immune system. Nat Rev Immunol. 2010;10(7):479-89.