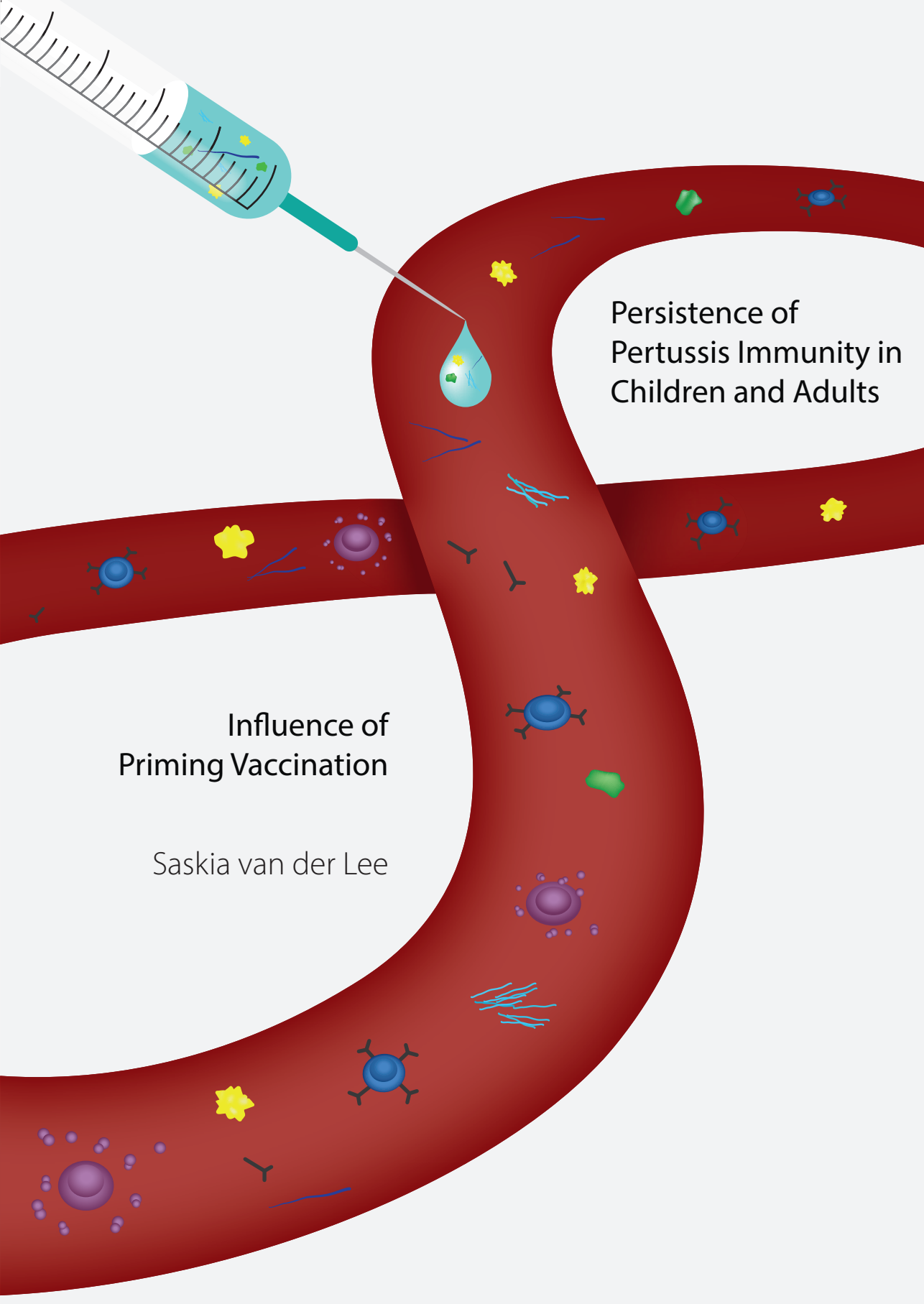

Persistence of Pertussis Immunity in Children and Adults

Influence of Priming Vaccination

Saskia van der Lee

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Persistence of pertussis immunity in children and adults

Influence of priming vaccination

De afweer tegen kinkhoest bij kinderen en volwassenen

Het belang van het type vaccin bij zuigelingen

(met een samenvatting in het Nederlands)

Proefschrift

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Dr. G.A.M. Berbers

Voor mijn ouders

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Chapter 1

General introduction

Clinical manifestation of pertussis

Pertussis, or whooping cough, is a highly contagious infection of the upper respiratory tract and may cause severe clinical disease, particularly in young unvaccinated infants. During a classic pertussis illness, after an asymptomatic incubation period of 7 to 10 days, the catarrhal stage begins with a-specific flu-like symptoms like rhinorrhoea, mild cough and low-grade fever. This first stage lasts for 1 to 2 weeks, during which the frequency and intensity of the cough episodes intensifies and in blood leucocytosis starts. The following paroxysmal stage is characterized by severe and spasmodic cough episodes, with 5 to 10 or more forceful coughs during an episode, followed by an inhalation effort with the classic whoop (from which pertussis derives the name 'whooping cough') and can result in post-cough vomiting. Complications occurring particularly in infants and children include pneumonia, otitis media, seizures and encephalopathy amongst many others, that may sometimes result in death. After several weeks or even months, a gradual transition from the paroxysmal stage to the convalescent stage is observed when a decrease in frequency of the cough episodes occurs followed by a decrease in intensity of these episodes (1, 2). The morbidity and mortality caused by pertussis is highest in infants too young to be vaccinated (<2 months of age), followed by (partially) immunised infants younger than 12 months of age. After infection, children, adolescents and adults can also present classical pertussis symptoms or prolonged coughing for weeks to months, although the majority after previous infections or vaccinations will have subclinical or mild symptoms (1).

Bordetella pertussis

Pertussis is caused by the gram negative bacterium *Bordetella pertussis*, which is a strictly human pathogen and belongs to the genus Bordetella. To a lesser extent, *B. parapertussis*, *B. bronchiseptica* and *B. holmesii* can also cause pertussis-like symptoms in humans (3). The transmission of *B. pertussis* occurs via respiratory droplets and close contact (4), which results in an estimated basic reproductive number of 5.5 (5). *B. pertussis* adheres to the respiratory epithelium using the adhesion molecules filamentous hemagglutinin (FHA), pertactin (Prn) and fimbriae (FIM). After attachment, *B. pertussis* produces several toxins, the most important one being pertussis toxin (PT), an antigen only expressed by *B. pertussis*. Other toxins include adenylate cyclase toxin (ACT), tracheal cytotoxin (TCT), dermonecrotic toxin (DNT), and the endotoxin lipopolysaccharide (LPS) (6).

Pertussis vaccines

In the pre-vaccination era, pertussis was a major cause of childhood morbidity and mortality throughout the world (7). The mean annual pertussis death rate between 1940 and 1948 was 64/100,000 for infants in the USA, this number being higher than

the combined number of deaths in the first year of life resulting from an infection with measles, scarlet fever, diphtheria, polio or meningitis (1). The average total number yearly reported pertussis cases in the USA was 157/100,000 (8), similar to that in Canada (156/100,000) (9), and somewhat lower than in England (230/100,000) (8). Since the 1940s and 1950s, the introduction of whole-cell pertussis (wP) vaccines in infant national immunisation programmes (NIP) dramatically reduced the incidence of pertussis (1, 3, 9, 10). Before the introduction of pertussis vaccines in the Netherlands, pertussis mortality burden contributed to about 3.8% of the total mortality causes, which dropped to nearly zero after the introduction (11). The majority of the wP vaccines showed good efficacy, induced herd protection and proved successful in the prevention of clinical pertussis. However, adverse events like persistent crying, fever, local swelling and pain following wP vaccination were frequently reported (12, 13), which lead to the development of purified acellular pertussis (aP) vaccines, causing far less adverse events (12). Several aP vaccine efficacy trials were conducted in the 1980s and early 1990s, showing high immunogenicity for the aP vaccine antigens and less reactogenicity compared with wP vaccines (14-19). Moreover, aP vaccines were found to be safe to administer as a booster vaccination to children, adolescents and adults (20, 21). The aP vaccines contain between 1 and 5 pertussis-specific antigens, including PT (monovalent vaccine), PT and FHA (bivalent vaccines), PT, FHA and Prn (trivalent vaccines), and PT, FHA, Prn together with two types of fimbriae (FIM2/3) (pentavalent vaccines). Similar to the wP vaccines, aP vaccines are administered in combination with diphtheria toxoid and tetanus toxoid (respectively DTwP and DTaP combination vaccines). From the mid 1990s, aP vaccines replaced the wP vaccines in the infant primary vaccination series in the USA, followed over the next decades by most other high income countries (14).

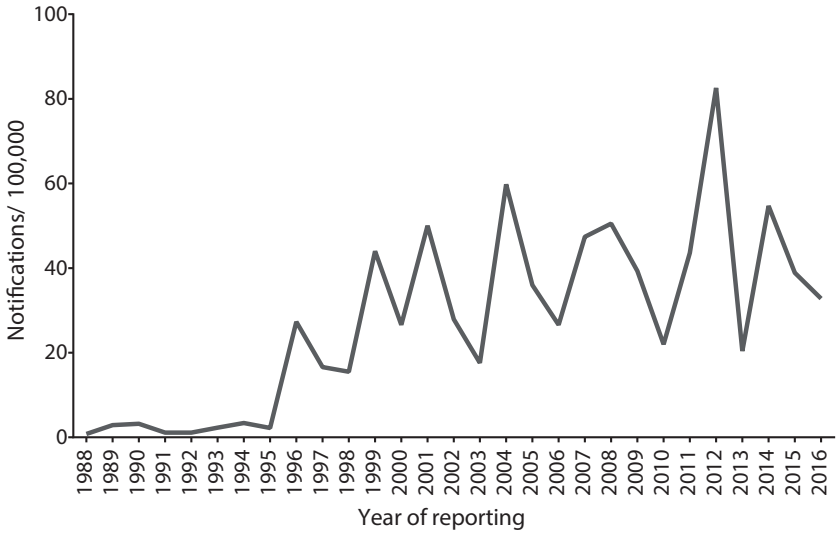

Figure 1. Pertussis notifications in the Netherlands. The yearly incidence (all ages) of reported pertussis cases in the Netherlands per 100,000 from 1988 to 2016.

The resurgence of pertussis and vaccine schedule adaptations

Despite a consistent high pertussis vaccination coverage (>95% in the Netherlands and >90% in other high income countries (22)), pertussis re-emerged in the late 1990s, with cyclic outbreaks reported every 2–3 years in the Netherlands (23) (**Figure 1**), and similar outbreaks occurring in the USA (24), Australia (25) and other countries (9, 26-29).

To reduce the pertussis disease burden, several changes regarding the pertussis vaccines and vaccinations were implemented in the Dutch NIP (**Table 1**). First, in 1997, the potency of the Dutch wP vaccine was increased from 4 to 7 IU. Additionally, to improve early protection of newborns in the first months of life, the vaccination schedule was accelerated in 1999, with primary immunisations starting at 2 months of life instead of 3 months (2, 3, 4, and 11 months of age instead of 3, 4, 5, and 11 months of age). Since older siblings aged 4–6 years showed higher pertussis incidence and were considered main transmitters to infants (30), a pre-school aP booster vaccination was introduced in 2001 in the Dutch NIP for children 4 years of age. Finally, the wP vaccine in the primary vaccination series at 2, 3, 4, and 11 months of age was replaced by the aP vaccine in 2005, in order to reduce adverse events and to induce higher immunogenicity against vaccine antigens. The aP booster vaccination for pre-school children had been implemented in the NIPs of the USA and most other European countries well before the Netherlands (31-34).

Unfortunately, the changes implemented in the NIP did not diminish the increased incidence of pertussis, with the baseline of pertussis notifications increasing over time and in particular incidence rates in newborns in the first months of life. The largest outbreak since the introduction of pertussis vaccines in the Netherlands was reported in 2012. During that epidemic, more than 13,000 pertussis cases were reported and 3 infants, too young to be fully protected by vaccination, died due to a pertussis infection (35). The increase in pertussis cases was especially observed in (pre-) adolescents and also in adults (23). In the Netherlands, an increase in

Table 1. Overview of the Dutch national immunisation programme and changes implemented with regard to pertussis vaccinations.

Year	Priming	Pre-school booster	Remarks
1957	DTwP		Start of Dutch NIP at 3, 4, 5, and 11 months of age
1997	DTwP		Increase of wP potency
1999	DTwP		Acceleration of NIP to 2, 3, 4, and 11 months of age
2001	DTwP	DTaP	Introduction of aP pre-school booster at 4 years of age
2005	DTaP	DTaP	Replacement of wP- by aP-priming vaccines
2017	DTaP	Tdap	Replacement of high-dose by low-dose pre-school booster

Abbreviations: DTwP: diphtheria, tetanus and whole-cell pertussis; DTaP: diphtheria, tetanus and acellular pertussis; NIP: national immunisation programme.

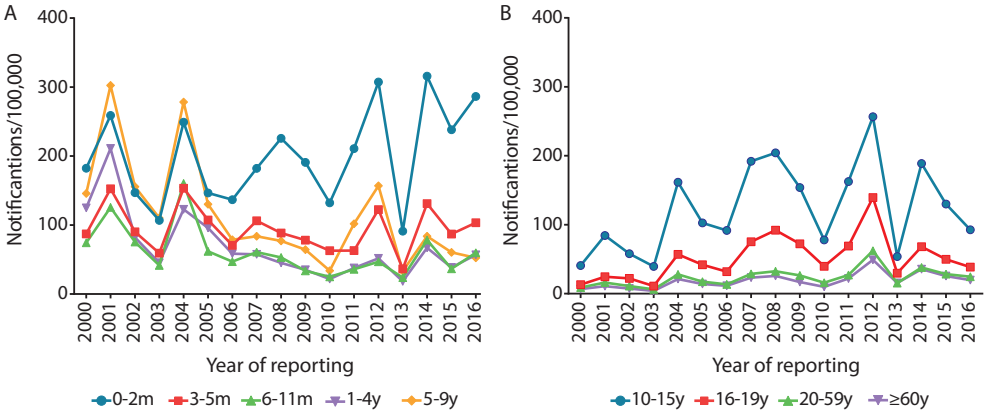

Figure 2. Pertussis notification in the Netherlands by age group. The yearly incidence of reported pertussis cases in the Netherlands per 100,000 **(A)** for children up to 9 years of age, and **(B)** for adolescents and adults.

pertussis cases in children 7–9 years of age was reported from 2011 onwards, 3–5 years after their pre-school aP booster vaccination at 4 years of age (71/100,000 in 2008–2010 and 185/100,000 in 2011–2012) (**Figure 2A**) (23). An increase was also noted in individuals above 10 years of age (35/100,000 in 2008–2010 and 59/100,000 in 2011–2012) (**Figure 2B**). In that same period, the USA reported almost 50,000 pertussis cases, with 16 pertussis related infant deaths (36), and the UK reported >9,000 cases and 14 infant deaths (37). In addition, Dutch serosurveillance studies reported an increase in (sub-) clinical pertussis infections. Two large cross-sectional population-based serosurveillance studies, conducted in 1995–1996 (n = 7,735) and in 2006–2007 (n = 7,903) in the Netherlands, investigated the prevalence of IgG antibodies against PT, since these antibody levels are specific for *B. pertussis* (38, 39). These studies reported PT-IgG levels ≥50 IU/mL, which is indicative for a pertussis infection in the previous year, in 4% of the participants in the first and 9.3% in the second study period, thereby demonstrating that *B. pertussis* circulation and (sub-) clinical pertussis infections showed a twofold increase in only 1 decade.

To protect infants in particular from clinical pertussis until their routine immunisations, several possible vaccination strategies could be considered. Vaccination of neonates directly after birth could induce protective antibodies against pertussis (40). However, two studies showed low efficacy and even an interference with immune responses to subsequent routine immunisations (41, 42). A large observational study in the UK investigated the safety of a maternal Tdap vaccination during pregnancy (43). They did not observe an increase in the risk of stillbirth or other adverse events related to pregnancy compared with matched controls. High maternally derived antibodies protect the infants against clinical pertussis until primary infant vaccinations offer protection (44, 45). The high pertussis related infant mortality in California in 2010 lead to the accelerated introduction of aP vaccinations for pregnant woman in 2011

in the USA (46). The UK implemented maternal pertussis vaccination during the 2012 outbreak, which is currently still advised (43). Already more than 25 countries worldwide have implemented maternal pertussis vaccinations in their NIPs (personal communication H. Campbell, PHE, UK).

Causes of pertussis resurgence

The resurgence of pertussis is multifactorial, and several explanations have been proposed, such as rapid waning immune responses within a few years after vaccination and infection, the switch from wP to aP vaccines with less clearance from *B. pertussis* from the upper airways as compared to wP vaccination and hence more transmission, improved molecular diagnostics, pathogen adaptation, and enhanced surveillance (24, 47-49). In the Netherlands, the resurgence of pertussis was already observed before replacement of wP vaccines by aP vaccines. However, a further increase in the incidence of pertussis notifications was observed several years after the implementation of aP vaccines in infant vaccination series (50-52). This may be related to the fact that though pertussis disease is prevented by aP vaccines in the first years after vaccination (23, 53), protection wanes substantially within 5 years after a pre-school aP booster vaccination (54). The duration of vaccine acquired protection is even more limited after an adolescent aP booster vaccination, dropping to 25% already within 3 years (55). The limited duration of protection after aP boosting was especially observed in (pre-) adolescents who exclusively received aP-priming vaccines during infancy (56, 57). The importance of wP-priming is further illustrated by a reduced risk of pertussis acquisition in wP-primed Australian adolescents compared with aP-primed adolescents, even if they had only received one initial wP vaccine followed by two or more aP vaccines in the infant immunisation series (58). Waning immunity after a natural pertussis infection is also reported with a new clinical pertussis infection after 7–20 years (59). Therefore, vaccine induced life-long protection against pertussis is most likely (very) difficult to achieve.

The pertussis baboon model has demonstrated that aP vaccines do not preclude pertussis colonization and transmission; aP vaccinated baboons, though protected against whooping cough and leucocytosis, can still transmit *B. pertussis* to naïve baboons after inoculation challenge with *B. pertussis* (60). The decreased clearance of *B. pertussis* after aP vaccinations as compared to clinical whooping cough or even wP vaccinations may contribute to the new age groups that represent the source for *B. pertussis* transmission to newborns. Previously, parents were the main source of infection in infants, followed by wP-primed siblings (61-63). In countries who use aP vaccines as priming vaccine for more than 2 decades like the USA (56), aP-primed siblings are now considered the main source of infant pertussis, followed by parents and other relatives (64).

Immunity against *B. pertussis*

Humoral and B-cell immune responses to pertussis vaccines

Pertussis-specific IgG antibodies are induced by natural infection, wP vaccines and aP vaccines. These antibodies play an important role in protection against a (subsequent) clinical symptomatic infection with *B. pertussis* (65, 66), by inhibiting bacterial binding to the epithelium, neutralizing bacterial toxins like PT, enabling bacterial uptake by macrophages and neutrophils and/or by activating the complement system (67). Children with pre-existing high levels of pertussis-specific IgG antibodies were protected against (severe) clinical pertussis when their family members were exposed to *B. pertussis* (68, 69). Furthermore, maternal pertussis-specific IgG antibodies transferred to infants during pregnancy also resulted in protection against clinical pertussis in infants (44, 45). The two types of pertussis vaccines elicit different immune responses after the primary immunisation series: aP-priming vaccines induce higher IgG antibody levels in comparison with wP-priming vaccines (70). This difference in IgG levels is still present at 4 years of age (71). A pre-school DTaP booster vaccination administered at 4 years of age also resulted in higher IgG levels in aP-primed children in comparison with wP-primed children (71). Moreover, children primed with aP vaccines had higher proportions of pertussis-specific IgG4 antibodies compared with wP-primed children, both before and after the pre-school DTaP booster vaccination (72). In line with the observed IgG responses, numbers of pertussis-specific IgG memory B-cells are higher in children 4 years of age and primed with aP vaccines, both before and after the pre-school booster vaccination compared with wP-primed children (73). Systemic specific memory B-cells were even observed in the absence of specific IgG antibody levels. Although antibody levels and in particular PT antibodies rapidly decline after vaccination and also after infection (71, 74, 75), individuals are not directly susceptible to re-infection with pertussis (59). This suggests a potential and important role of vaccine induced memory B-cells in protection against pertussis. In contrast to IgG, systemic pertussis-specific IgA antibodies and IgA memory B-cells are not observed after the infant primary vaccination series, nor after a pre-school DTaP booster vaccination (76). Vaccine studies mostly use IgG responses to the aP vaccine antigens as main outcome to determine immunogenicity of the pertussis vaccines.

T-cell immune responses to pertussis vaccines

Apart from humoral immunity, T-cellular immunity upon infection and vaccination also contributes to protection. Naïve $CD4^+$ T-cells are activated upon the recognition of antigen by the T-cell receptor. Antigen presenting cells present antigens to $CD4^+$ T-cells and secrete cytokines that determine to which lineage the activated $CD4^+$ T-cell will differentiate (77). Each $CD4^+$ T-cell lineage has its own effector function with cytokine secretion profiles (**Figure 3**). The main effector function of T helper 1 (Th1) cells is the clearance of intracellular pathogens, of Th2 cells the clearance

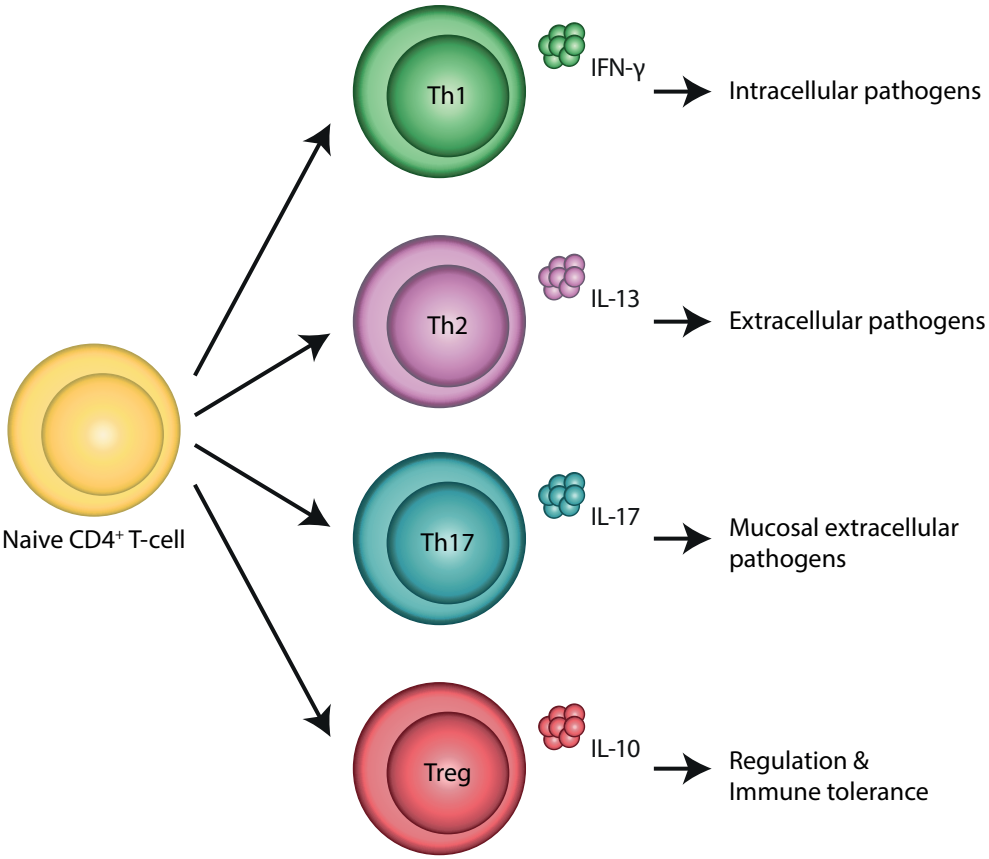

Figure 3. CD4⁺ T-cell differentiation. After activation, a naïve CD4⁺ T-cell will differentiate into one of several distinctive T-cell lineage, of which four are depicted in this figure. Upon activation and differentiation, each T-cell lineage produces a combination of several cytokines. The cytokines indicated in this figure, was used in this thesis as the representative cytokine to characterize the type of T-cell immune response following an acellular pertussis booster vaccination.

of extracellular pathogens, and of Th17 cells the clearance of mucosal extracellular pathogens. T regulatory (Treg) cells are important for the regulation of the immune response and for immune tolerance (78, 79).

Mouse and baboon models show an induction of pertussis-specific Th1 and Th17 cells after an infection with *B. pertussis*, while virtually no increase in pertussis-specific Th2 cells has been observed (80, 81). In humans, a *B. pertussis* infection also results in the induction of pertussis-specific Th1 cells, while less is known about Th17 induction during infection (82-84). With regard to the pertussis vaccines, infants 12 months of age primed with wP vaccines showed a strong Th1 response, similar to pertussis infected infants, while aP-primed infants showed a mixed Th1/Th2 response, though without an outspoken Th17 response (85-87). The difference in Th subsets induced by wP- and aP-priming vaccines was found to persist during early childhood, and

was still observed in children 4 years of age before the pre-school booster (87, 88). This mixed Th1/Th2 immune response in aP-primed children in contrast to wP-primed children is consistent with the higher proportion of pertussis-specific IgG4 levels observed after aP-priming in infancy, since B-cell class switching to IgG4 is stimulated by the Th2 cytokines IL-4 and IL-13 (75, 89).

Scope and outline of this thesis

Epidemiological studies indicate a more limited protection against clinical pertussis in young individuals primed with aP vaccines in infancy compared with wP-primed youngsters. The emerging pertussis outbreaks, the steadily increased notifications in all age groups and increasing number of pertussis related deaths in unvaccinated infants stress the importance of improved pertussis vaccines. Since the development of new vaccines will take many years, adaptation of the current pertussis vaccination schedules may contribute to reduce the current pertussis burden. Several countries recommend aP booster vaccinations for adolescents, adults and pregnant women, in addition to the pre-school aP booster. The long-term immunological responses to booster vaccinations in these age groups are however currently unknown.

Research aims of this thesis

The main objective of the studies described in this thesis is to determine and compare the long-term humoral and cellular immune responses after one or more aP booster vaccinations in (pre-) adolescents, primed in infancy with wP or with aP vaccines, and in wP-primed adults.

The following questions are addressed in this thesis:

1. *What is the (sub-) clinical incidence of pertussis in adolescents during a pertussis outbreak in the Netherlands? Is the current presumed indicator for recent infection discriminative?*
2. *What is the difference between cellular and humoral immune responses induced by a pre-adolescent Tdap booster vaccination after aP vaccinations as compared to wP vaccinations in infancy?*
3. *Is there a difference in vaccine induced humoral and/or cellular immunity between children with and without a clinically severe adverse event following the pre-school DTaP booster vaccination?*
4. *What is the humoral and cellular immune response to a first adult Tdap booster vaccination and what is the duration of high antibody levels after adult Tdap booster vaccination?*

Study outline

Different clinical studies have been used to address each research question. For the first question, samples were used from a longitudinal intervention study

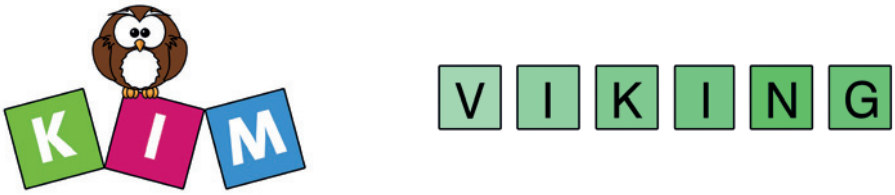

Figure 4. Study logos of the KIM and VIKING study.

which investigated meningococcal serogroup C conjugate booster vaccination in adolescents 10 to 15 years of age (TIM study). The longitudinal samples were collected in 2011, 2012 and 2014, just before and after the largest pertussis outbreak in the Netherlands since the implementation of pertussis vaccines in 1957.

For the second question, children 9 years of age who all had received aP vaccines in infancy were recruited to participate in a longitudinal intervention study (KIM study, **Figure 4**). At the start of the study, they received a Tdap booster immunisation. Blood samples were collected just before, 1 month and 1 year after this pre-adolescent aP booster vaccination. Tdap induced immune responses were compared with results from two previous immunology studies, the Memory and the Booster study. For all three studies, children eligible to participate had to be vaccinated according to the Dutch NIP, i.e. they received either DTwP or DTaP combination vaccines in the first year of life at 2, 3, 4, and 11 months of age. In the Memory study, blood was collected in cross-sectional groups of children, just before the pre-school DTaP booster vaccination at 4 years of age, and 1 month and 2 years (at age 6) after the booster. Similar to the KIM study, in the second longitudinal Booster study, children 9 years of age, primed with wP vaccines, received an additional Tdap booster vaccination. Again, blood samples were collected just before, 1 month and 1 year after this pre-adolescent booster and immune responses were compared.

More adverse events are reported after the pre-school aP booster vaccination since children are primed with aP vaccines compared with wP-primed children. To address the third question, Dutch children whose parents reported a severe adverse event following the pre-school DTaP booster vaccination at 4 years of age (PerVac study), were recruited for this study. Age-matched children without severe adverse events participated as controls.

For the fourth question, adults 25 to 29 years of age were recruited to participate in open-label, phase IV clinical intervention study (VIKING study, **Figure 4**). At the start of the study, the participants received a Tdap booster vaccination and blood samples were collected just before, 2 weeks, 4 weeks, 1 year and 2 years after the Tdap booster vaccination.

In **Chapter 2**, the *B. pertussis* acquisition rate is determined during the pertussis outbreak in 2012 in Dutch adolescents primed with wP vaccines in the first year of

life based on PT-IgG antibodies in blood. The kinetics of the PT-IgG antibody decay is determined after infection to verify the currently used level of PT-IgG antibody that is presumed to be indicative for a recent pertussis infection.

Chapter 3 describes the vaccine antigen-specific humoral IgG responses in children 4 to 10 years of age primed with either wP or aP vaccines in the first year of life, before and after 2 successive aP booster vaccinations at 4 and 9 years of age. Furthermore, vaccine antigen-specific B- and T-cell responses induced by the pre-adolescent Tdap booster vaccination are compared in children 9 years of age. In **Chapter 4**, we determined the specific IgG responses in more detail. IgG subclass responses against the vaccine antigens are determined for children 4 and 9 years of age before and after their respective booster vaccinations. Together, these 2 chapters provide valuable information regarding long-term immunity to pertussis induced by aP booster vaccines and the influence of wP- and aP-priming on these responses. These data corroborate epidemiological findings describing differences in protection against pertussis in adolescents primed with either of these pertussis vaccines.

Chapter 5 compares diphtheria, tetanus and pertussis-specific humoral and cellular immune responses in children 4 years of age with and without a reported severe adverse event after the pre-school DTaP booster vaccination.

In **Chapter 6**, we describe the humoral and cellular immune responses after a first adult Tdap booster vaccination. We estimate the antibody decay of serum PT-IgG antibody levels using a bi-exponential model. With this study, we provide valuable information for public health agencies to optimize vaccination recommendations for adults and pregnant women.

Finally, this thesis concludes with a general discussion summarizing and evaluating the main findings of these clinical studies and discusses recommendations to optimize the national immunisation programmes with regard to pertussis vaccinations (**Chapter 7**).

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Chapter 2

Enhanced *Bordetella pertussis* acquisition rate in adolescents during the 2012 epidemic in the Netherlands and evidence for prolonged antibody persistence after infection

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Abstract

Introduction

In 2012 a large epidemic of pertussis occurred in the Netherlands. We assessed pertussis toxin (PT) antibody levels in longitudinal serum samples from Dutch 10–18 year-olds, encompassing the epidemic, to investigate pertussis infection incidence.

Methods

Blood was sampled in October 2011 (n = 239 adolescents), then 1 year (2012; n = 228) and 3 years (2014; n = 167) later. PT-IgG concentrations were measured by immunoassay and concentrations ≥50 IU/mL (seropositive) assumed indicative of an infection within the preceding year.

Results

During the 2012 epidemic, 10% of participants became seropositive, while this was just 3% after the epidemic. The pertussis acquisition rate proved to be sixfold higher during the epidemic (97 per 1,000 person-years) compared with 2012–2014 (16 per 1,000 person-years). In 2012, pertussis notifications among adolescents nationwide were 228/100,000 (0.23%), which is at least 40 times lower than the seropositivity percentage. Remarkably, 17 of the 22 seropositive participants in 2011, were still seropositive in 2012 and nine remained seropositive for at least 3 years.

Discussion

Longitudinal studies allow a better estimation of pertussis infections in the population. A PT-IgG concentration ≥50 IU/mL as indication of recent infection may overestimate these numbers in cross-sectional serosurveillance and should be used carefully.

Introduction

Pertussis, caused by the bacterium *Bordetella pertussis*, is a vaccine preventable infection of the upper respiratory tract, which is particularly severe in young infants (1, 2). Despite high vaccination coverage, pertussis has re-emerged since the 1990s in most high income countries (3-7).

Serological surveillance studies are a tool to investigate the pertussis infection rate in the population by determining serum IgG antibody concentrations against pertussis toxin (PT). PT is one of the major virulence factors during infection and is only expressed by *B. pertussis* (8-11). A cross-sectional population-based serosurveillance study conducted in the Netherlands in 2006–2007, showed that 9% of adolescents (10–18 years of age) and adults (≥18 years of age) contained a PT serum antibody concentration ≥50 IU/mL, suggestive for a pertussis infection in the preceding year (12). In contrast, the number of reported pertussis cases during that same period was only 0.03% (29.8 cases/100,000 persons) (13). More accurate information regarding pertussis acquisition can be provided by longitudinal studies. Furthermore, longitudinal studies may also illustrate the course of antibody kinetics during pertussis infections and between epidemics.

In 2011–2012, a longitudinal meningococcal vaccination trial was conducted among Dutch adolescents (14). By coincidence, two blood samples from that study were collected encompassing the largest pertussis epidemic in the Netherlands since its resurgence. During that 2012 epidemic, the number of reported pertussis cases increased to 82.8/100,000 persons in the whole Dutch population (13). In addition to the samples from 2011 and 2012, a follow-up sample was collected in 2014, resulting in three longitudinal samples over a period of 3 years. We determined PT-specific IgG antibody concentrations in these samples to investigate the (sub-clinical) pertussis infection rate among adolescents during and after the 2012 epidemic and to explore antibody kinetics after pertussis infection.

Methods

Study design and participants

The samples used in this study originated from a phase IV meningococcal serogroup C conjugated (MenCC) booster vaccine trial (14, 15). In short, adolescents aged 10, 12 and 15 years were vaccinated in October 2011 with a MenCC booster vaccination and blood samples were collected before, 1 month, 1 year and 3 years following vaccination. Participants were randomly selected from four different municipalities in the Netherlands. Samples collected 1 month post-booster vaccination were excluded in the current study. Time of sampling is further indicated by year of sampling.

The study was approved by the Medical research Ethics Committees United (MEC-U, Nieuwegein, the Netherlands). Written informed consent was obtained from both

parents and from participants 12 years and older. The trial was registered at the European Clinical Trials Database (2011-000375-13) and at the Dutch Trial Register (www.trialregister.nl; NTR3521).

Vaccination background

All participants were vaccinated according to the Dutch national immunisation programme (NIP), including four vaccinations with the whole-cell pertussis combination vaccine (DTwP-IPV-Hib, the Netherlands Vaccine Institute (NVI), Bilthoven, the Netherlands) in a 3 + 1 schedule in the first year of life. In addition, the 10 and 12 year-olds received an acellular pertussis vaccine in combination with a DT-IPV booster (NVI) at 4 years of age.

Pertussis toxin specific antibody concentration

Serum PT-IgG antibody concentrations were measured using the fluorescent-bead-based multiplex immunoassay as described (16). The in-house pertussis reference sample was previously calibrated to United States reference pertussis antiserum human lot 3 (Centre for Biologics Evaluation and Research, Food and Drug Administration, Silver Spring, MD). To express PT-IgG concentration in international units (IU) per mL, we extensively compared the in-house reference to the World Health Organization international standard (pertussis antiserum 1st international standard, 06/140, National Institute for Biological Standards and Control, Potters Bar, United Kingdom (17)). We found a small difference with the previous calibration resulting in a correction of a factor 0.8 for PT antibody levels. This implies that the level for recent *B. pertussis* infection changes from 62.5 enzyme-linked immunosorbent assay units (EU) per mL to 50 IU/mL in our laboratory (12, 18). A PT-IgG concentration of ≥50 IU/mL was used as a cut-off for pertussis infection in the preceding year (12) with a specificity of 95% and a sensitivity of 80%, and indicated here as seropositive. Furthermore, a PT-IgG concentration of ≥100 IU/mL was used as cut-off for pertussis infection in the preceding 6 months (12), with a specificity of 99% and sensitivity of 70% (19).

Pertussis surveillance data

To compare the number of seropositive study participants with the reported notifications of all Dutch adolescents of 10–18 years of age, pertussis notification data were extracted from the mandatory national surveillance notification system for vaccine-preventable diseases in the Netherlands, as previously described (7). Then, based on the age of the study participants, all cases who matched in age were grouped per year. In this study, the provided pertussis incidence data were obtained from November to October, as the serology data were also determined in the month October. For example, incidence data of all Dutch 11, 13 and 16 year-olds, which were reported between November 2011 and October 2012, were used for the comparison with serology data at sampling time point October 2012, while incidence data of all

Dutch 13, 15 and 18 year-olds reported between November 2013 and October 2014 were used to compare with the serology data at sampling time point October 2014.

Statistical analyses

Differences in PT-IgG antibody concentrations between the age groups were tested with one-way analysis of variance (ANOVA). PT-IgG antibody concentrations were also dichotomised to study differences in proportion of participants with a PT-IgG concentration ≥50 IU/mL or with a PT-IgG ≥100 IU/mL between time points, and were tested using McNemar tests. A p-value <.05 was considered statistically significant. Chi-squared tests were used to assess difference according to sex within the study population.

The participants who became seropositive during the course of the study were considered to have been infected with *B. pertussis*. The acquisition rates per 1,000 person-years between October 2011 and October 2012 and between October 2012 and October 2014 were determined by dividing the number of new pertussis infected individuals by the total person-time in years of all initially negative individuals (20).

Results

Study population

Of the 268 participants enrolled in the original study, 29 had not given permission for sample analysis beyond the objectives for the meningococcal vaccine trial and were therefore excluded from analysis (14). From the 239 participants available for analysis, blood samples had been collected in 2011 (n = 239), 2012 (n = 228; 95.4%) and 2014 (n = 167; 69.9%). In total, 72 participants were lost to follow-up during the course of the study. These were distributed evenly across all age groups. Baseline characteristics are listed in **Table 1**. There were no differences according to sex within the overall study population (p-value = .134). At the beginning of the study (2011), the PT-IgG geometric mean concentrations (GMCs) were similar between the age groups (GMC of the 10, 12 and 15 year-olds was 5.4, 6.8, and 6.7 IU/mL respectively, p-value = .420), and between males and females (GMCs were 5.8 and 6.7 IU/mL, respectively, p-value = .276).

Table 1. Characteristics of participants at the beginning of the study, the Netherlands, October 2011 (n = 239 participants).

Characteristics	Overall	10 year-olds	12 year-olds	15 year-olds
Number of participants[#]	239	81	82	76
Mean age in years in October 2011 (SD)	NA	9.9 (0.3)	12.0 (0.3)	15.0 (0.3)
Number of participants of male sex (%)	120 (50)	34 (42)	42 (51)	44 (58)

Abbreviations: NA: not applicable; SD: standard deviation.

[#] All participants were primed with a whole-cell pertussis combination vaccine four times in the first year of life (3 + 1 schedule). Ten and 12 year-old participants received an acellular pertussis booster vaccine at 4 years of age.

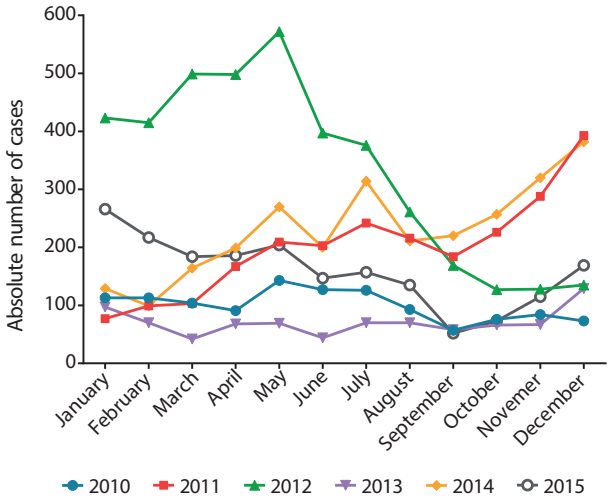

Figure 1. Absolute number of reported pertussis cases for Dutch adolescents 10–18 years of age, January 2010–December 2015 (n = 13,127 total cases).

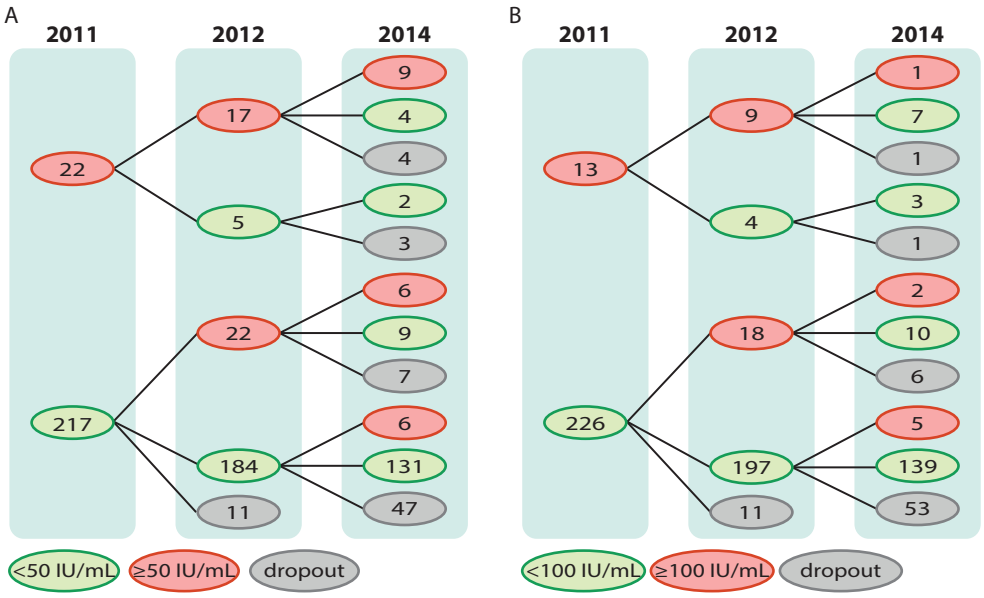

Figure 2. Follow-up of Dutch study participants aged 10–18 years, the Netherlands, 2011–2014 (n = 239 initial participants[#]). (A) Pertussis toxin (PT) IgG concentration ≥50 IU/mL was taken as indicative of a pertussis infection in the preceding year, **(B)** PT-IgG concentration ≥100 IU/mL was taken as indicative of a pertussis infection in the preceding 6 months. Note, the red circles indicate the number of participants above the cut-off; the green circles the number of participants below the cut-off. The number of participants lost to follow-up (dropout) are depicted in grey. [#] A total of 72 participants were lost to follow-up during the course of the study.

Pertussis surveillance data

The number of notified pertussis cases in Dutch adolescents aged 10–18 years for the period 2010–2015 is depicted in **Figure 1**. During the 2012 epidemic, a two fold increase in the number of reported pertussis cases was seen in the respective age range (10–18 years of age) of the study participants, with 115/100,000 (0.12%) cases between November 2010 and October 2011, and 228/100,000 cases (0.23%) between November 2011 and October 2012. After the epidemic, between November 2013 and October 2014, the number of reported cases was 80/100,000 (0.08%).

Pertussis toxin IgG seropositivity per year

In October 2011, 9% (22/239) of the participants had a PT-IgG concentration ≥50 IU/mL. The proportion of participants with a PT-IgG concentration ≥50 IU/mL in 2012 was significantly higher compared with 2011 (17%, 39/228; p-value = .002). In 2014, 13% (21/167) of the participants had a PT-IgG concentration ≥50 IU/mL (p-value = .238 and p-value = .167 compared with 2011 and 2012 respectively). The proportion of participants with a PT-IgG concentration ≥100 IU/mL was highest in 2012 (12%, 27/228) compared with 2011 and 2014 (5% (13/239), p-value = .004 and 5% (8/167), p-value = .017, respectively). For both cut-off PT-IgG concentrations, no differences in seropositivity were found according to sex.

New pertussis infections and acquisition

Of the initially negative participants in 2011, 10% (22/217) were seropositive in 2012, resulting in an acquisition rate of 97 per 1,000 person-years (**Figure 2A**). Of the initially negative participants in 2012, 3% (6/184) became seropositive in 2014 (Figure 2A). The acquisition rate for the period 2012–2014 was 16 per 1,000 person-years. During the 2012 epidemic, the percentage of newly seropositive participants was 44 times higher than the percentage of reported pertussis cases in these age groups.

Kinetics of pertussis toxin specific antibody concentrations

Of the 22 participants who became seropositive (≥50 IU/mL) in 2012, 20 had a PT-IgG concentration <10 IU/mL in 2011. Eighteen of these 22 participants had a PT-IgG concentration >100 IU/mL in 2012 (red dots **Figure 3A**). All participants who became seropositive between 2012 and 2014 (n = 6) had PT-IgG concentrations <10 IU/mL in 2012 (red dots in Figure 3A).

PT-IgG levels in the samples of the 22 seropositive participants in 2011 declined on average 1.8 fold between 2011 and 2012 (orange and green dots in Figure 3A), and five samples dropped below 50 IU/mL in 2012 (green dots in Figure 3A). Three seropositive participants in 2011 showed a 1.2–1.3 fold increase in PT-IgG concentrations in 2012. An average of 3.0 fold antibody decay was observed between 2012 and 2014 in the 39 seropositive participants of 2012 (orange and green dots in Figure 3A).

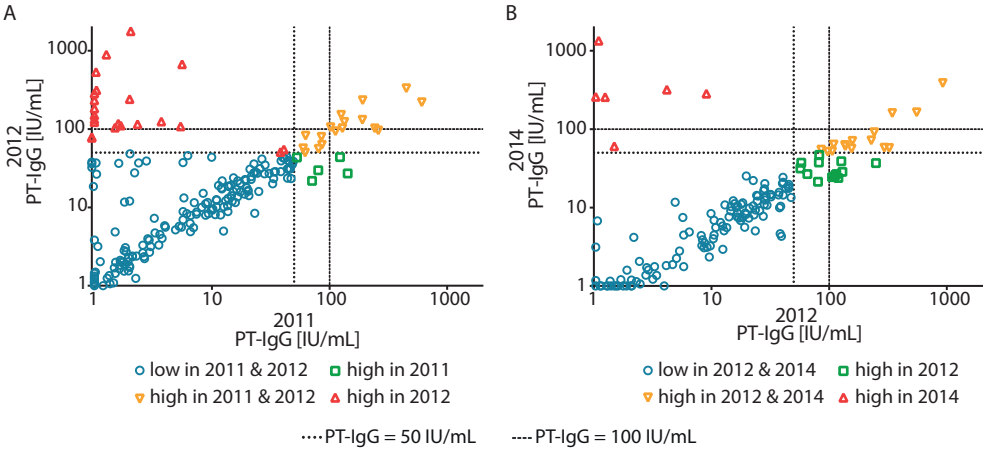

Figure 3. Individuals' paired pertussis toxin (PT) specific IgG concentrations (IU/mL), the Netherlands, 2011–2014 (n = 239 initial participants[#]). (A) 2011 vs 2012, and **(B)** 2012 vs 2014, Note, 'High' is ≥50 IU/mL, 'low' is <50 IU/mL, IU: international units. [#] A total of 72 participants were lost to follow-up during the course of the study.

Duration of seropositivity

In 2012, 17 of the 22 participants with a PT-IgG concentration ≥50 IU/mL in 2011 maintained a PT-IgG concentration ≥50 IU/mL (**Figure 2A**). Of the 39 participants with a PT-IgG concentration ≥50 IU/mL in 2012, 15 maintained this PT-IgG concentration ≥50 IU/mL in 2014. Furthermore, nine of the 22 participants with a PT-IgG concentration ≥50 IU/mL in 2011 maintained a PT-IgG concentration ≥50 IU/mL over the 3-year course of the study (Figure 2A). One participant (1/13) maintained a PT-IgG concentration ≥100 IU/mL for 3 years (**Figure 2B**). In 2012, nine of the 13 participants in 2011 maintained a PT-IgG concentration ≥100 IU/mL and three of the 27 participants in 2012 maintained a PT-IgG concentration ≥100 IU/mL in 2014 (Figure 2B).

Discussion

In this study, PT-specific antibody levels in longitudinal samples from adolescents aged 10–18 years encompassing the pertussis epidemic of 2012 in the Netherlands were assessed. The pertussis infection acquisition rate in the study population was six times higher during the 2012 epidemic (97 per 1,000 person-years), compared with the low-epidemic period of 2013–2014 (16 per 1,000 person-years). Interestingly, 17 of the 22 seropositive participants in 2011 were still seropositive in 2012 and nine remained seropositive for 3 years.

In cross-sectional serosurveillance studies a PT-IgG concentration of ≥50 IU/mL is defined as cut-off for *B. pertussis* infection in the preceding year (11). By applying this cut-off to our cohort, even in low-epidemic years (2011 and 2014) compared with 2012, already 9 (22/239) to 13% (21/167) of the study participants could be considered to be recently infected with *B. pertussis*. This was in line with the cross-sectional serosurveillance study conducted in 2006–2007 in the Netherlands (12) and with other studies from Australia (2007) (21), Belgium (2012) (22), and Norway (2004) (23). At the end of the pertussis epidemic in 2012, the proportion of seropositive adolescents in our cohort almost doubled to 17% (39/228). Moreover, 10% (22/217) of the participants had become seropositive in 2012. This indicates that these participants were actually infected with *B. pertussis* during the epidemic and that pertussis circulation was high. In contrast, only 3% (6/184) of the participants became seropositive between 2012 and 2014. Acquisition rates were determined based on initial seronegative participants, which included also the participants who were unavailable for additional blood samplings during the rest of the study period. Approximately 30% (72/239) of the recruited participants were lost to follow-up between 2011 and 2014. The percentage of participants who became seropositive during the study could possibly have been even higher than indicated, and thereby also the pertussis acquisition rate, as we do not know the status of these participants.

The high pertussis acquisition rate in adolescents observed during the epidemic could be caused by the relatively easy spreading of *B. pertussis* via respiratory droplets (24) and the tendency of adolescents to mix especially with people of the same age (25). However, in a cross-sectional population based serosurveillance study no differences were found in the percentage of seropositive participants between adolescents, adults and elderly (12). This suggests that pertussis acquisition might be comparable for all individuals above 9 years of age due to high transmission through all kinds of routes.

Following individual antibody concentrations, we noticed a limited antibody decay in seropositive samples during the 3 year follow-up. Nine of the 22 initially seropositive participants in 2011 remained seropositive for at least 3 years. This was in agreement with a previous study, where the half-life of PT-IgG antibodies after infection was estimated to be 17 months (26). Although natural boosting of these nine participants

during the study period cannot be excluded, particularly during the epidemic, no increase in PT-IgG antibody concentration was found between 2012 and 2014. This finding emphasises that the use of a PT-IgG concentration off ≥50 IU/mL as a cut-off for pertussis infection in the preceding year leads to an overestimation of pertussis infections. Moreover, a PT-IgG antibody concentration above 100 IU/mL, used as indication of pertussis infection in the preceding 6 months (11, 12), will also result in an overestimation of pertussis infections. At this moment, it seems unclear which PT-IgG cut-off is appropriate to use as an indication for recent pertussis infection.

Since the scope of the original study was to investigate immune responses to a meningococcal serogroup C booster vaccination, information about clinical manifestation and PCR confirmation of pertussis was unavailable. Therefore, we do not know from the seropositive participants if the pertussis infection involved symptoms or not and if they were able to transmit *B. pertussis* to others. Presumably most of the infected participants were asymptomatic or only had mild symptoms, as the majority of pertussis infections do not cause severe morbidity in adolescents (27). It should be noted that pertussis immunisation schedules differed among the 10, 12 and 15 year-old age groups in this study. In 2001, a booster dose with the acellular pertussis vaccine at the age of four years was introduced into the Dutch NIP. Participants aged 15 years at the beginning of the study, were 5 years of age in 2001 and therefore did not receive this booster. However, PT-specific antibodies wane rapidly after acellular pertussis booster vaccination (28, 29), and the booster vaccination has a limited duration of protection (30, 31). Moreover, no differences were found in PT-IgG concentrations between the three age groups at the beginning of the study (2011). Therefore, the effect of this acellular pertussis booster vaccination on the pertussis susceptibility of the participants in our study is likely limited.

Although our findings suggest that numbers of recent pertussis infections indicated in cross-sectional serosurveillance studies could be overestimated, we have provided evidence that the pertussis acquisition rate was 97 per 1,000 person-years during the 2012 pertussis epidemic in our participants, over 40 times higher than the actually reported pertussis cases in these age groups. This suggests that also adolescents could form a large reservoir for *B. pertussis*, which poses a possible threat for young unvaccinated infants who are especially at risk of developing severe illness (32). In order to reduce this risk in neonates, several countries have implemented adolescent pertussis booster vaccinations next to maternal vaccination (33, 34). Unfortunately, whether (repeated) administrations of acellular pertussis booster vaccines can reduce the circulation of *B. pertussis* remains uncertain. In a baboon model, Warfel et al. demonstrated that acellular pertussis vaccines protected against disease, but did not stop transmission and colonisation, while whole-cell pertussis vaccines protected against disease with rapid clearance of *B. pertussis* (35). Furthermore, individuals primed only with acellular pertussis vaccines in the first year of life have an increased risk of acquiring pertussis compared with individuals vaccinated with at least one

whole-cell pertussis vaccine (36-38). Nowadays, industrialised countries use acellular pertussis vaccines in the first year of life for priming. Therefore, enhanced surveillance of pertussis acquisition rates is crucial to monitor pertussis circulation in a population increasingly immunised with the acellular pertussis vaccine.

In conclusion, our results demonstrate that using longitudinal serological studies the acquisition rate of (sub-clinical) pertussis infections can be determined. Thereby, these studies can contribute to a better estimation of the true pertussis incidence in the population. Pertussis incidence in adolescents proved much higher than the number of reported pertussis cases, especially during an epidemic. This indicates that protection against infection conferred by the Dutch national immunisation programme is limited at that age. Furthermore, we highlighted to be cautious applying the current PT-IgG cut-off values in serosurveillance studies, as this will result in an overestimation of the numbers of pertussis infections.

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Chapter 3

Whole-cell or acellular pertussis primary immunisations in infancy determines adolescent cellular immune profiles

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Abstract

Introduction

Pertussis is re-emerging worldwide, despite effective immunisation programmes for infants and children. Epidemiological studies show a more limited duration of protection against clinical pertussis in adolescents primed with acellular pertussis (aP) vaccines during infancy than those who have been primed with whole-cell pertussis (wP) vaccines. This study aimed to determine whether memory immune responses to aP, diphtheria, and tetanus vaccine antigens following booster vaccinations at 4 and 9 years of age differ between wP- versus aP-primed children.

Methods

In a cross-sectional study, blood was collected of DTwP- or DTaP-primed children before, 1 month and 2 years after the pre-school DTaP booster administered at 4 years of age (n = 41–63 per time point). In a longitudinal study, blood was sampled of DTwP- or DTaP-primed children before, 1 month and 1 year after a pre-adolescent Tdap booster at 9 years of age (n = 79–83 per time point). Pertussis, diphtheria and tetanus vaccine antigen-specific IgG levels, B-cell and T-cell responses were determined.

Results

After the pre-school booster vaccination, IgG levels were significantly higher in aP-primed as compared with wP-primed children up till 6 years of age. Before the pre-adolescent Tdap booster vaccination, humoral and cellular immune responses were similar in aP- and wP-primed children. However, the Tdap booster vaccination induced lower vaccine antigen-specific humoral, B-cell and T-helper 1 (Th1) cell responses resulting in significantly lower Th1/Th2 ratios in aP-primed compared with wP-primed children.

Conclusions

The memory immune profiles at pre-adolescent age to all DTaP vaccine antigens are already determined by the wP or aP combination vaccines given in infancy, showing a beneficial Th1-dominated response after wP-priming. These immunological data corroborate epidemiological data showing that DTaP-primed adolescents are less protected against clinical pertussis than DTwP-primed children.

Introduction

Since the introduction of whole-cell pertussis (wP) vaccines in the 1940s, widespread vaccination of children strongly reduced the incidence of clinical pertussis (1). Despite effective infant immunisation programmes and high vaccination coverage in developed countries, pertussis is re-emerging worldwide, not only in non- or partially vaccinated infants, but also in adolescents and adults (2-8). Estimates from the WHO suggest that approximately 16 million cases and 200,000 deaths are due to pertussis annually (9). Large population-based serosurveillance studies conducted in the Netherlands reported up to 9% of asymptomatic pertussis infections in the population above 9 years of age (10, 11).

In a response to the high number of adverse reactions after wP vaccination, acellular pertussis (aP) vaccines have been developed and implemented in the USA from the mid 1990s and subsequently in many national immunization programs (NIP) of high income countries in the following decades (12). In the Netherlands the wP vaccine was replaced by the aP vaccines for the infant priming vaccination series in 2005. The switch from wP to aP vaccines did not cease pertussis re-emergence but rather contributed to the increased incidence of pertussis. Cyclic outbreaks of pertussis are being reported regularly, with the epidemic of 2012 being the largest one since pertussis vaccine introduction (6, 13). The reason of this resurgence is multifactorial, with improved diagnostics, enhanced surveillance and the switch from wP to aP vaccines, but also changes in genetic composition of pertussis strains and especially rapidly waning immunity after vaccination or even after natural infection. Epidemiological studies conducted in the USA and Australia indicated that pre-school (at age 4-6 years) and adolescent (at age 11-13 years) aP booster vaccinations only protect for a few years against clinical pertussis, and that vaccine acquired protection wanes more rapidly in individuals primed with aP vaccines at infancy than individuals who were primed with wP vaccines at infancy (14-16). Furthermore, another study also indicated that the increased clinical pertussis incidence in aP-primed adolescents was not cofounded by age or time since last booster vaccination (17).

This means that significant numbers of aP-primed individuals become susceptible to infection within a few years after a booster vaccination. Pertussis vaccine studies in the baboon model have demonstrated that aP vaccines prevent clinical disease, but do not preclude asymptomatic infection, colonisation and transmission, which is associated with a lack of T helper 1 (Th1) immune responses (18).

The immune mechanisms important for protection against pertussis in humans still remain elusive. Protection is suggested to be mediated by both humoral and cellular immunity (19, 20). Higher pertussis-specific antibody levels and memory B-cell responses have been reported in aP-primed versus wP-primed children after a diphtheria, tetanus and aP (DTaP) booster at age 4 years (21, 22). Additionally, DTaP booster vaccination induced higher Th1 and Th2 T-cell responses in pre-school aP-

primed children compared with wP-primed children (23). Th1 cells are crucial for bacterial clearance and therefore more associated with protection against pertussis than Th2 cells (24).

To elucidate the immune mechanism in relation to long-term protection against pertussis, it is important to evaluate pertussis-specific immune responses over time. In this study, long-term humoral and cellular immune responses to pertussis have been determined in groups of children till adolescent age who received either DTwP or DTaP combination vaccines in infancy following two successive pertussis booster vaccinations at the age of 4 and 9 years.

Methods

Study design and participants

For this study, blood samples were collected at 6 different time points from children primed with either wP or aP combination vaccines in the first year of life (**Figure 1**). The participants received their vaccinations according to the Dutch NIP: DTwP or DTaP at 2, 3, 4, and 11 months of age and DTaP at 4 years of age. Blood was cross-sectionally sampled before the pre-school DTaP booster vaccination at age 4 years, and 1 month and 2 years after the pre-school booster. After an additional Tdap booster vaccination at 9 years of age, blood was longitudinally sampled before, 1 month and 1 year from groups of wP- or aP-primed children. This aP booster is not implemented in the Dutch NIP, therefore, these children participated in a longitudinal phase IV intervention study. All information regarding study recruitment, characteristics and flow charts were previously described (ISRCTN65428640 (25) and ISRCTN64117538 (26, 27)), except for the aP-primed children 9 years of age. Information regarding the recruitment and flow chart of these aP-primed 9-years-olds is given in supplementary Method 1 and supplementary Figure 1 (2013-001864-50 [eudract.ema.europa.eu]; NTR4089 [trialregister.nl]). Sex distribution between the wP-primed and aP-primed groups was similar at all time points. For all participants, written informed consent was obtained from both parents or legal representatives in accordance with the Declaration of Helsinki.

Vaccination background

During infancy, children received either a diphtheria, tetanus, whole-cell pertussis, inactivated polio virus, Haemophilus influenza type b (DTwP-IPV-Hib; Netherlands vaccine institute, Bilthoven, the Netherlands) (wP-primed children), or a DTaP-IPV-Hib (Infanrix-IPV-HibTM, GlaxoSmithKline (GSK), Rixensart, Belgium) (aP-primed children) combination vaccine at 2, 3, 4, and 11 months of age. Children received a paediatric DTaP booster vaccination at 4 years of age (Infanrix-IPVTM, GSK; containing 25 µg pertussis toxin (PT) and filamentous hemagglutinin (FHA), 8 µg pertactin (Prn), ≥30 IU (international units) diphtheria toxoid (Dd) and ≥40 IU tetanus toxoid (Td)). In addition, a Tdap booster vaccination was administered to children 9 years of age

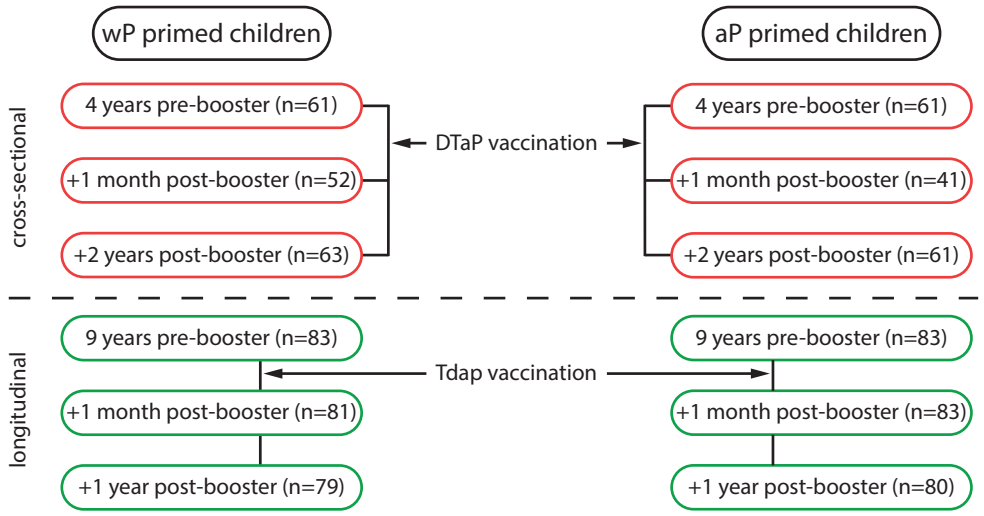

Figure 1. Overview of the study groups. Schematic overview of the 6 blood sampling time points. Children were primed with either whole-cell pertussis (wP) or acellular pertussis (aP) combination vaccines in the first year of life (2, 3, 4, 11 months of age) and received a DTaP booster vaccination at 4 years of age, all according to the Dutch national immunisation programme. Groups of children were sampled cross-sectionally pre-booster, 1 month and 2 years (at age 6 years) post DTaP booster vaccination at 4 years of age (indicated in red) (25). In addition, children 9 years of age, primed with either wP vaccines (26, 27) or with aP vaccines (NTR4089, supplementary Methods 1 and supplementary Figure 1) received an additional Tdap booster vaccination and were sampled longitudinally before, 1 month and 1 year after the booster (indicated in green).

(Boostrix-IPVTM, GSK; containing 8 µg PT and FHA, 2.5 µg Prn, ≥2 IU Dd and ≥20 IU Td).

Blood samples

Blood was sampled at 4 years of age, before the DTaP booster vaccination, 1 month (28 days ± 2 days) and 2 years (± 2 weeks) after the DTaP booster. From children 9 years of age, blood was sampled just before, 1 month (28 days ± 2 days) and 1 year (± 2 weeks) after a Tdap booster vaccination. Vacutainer cell preparation tubes containing sodium citrate (Becton Dickinson Biosciences, San Diego, CA) were used for all blood samplings. Peripheral blood mononuclear cells (PMBCs) were isolated within 18 hours and stored at –135ºC, and plasma was stored at –20ºC as described (28).

Serological analysis

Plasma IgG antibody concentrations against PT, FHA, Prn, Dd and tetanus toxin were quantified using the fluorescent-bead-based multiplex immunoassay as described (2, 29). The WHO international standard (pertussis antiserum 1st international standard, 06/140, NIBSC, Potters Bar, UK) was used to express pertussis IgG concentrations in IU/mL.

Memory B- and T-cell responses

From a randomly selected subset of 20 longitudinal blood samples of the children aged 9 years, PT-, FHA-, Prn- and Td-specific enzyme-linked immunospot (ELISPOT) assays were performed on purified B-cells to determine the numbers of antigen-specific IgG producing memory B-cells (22, 28). The same PBMC samples, depleted of CD19+ cells, were stimulated for 5 days with PT (heat inactivated), FHA, Prn, Td or pokeweed mitogen and culture supernatants were stored at –80ºC (23). In these supernatants, the cytokines interferon-gamma (IFN-γ) (Th1), interleukin-13 (IL-13) (Th2), IL-17 (Th17), and IL-10 (Treg) were quantified using the Bio-Plex cytokine assay kits (Bio-Rad Laboratories, Hercules, CA) (27).

Statistical analyses

Geometric mean concentrations (GMC) with corresponding 95% confidence intervals were calculated for antigen-specific IgG and cytokine responses. Numbers of antigen-specific memory B-cells were counted per 105 B-cells and corresponding geometric mean values were calculated. Normal distribution of (log-transformed) data was checked prior to analysis. Differences in IgG concentrations between independent or paired samples were tested with corresponding t-tests. Differences in not normally distributed B-cell numbers and cytokine concentrations were tested with Mann-Whitney U (independent samples) or Wilcoxon Signed Ranks (paired samples) tests. Differences in proportion of children with a PT-IgG concentration ≥50 IU/mL were tested with Chi-Square tests. Within the longitudinal studies, p-values were corrected for multiple testing according to the Bonferroni test. P-value <.05 was considered statistically significant. Data were analysed using GraphPad Prism 6 (GraphPad Software, La Jolla, CA), and SPSS statistics 22 (IBM, Armonk, NY).

Results

Study groups

The study characteristics of wP- and aP-primed children 4–6 years of age and wP-primed children 9 years of age have been described previously (25-27). The flow chart of aP-primed children 9 years of age are depicted in supplementary Figure 1. A schematic overview of all groups is depicted in Figure 1.

IgG antibody levels after the pre-school DTaP booster vaccination in children 4 to 9 years of age

After the pre-school DTaP booster, pertussis-specific IgG levels were significantly higher in aP-primed compared with wP-primed children up till 6 years of age (**Figure 2** and supplementary Table 1). Moreover, Prn-IgG levels remained significantly higher in aP-primed children up till 9 years of age (Figure 2). In contrast, diphtheria-IgG levels were significantly lower up till 6 years of age in aP-primed as compared with wP-primed children, whereas no differences were observed for tetanus-IgG levels

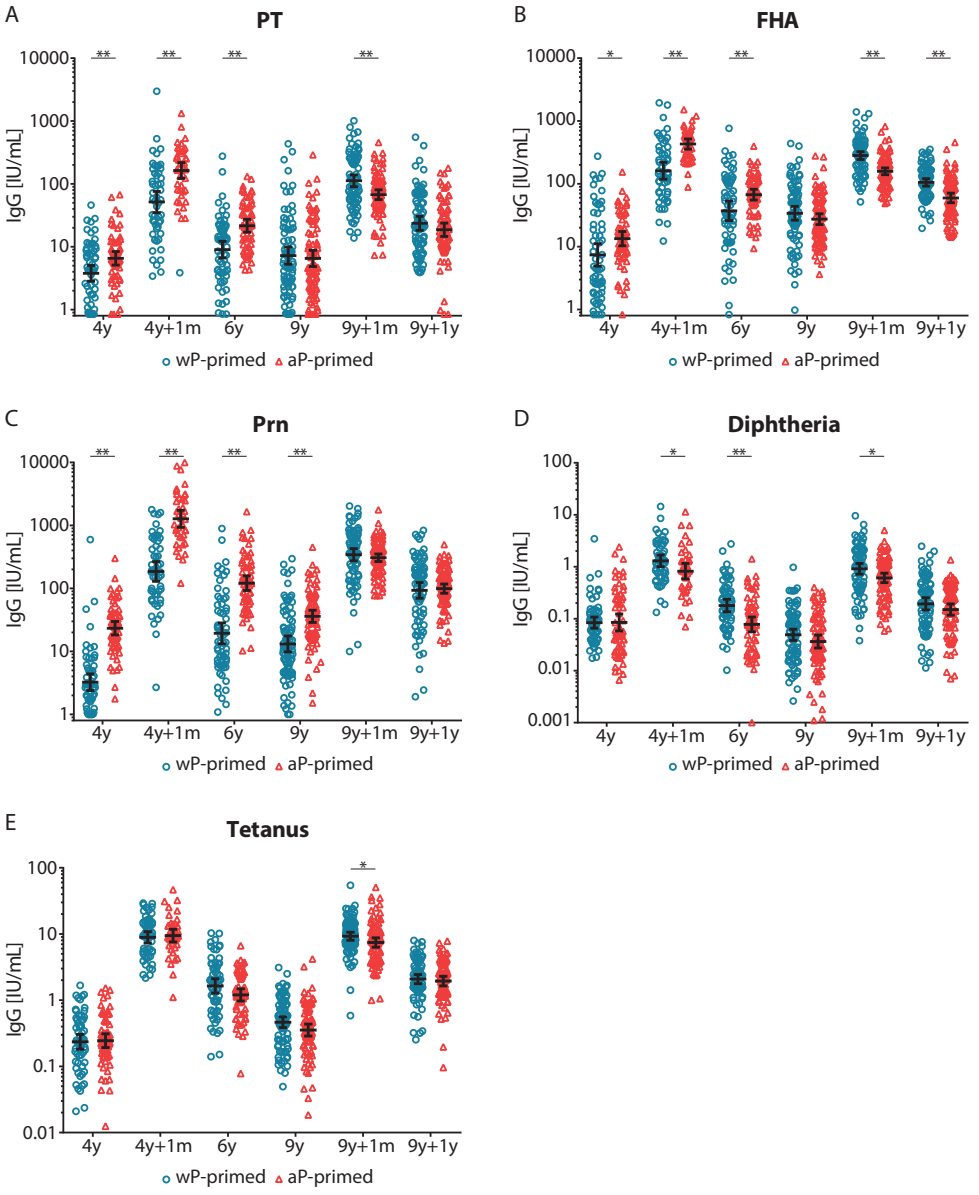

Figure 2. IgG antibody levels in children 4–9 years of age covering 2 booster vaccinations. (A) pertussis toxin (PT), **(B)** filamentous hemagglutinin (FHA), **(C)** pertactin (Prn), **(D)** diphtheria toxoid and **(E)** tetanus toxin specific IgG levels (IU/mL) of 4 to 9 year old wP- (blue circles) or aP-primed (red triangles) children. Blood was sampled cross-sectionally at 4 years of age before, 1 month and 2 years (at age 6 years) after the pre-school DTaP booster and from different children longitudinally at 9 years of age before, 1 month and 1 year after the pre-adolescent Tdap booster. Note, black lines represents the geometric mean concentration with 95% confidence interval, * = p-value <.05 and ** = p-value <.01. Data of PT, FHA and Prn for wP-primed children aged 4–9 years and aP-primed children aged 4–6 years were previously published (25, 26).

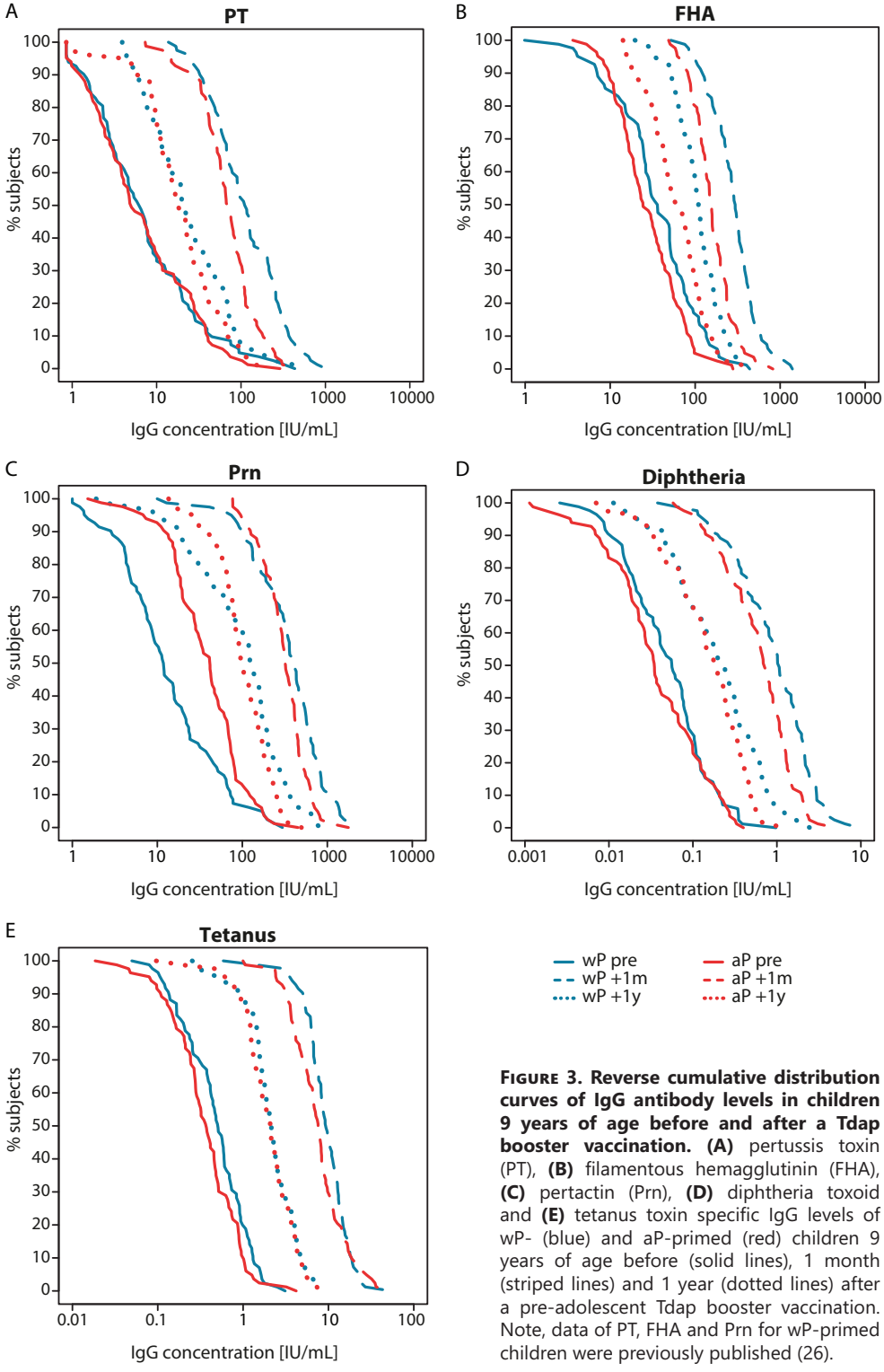

Figure 3. Reverse cumulative distribution curves of IgG antibody levels in children 9 years of age before and after a Tdap booster vaccination. (A) pertussis toxin (PT), **(B)** filamentous hemagglutinin (FHA), **(C)** pertactin (Prn), **(D)** diphtheria toxoid and **(E)** tetanus toxin specific IgG levels of wP- (blue) and aP-primed (red) children 9 years of age before (solid lines), 1 month (striped lines) and 1 year (dotted lines) after a pre-adolescent Tdap booster vaccination. Note, data of PT, FHA and Prn for wP-primed children were previously published (26).

between the two groups. In general, all pertussis-specific IgG levels were low at 9 years of age, although significantly higher in both groups as compared to pre-school booster levels at 4 years of age, with the exception of PT-IgG levels in aP-primed children aged 9 years (supplementary Table 1). Diphtheria-IgG levels were significantly lower in both wP- and aP-primed children aged 9 years compared with levels before the pre-school booster.

IgG antibody kinetics after a pre-adolescent Tdap booster vaccination in children 9 years of age

IgG antibody levels specific for all 5 vaccine antigens (PT, FHA, Prn, diphtheria and tetanus) were significantly higher 1 month and 1 year after the pre-adolescent Tdap booster at 9 years of age compared with pre-booster levels for all children (supplementary Table 1). However, we observed differences in dynamics between wP- and aP-primed children. One month after the pre-adolescent Tdap booster, aP-primed children had significantly lower PT-, FHA-, diphtheria- and tetanus-IgG levels compared with wP-primed children (all p-values <.001), a difference which remained for at least 1 year for FHA-IgG (p-value <.001) (**Figure 2** and **Figure 3**, supplementary Table 1). The increase in IgG levels for the three pertussis antigens after the pre-adolescent booster was significantly less in aP-primed children compared with wP-primed children, while no differences were observed for diphtheria and tetanus (supplementary Table 2).

To exclude possible differences in exposure to pertussis between wP- and aP-primed groups, we compared the IgG levels against PT, which is the specific antigen for *B. pertussis*. We found no difference in the proportion of children 9 years of age with a PT-IgG concentration ≥50 IU/mL (suggestive for recent pertussis infection (11, 30)) before the Tdap booster (p = .576) (Figure 3A). None of the children indicated to have had clinical pertussis in the year preceding the pre-adolescent booster vaccination.

B-cell responses after a pre-adolescent Tdap booster vaccination in children 9 years of age

No differences were observed in the number of antigen-specific memory B-cells between wP- and aP-primed children before the pre-adolescent booster (**Figure 4**). Similar to PT-, and FHA-IgG levels, the numbers of PT- and FHA-specific memory B-cells were significantly lower 1 month after the booster in aP- compared with wP-primed children (p-value = .005 and .018 respectively). This trend was also observed for Prn and tetanus, but failed to reach significance. One year after the booster, differences in numbers of memory B-cells had disappeared. Although numbers of Prn-specific memory B-cells were not significantly different, a significant smaller increase in numbers between pre (T0) and 1 month post-booster (T1) vaccination was observed in aP- versus wP-primed children (GM T1/T0 ratio = 4.40 and 15.96 respectively; p-value = .001).

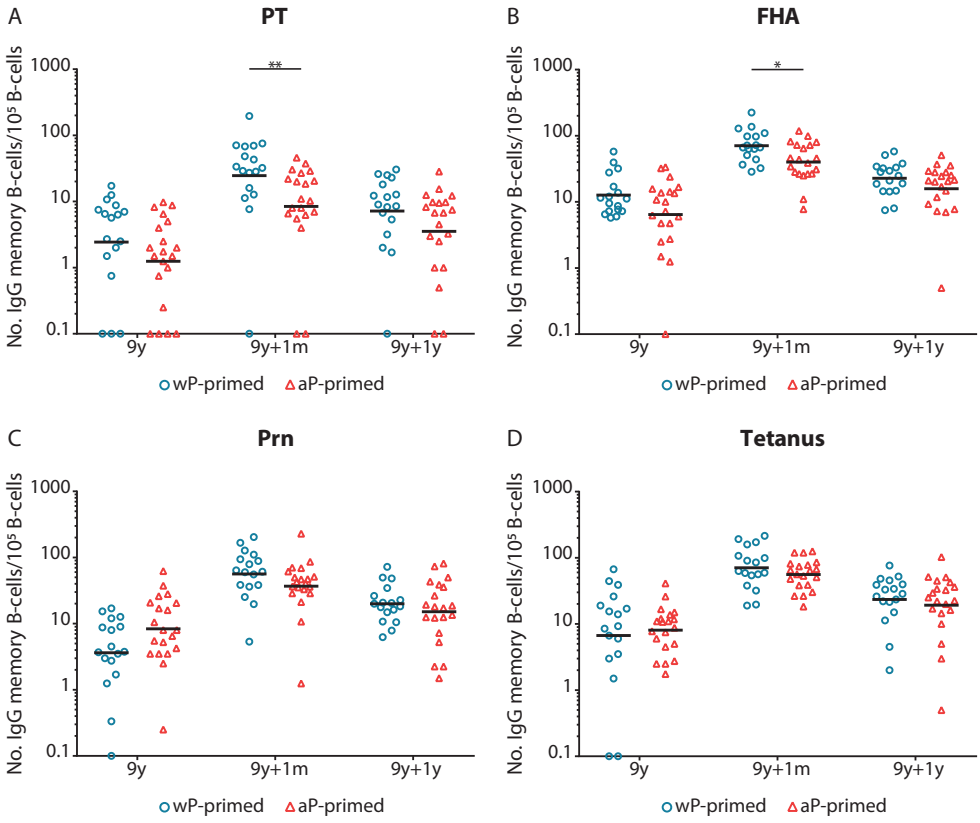

Figure 4. Numbers of memory B-cells in children 9 years of age before and after a Tdap booster vaccination. (A) pertussis toxin (PT), **(B)** filamentous hemagglutinin (FHA), **(C)** pertactin (Prn), and **(D)** tetanus toxoid specific IgG producing memory B-cells per 105 IgG producing B-cells of wP- (blue circles) or aP-primed (red triangles) children 9 years of age before, 1 month and 1 year after a pre-adolescent Tdap booster vaccination. Note, black lines represents the geometric mean numbers, * = p-value <.05 and ** = p-value <.01. For all antigens, values of pre vs 1 month, pre vs 1 year, and 1 month vs 1 year were all significantly different within the wP- and aP-primed longitudinal groups of children (all p-values <.05). Data of wP-primed children were previously published (26).

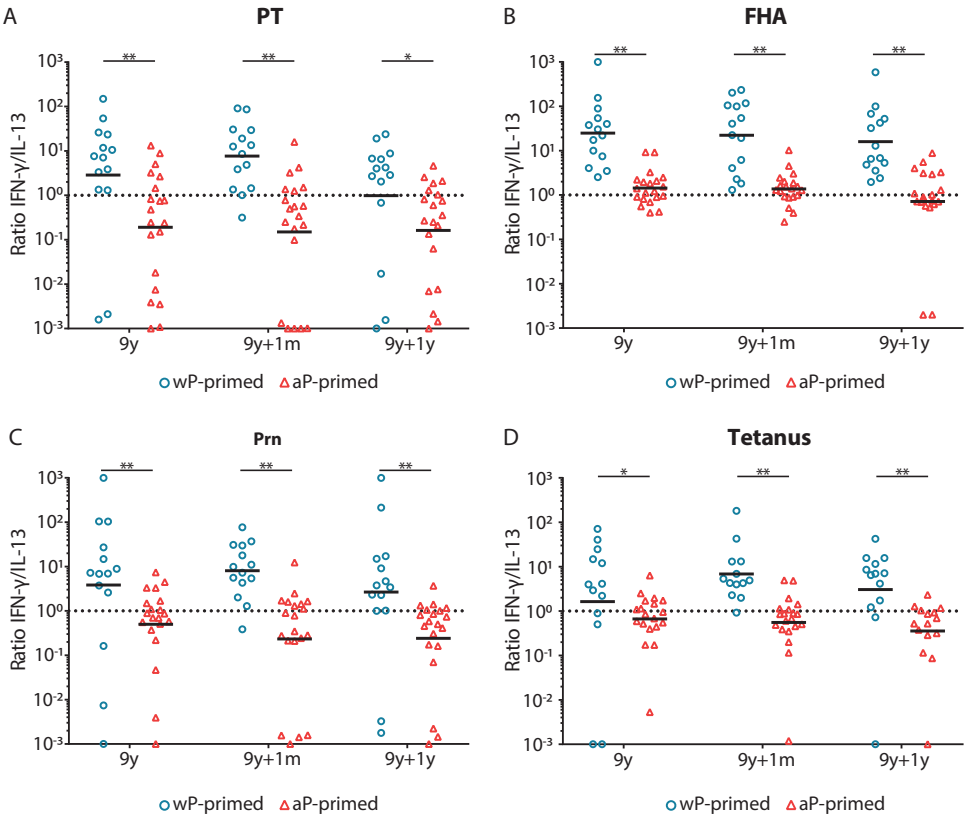

Figure 5. Th1/Th2 ratio in children 9 years of age before and after a Tdap booster vaccination. The IFN-γ (Th1)/ IL-13 (Th2) ratio in supernatants of **(A)** pertussis toxin (PT), **(B)** filamentous hemagglutinin (FHA), **(C)** pertactin (Prn), or **(D)** tetanus toxoid stimulated T-cells of wP- (blue circles) and aP-primed (red triangles) children 9 years of age, before, 1 month and 1 year after a pre-adolescent Tdap booster vaccination. Note, black lines represents the geometric mean IFN-γ/IL-13 ratio and dotted lines indicates an IFN-γ/IL-13 ratio of 1. * = p-value <.05 and ** = p-value <.01; within the longitudinal study groups, there was only a significant difference for PT-stimulated T-cells between pre- and 1 year post-booster in wP-primed children (p-value <.05).

Overall, the number of antigen-specific memory B-cells was significantly higher for all 4 antigens (PT, FHA, Prn, tetanus) in both groups at 1 month and 1 year post-booster compared with pre-booster (Figure 4). However, the number of circulating memory B-cells 1 year post-booster had significantly declined in both groups.

T-cell responses after a pre-adolescent Tdap booster vaccination in children 9 years of age

We found significantly lower IFN-γ levels in aP-primed compared with wP-primed children for FHA before the booster, for all four antigens (PT, FHA, Prn and tetanus) at 1 month and for PT, FHA and tetanus 1 year post-booster (supplementary Figure 2), while IL-13 levels only differed for Prn before the booster vaccination between wP- and aP-primed children. Although IL-17 cytokine levels were significantly higher in wP-primed children for FHA and tetanus 1 month post-booster compared with aP-primed children, all IL-17 levels remained low, especially in relation to IFN-γ and IL-13 levels. The IL-10 cytokine levels of vaccine antigen stimulated T-cells were low and still showed a high variability at all time points (supplementary Figure 2).

With higher IFN-γ levels, the ratio of IFN-γ/IL-13 (Th1/Th2 cells) was >1 for all antigens and at all time points in wP-primed children (**Figure 5**), while this ratio was always <1 in aP-primed children, except for FHA both before and 1 month after the pre-adolescent booster (IFN-γ/IL-13 ratio at both time points = 1.4). This resulted in significantly lower Th1/Th2 (IFN-γ/IL-13) ratios at all time points and for all antigens in aP-primed compared with wP-primed children (Figure 5).

No differences in IFN-γ, IL-13, IL-17 and IL-10 cytokine levels were observed between the time points pre- and post-booster within the wP- and aP-primed groups of children, except for lower PT-specific IL-10 production and FHA-specific IL-17 production in wP-primed children 1 year after the booster compared with 1 month (supplementary Figure 2).

Discussion

Based on epidemiological data, waning protection of pertussis-specific immunity seems to occur more rapidly in children who received aP vaccines at infant age in comparison with those who received wP vaccines. In this study, for the first time, we corroborate these epidemiological data with long-term immunological data in groups of children up to 10 years of age showing that priming during infancy with either wP or aP vaccines determines the humoral and cellular immune responsiveness to additional aP booster vaccinations until at least pre-adolescent age. We clearly showed that replacement of a DTwP combination vaccine by DTaP combination vaccines has enhanced pertussis immune responses on the short term till 6 years of age, 2 years after the 5th DTaP booster at pre-school age. However, after the pre-adolescent Tdap booster vaccination humoral and cellular immunity showed a shift. While Th2 responses were similar between the 2 groups, pre-adolescents primed with wP vaccines during infancy had the more favourable Th1 dominated immune response compared to aP-primed pre-adolescents for at least 1 year after the booster vaccination. This resulted in a Th2-skewed profile still present in adolescents primed with repeated aP vaccines during infancy that corroborates the increased pertussis incidence in aP-primed adolescents during pertussis outbreaks.

Although the resurgence of pertussis had already started before the introduction of aP vaccines, the incidence of pertussis has sharply increased since aP vaccines have been implemented in all primary and booster vaccination schedules in many countries worldwide (31). In the last years, the majority of the pertussis scientific community has reached consensus that priming with aP vaccines is less effective than with wP vaccines in maintaining pertussis immunity (31-33). It has been demonstrated that growing cohorts of older children and adolescents who only have received aP vaccines continue to be at higher risk of contracting pertussis than wP-primed individuals and thereby sustaining epidemics (34). In the Netherlands, the wP vaccines were replaced by aP vaccines in 2005. Therefore, we have not yet observed a difference in adolescent pertussis cases based on a different priming vaccine regime, as the fully aP vaccinated adolescents now are reaching the age of 13 years.

We showed that the aP-primed children 6 years of age had the advantage of having higher pertussis-specific antibody levels 2 years after the pre-school booster compared with wP-primed children. This effect was lost at 9 years of age, with low antibody levels in both wP- and aP-primed children, although Prn-IgG levels remained higher in aP-primed children due to the much higher antibody response shortly after the pre-school booster vaccination. Subsequently, the pre-adolescent booster vaccination induced lower pertussis-specific antibody levels and numbers of memory B-cells in aP-primed compared with wP-primed children. This implies that germinal centre responses after wP-priming lead to a more sustainable memory B-cell compartment that is longer boostable during life. Interestingly, we showed

that the rise in antibody levels upon a 6th aP vaccination was less than after the 5th aP vaccination, indicating that the degree of antibody production does not persist at the same level despite several vaccine doses. This might be explained by the lower pertussis antigen concentration in the pre-adolescent Tdap booster vaccination compared with the pre-school DTaP booster vaccination. However, wP-primed children, vaccinated with the same DTaP and Tdap booster vaccines as the aP-primed children, showed higher pertussis-specific IgG levels 1 month after the pre-adolescent booster vaccination compared with 1 month after the pre-school booster vaccination. This is in line with Eberhardt et al., who suggests that germinal centre reactions induced by aP-priming vaccines are not effective enough to confer long-term memory responses (35). Furthermore, the more durable priming upon wP vaccination might especially be due to the presence of lipopolysaccharide, a highly immune stimulatory bacterial cell wall component, operating as an adjuvant by activating innate immune cells via toll-like receptor 4 (36). The lack of immune stimulatory components in the aP vaccines might explain the limited duration of effective immune responses against pertussis.

Importantly, the T-cell responses of aP vaccinated children showed a clear Th2-skewed profile, especially after the 6th aP vaccination at 9 years of age for the three pertussis vaccine antigens, as well as for the co-administered tetanus toxoid. Previous studies indicated more Th2 polarization in infants, children and even in adults who have been primed with aP vaccines compared with wP-priming (37, 38). In contrast, we now show that the polarization towards a Th2 profile in aP-primed pre-adolescents was caused by a decreased production of the Th1 cytokines (IFN-γ), and not by a higher Th2 cytokine (IL-13) production. Since Th1 and Th2 cytokine levels remained unchanged upon the pre-adolescent booster vaccination in both groups of children, Th1/Th2 ratios did not substantially change following the booster. This is in agreement with our earlier findings that aP-primed children 4 years of age showed high Th1 and Th2 responses, more effector memory and terminally differentiated CD4+ T-cells that remained unchanged or even decreased upon the pre-school booster, whereas those in wP-primed children were low but increased upon a pre-school booster vaccination (23, 39). Others also described that wP-primed adolescents showed less terminally differentiated pertussis-specific CD4+ T-cell responses than aP-primed adolescents (40). Natural boosting, due to the high circulation of pertussis, will enhance the vaccine induced T-cell responses during life. So, once a Th1 and/or a Th2 T-cell response has been induced by primary immunisations early in life, it most likely remains stable during life. Since Th1 cytokines play an important role in protection against pertussis (41), this might, at least in part, explain the better protection found in wP-primed individuals. Therefore, in future vaccine development strategies, it is advisable to change the Th2-skewed T-cell responses upon aP-priming in infancy into a more Th1-skewed response by introducing other adjuvants in the priming combination vaccines that stimulate the innate immune response towards a more Th1 profile (42). Another possible vaccination strategy could be to use a wP vaccine

as a first priming vaccination to establish a Th1 dominated immune response, that is subsequently followed by aP vaccines to boost the Th1 primed immunity (43).

Although Th17 responses have been reported to play an important role in protection against pertussis in mice and baboons (44, 45), we found just low Th17 responses in both wP- and aP-primed pre-adolescents, indicating that these responses may be far less pronounced upon vaccination in humans than in animal models. IL-10 might be involved in the regulatory T-cell response, however, we found similarly low values of IL-10 in both groups.

The recruitment of the two groups of 9-year-old pre-adolescents was conducted in two different years. This could introduce a different epidemiological background between the cohorts, given the frequent pertussis epidemics in the Netherlands. However, the proportion of PT-IgG seropositive participants (≥50 IU/mL as indication of recent infection (11)) was similar in the two groups. This indicates that the exposure to *B. pertussis* was most likely similar and therefore did not affect our study results.

In conclusion, our pertussis immune responses in pre-adolescents corroborate the epidemiological data showing that adolescents primed with aP vaccines are less protected against pertussis than those being primed with wP vaccines. New pertussis vaccines should be developed that induce a more Th1 dominated immune response in the primary vaccination series.

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Supplementary data

Supplemenary methods 1. Recruitment procedures of children 9 years of age, primed with aP combination vaccines

Children 9 years of age were recruited by sending invitation letters to the parents of eligible, healthy children being vaccinated according to the Dutch national immunisation programme (DTaP at 2, 3, 4, and 11 months of age and DTaP at 4 years of age). Exclusion criteria were: serious adverse event after previous vaccination; severe disease or medical treatment possibly interfering with study results as well as use of plasma products within 6 months, other vaccinations within a month; or antibiotic use/ fever within 14 days prior to enrolment. This study was approved by the medical research ethics committees united (MEC-U, Nieuwegein, the Netherlands), and registered at the European clinical trials database (2013-001864-50) and the Dutch trial register (www.trialregister.nl; NTR4089). For all participants, written informed consent was obtained from both parents or legal representatives. In September 2013, all children were enrolled in the study and received the Tdap booster vaccination according to study protocol.

Supplementary Table 1. IgG geometric mean concentrations (GMC) with corresponding 95% confidence intervals before and after a pre-school DTaP booster vaccination in children aged 4 years and before and after an additional Tdap booster vaccination in children aged 9 years.

		wP-primed	aP-primed	p-value
PT IU/mL (95% CI)	4 years	3.8 (2.8 - 5.1)	6.5 (5.1 - 8.4)	**.005**
	4 years + 1 month	51.6 (34.9 - 76.1)	163.5 (122.6 - 218.2)	**<.001**
	6 years	9.0 (6.6 - 12.2)	21.6 (17.1 - 27.3)	**<.001**
	9 years	7.3[#] (5.3 - 10.0)	6.5 (4.8 - 8.8)	.635
	9 years + 1 month	112.4 (90.6 - 139.4)	67.6 (56.6 - 80.7)	**<.001**
	9 years + 1 year	23.6 (18.3 - 30.5)	18.7 (14.6 - 23.8)	.189
FHA IU/ml (95% CI)	4 years	7.4 (4.9 - 11.2)	13.4 (10.3 - 17.5)	**.016**
	4 years + 1 month	161.5 (118.7 - 219.9)	430.0 (356.4 - 518.7)	**<.001**
	6 years	37.3 (26.1 - 53.3)	67.2 (55.1 - 81.9)	**.005**
	9 years	34.0[#] (26.4 - 43.8)	27.4[#] (22.5 - 33.4)	.182
	9 years + 1 month	281.6 (244.0 - 325.1)	158.1 (139.7 - 179.0)	**<.001**
	9 years + 1 year	105.5 (92.0 - 120.9)	59.7 (50.1 - 71.1)	**<.001**
Prn IU/mL (95% CI)	4 years	3.2 (2.4 - 4.4)	23.4 (18.3 - 29.9)	**<.001**
	4 years + 1 month	186.2 (129.4 - 267.9)	1274.0 (932.6 – 1739.0)	**<.001**
	6 years	19.4 (13.3 - 28.4)	121.3 (92.7 - 158.6)	**<.001**
	9 years	13.2[#] (9.8 - 17.6)	36.1[#] (28.8 - 45.1)	**<.001**
	9 years + 1 month	343.3 (274.6 - 429.3)	306.8 (267.2 - 352.4)	.394
	9 years + 1 year	93.1 (69.8 - 124.1)	99.2 (83.8 - 117.5)	.703
Diphtheria IU/mL (95% CI)	4 years	0.08 (0.07 - 0.11)	0.08 (0.06 - 0.12)	.969
	4 years + 1 month	1.30 (1.00 - 1.69)	0.82 (0.58 - 1.16)	**.032**
	6 years	0.18 (0.14 - 0.24)	0.08 (0.06 - 0.11)	**<.001**
	9 years	0.05[$] (0.04 - 0.06)	0.04[$] (0.03 - 0.05)	.119
	9 years + 1 month	0.91 (0.72 - 1.16)	0.61 (0.49 - 0.75)	**.012**
	9 years + 1 year	0.19 (0.15 - 0.25)	0.15 (0.12 - 0.19)	.170
Tetanus IU/mL (95% CI)	4 years	0.24 (0.18 - 0.30)	0.24 (0.19 - 0.31)	.826
	4 years + 1 month	8.88 (7.27 - 10.84)	9.46 (7.59 - 11.79)	.671
	6 years	1.64 (1.28 - 2.11)	1.20 (0.97 - 1.49)	.062
	9 years	0.47[#] (0.39 - 0.56)	0.35[#] (0.29 - 0.44)	.053
	9 years + 1 month	9.24 (8.04 - 10.62)	7.44 (6.31 - 8.76)	**.046**
	9 years + 1 year	2.08 (1.78 - 2.44)	1.95 (1.65 - 2.30)	.550

Note: Bold p-values represent values <.05. Differences between pre and post IgG GMCs within the longitudinal studies (pre and post 9-year-old booster vaccination samples) were tested with paired samples t-tests and were corrected for multiple comparison. For all antigens, GMC's of pre vs 1 month, pre vs 1 year, and 1 month vs 1 year were all significantly different (all p-values <.001).

Abbreviations: GMC: geometric mean concentration; CI: confidence interval; wP: whole-cell pertussis vaccine; aP: acellular pertussis vaccine; PT: pertussis toxin; FHA: filamentous hemagglutinin; Prn: pertactin; IU: international units.

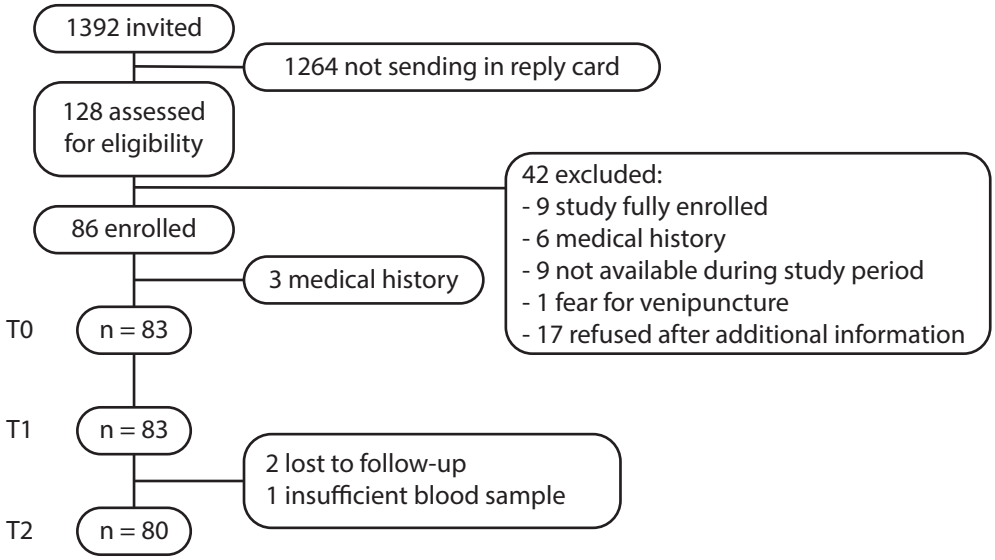
[#] GMC significant higher in 9-year-olds vs 4-year-olds (both before booster vaccination).

[$] GMC significant lower in 9-year-olds vs 4-year-olds (both before booster vaccination).

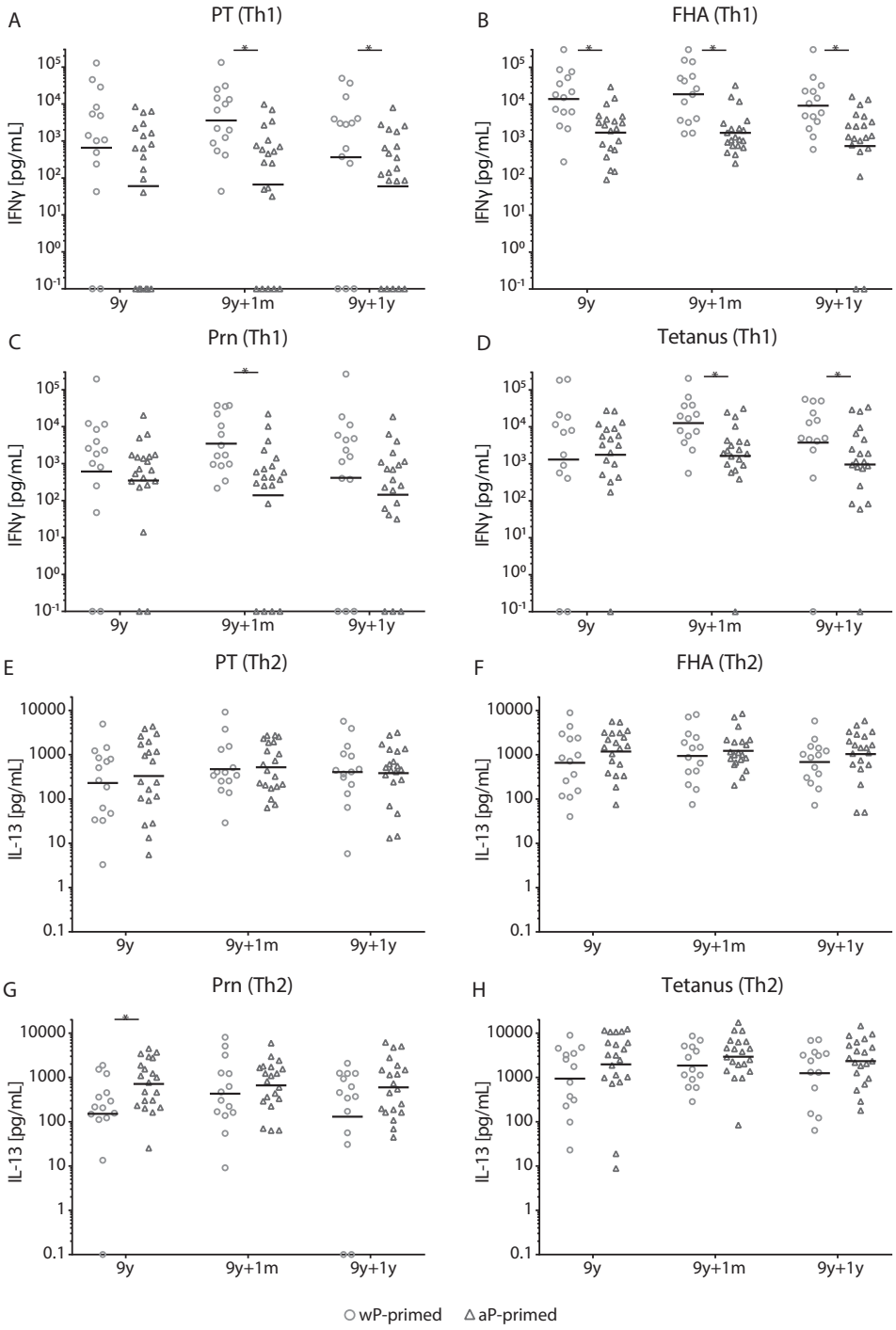
Supplementary Table 2. Ratio of IgG antibody increase between before and 1 month after a Tdap booster vaccination at age 9 years. Children were primed with wP or aP combination vaccines in the first year of life (2, 3, 4, 11 months of age) and received a DTaP booster at age 4 years.

	Antigen	wP-primed	aP-primed	p-value
GM Ratio T1/T0 (95% CI)	PT	15.1 (11.6 - 19.7)	10.3 (8.2 - 13.0)	**.031**
	FHA	8.1 (6.5 - 10.3)	5.8 (4.9 - 6.8)	**.016**
	Prn	25.0 (19.7 - 31.7)	8.5 (7.1 - 10.2)	**<.001**
	Diphtheria	18.6 (15.1 - 23.0)	16.7 (13.5 - 20.8)	.481
	Tetanus	19.8 (15.9 - 24.6)	21.0 (17.2 - 25.8)	.690

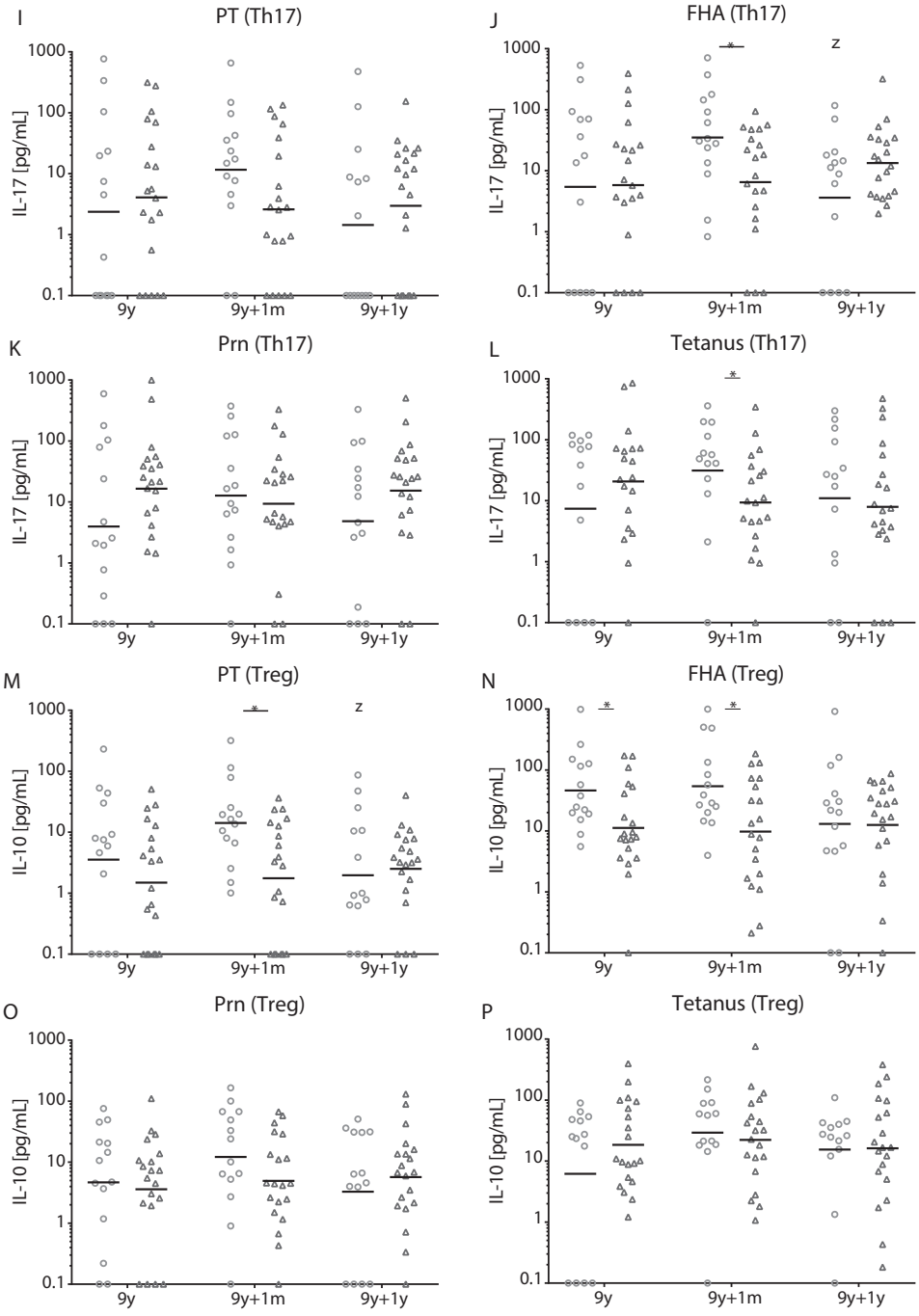
Note: the GM ratio T1/T0 indicates level of increase between before and 1 month after vaccination at age 9 years. Differences between the groups were determined with independent samples t-tests, bold p-values indicate a significant difference between wP- versus aP-primed children.
Abbreviations: GM: geometric mean; CI: confidence interval; wP: whole-cell pertussis vaccine; aP: acellular pertussis vaccine; T0: pre-booster; T1: 1 month post-booster; PT: pertussis toxin; FHA: filamentous hemagglutinin; Prn: pertactin.



Supplementary Figure 1. Flow chart recruitment of aP-primed children 9 years of age. Schematic overview of the recruitment, enrolment and follow-up of children 9 years of age who participated in a phase IV, longitudinal intervention study. Children were primed with diphtheria, tetanus and acellular pertussis (DTaP) combination vaccines in the first year of life (2, 3, 4, and 11 months of age) and received a DTaP booster vaccination at 4 years of age according to the Dutch national immunisation program. At the start of the study (T0), all 83 participants received a tetanus, reduced dosed diphtheria and acellular pertussis booster vaccination (Tdap; Boostrix-Polio; GlaxoSmithKline, Rixensart, Belgium). Blood samples were collected just before (T0), 1 month (T1) and 1 year (T2) after the Tdap booster vaccination.



Supplementary Figure 2. Cytokine levels of stimulated T-cells before and after a Tdap booster vaccination (left and right page)**.** IFN-γ **(A-D)**, IL-13 **(E-H)**, IL-17 **(I-L)** and IL-10 **(M-P)** cytokine concentrations (pg/mL) in the supernatants of T-cells stimulated with **(A, E, I, M)** heat inactivated pertussis toxin (PT); **(B, F, J, N)** filamentous hemagglutinin (FHA); **(C, G, K, O)** pertactin (Prn); or **(D, H, L, P)** tetanus



toxoid before, 1 month and 1 year after a Tdap booster vaccination in wP- (light grey circles) and aP-primed (dark grey triangles) children 9 years of age. Note, black lines represents the geometric mean concentration. * = p-value <.05 between wP- and aP-primed children. z = indicates a significant decrease between 1 month and 1 year post-booster in wP-primed children.

Chapter 4

Whole-cell or acellular pertussis vaccination in infancy determines IgG subclass profiles to DTaP booster vaccination

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Abstract

Introduction

Duration of protection against clinical pertussis is shorter in adolescents who have been immunised with acellular pertussis (aP) in infancy compared with adolescents who received whole-cell pertussis (wP) vaccines in infancy, which is related to immune responses elicited by these priming vaccines. To better understand differences in vaccine induced immunity, we determined pertussis, diphtheria, and tetanus (DTaP) vaccine antigen-specific IgG subclass responses in wP- and aP-primed children before and after two successive DTaP booster vaccinations.

Methods

Blood samples were collected in a cross-sectional study from wP- or aP-primed children before and 1 month after the pre-school DTaP booster vaccination at age 4 years. Blood samples were collected from two different wP- and aP-primed groups of children before, 1 month and 1 year after an additional pre-adolescent Tdap booster at age 9 years. IgG subclass levels against the antigens included in the DTaP vaccine have been determined with fluorescent-bead-based multiplex immunoassays.

Results

At 4 years of age, the IgG4 proportion and concentration for pertussis, diphtheria and tetanus vaccine antigens were significantly higher in aP-primed children compared with wP-primed children. IgG4 concentrations further increased upon the two successive booster vaccinations at 4 and 9 years of age in both wP- and aP-primed children, but remained significantly higher in aP-primed children.

Conclusions

The pertussis vaccinations administered in the primary series at infancy determine the vaccine antigen-specific IgG subclass profiles, not only against the pertussis vaccine antigens, but also against the co-administered diphtheria and tetanus vaccine antigens. These profiles did not change after DTaP booster vaccinations later in childhood. The different immune response with high proportions of specific IgG4 in some aP-primed children may contribute to a reduced protection against pertussis.

Introduction

Since the 1990s, pertussis is re-emerging in many countries, despite high coverage of pertussis vaccination in national immunisation programs (1-3). Pertussis outbreaks are reported every two to three years, not only in infants, but also in children, adolescents and adults (4-7). The replacement of whole-cell pertussis (wP) vaccines by acellular pertussis (aP) vaccines in the primary vaccination series during infancy is thought to contribute to the re-emergence of pertussis (8). In order to reduce the pertussis burden in children, aP booster vaccinations around 4–6 years of age were implemented in many countries (9, 10).

Epidemiological data indicate reduced vaccine-derived protection in aP-primed adolescents compared with adolescents who received at least one wP vaccine in the primary series in infancy (11, 12). Immunological studies comparing wP- and aP-primed children showed differences both in humoral and cellular pertussis-specific immune responses. After a pre-school DTaP booster vaccination at 4 years of age, aP-primed children showed higher pertussis-specific antibody levels and memory B- and T-cell responses compared with wP-primed children (13-17). Comparable differences with regard to antibody and T-cell responses between wP- and aP-primed infants were observed in children shortly after the primary vaccination series at 13 months of age (18). In contrast, an additional Tdap booster vaccination in pre-adolescents resulted in better DTaP-specific humoral and cellular immune responses in children who had received wP vaccinations in infancy (19).

Although an internationally accepted correlate of protection for pertussis has not been established, pertussis-specific IgG antibodies are involved in protection against clinical pertussis (20, 21). The high effectiveness of preventing pertussis in the first months of life following maternal pertussis vaccination also indicates protection mediated by IgG antibodies. (22, 23). Even though all four IgG subclasses are transported over the placenta, IgG4 antibodies are unable to bind complement which makes the contribution to protection against pertussis less likely (24). Previously, Hendrikx et al. reported a higher proportion of pertussis-specific IgG4 antibodies in 4 year old children primed with DTaP, and boosted with a 5th dose of the same vaccine, compared with children primed with wP vaccines (25). Increased levels of the IgG4 subclass are associated with a Th2-skewed immune response, which may influence the induced protection against pertussis in aP vaccinated children (26).

Nowadays, several countries have implemented a 6th DTaP booster vaccination in (pre-) adolescents in order to reduce the pertussis disease burden in adolescents (27). Information about the pertussis antigen-specific IgG subclass responses in this age group after such a booster is scarce. Furthermore, IgG subclass profiles specific for diphtheria and tetanus, the other components in the DTaP vaccine, have not been studied in aP-primed children.

In this study we determined the IgG subclass profiles for the pertussis, diphtheria and tetanus vaccine antigens in children around 4 and 9 years of age, before and after a pre-school DTaP and a pre-adolescent Tdap booster vaccination. We compared groups vaccinated with either wP or aP combination vaccines in the first year of life.

Methods

Study design and participants

The participants were primed according to the Dutch national immunisation program: DTwP or DTaP at 2, 3, 4, and 11 months of age and received a DTaP booster vaccination at 4 years of age (**Figure 1**). Blood samples were collected from children 4 years of age before and 1 month after the pre-school DTaP booster vaccination (cross-sectional study ISRCTN65428640) (28). Two groups of children 9 years of age were included in longitudinal intervention studies and received an additional Tdap booster vaccination. Blood samples were collected before, 1 month and 1 year after the Tdap vaccination and plasma was stored at -20ºC until analysis. These children were primed with either DTwP combination vaccines in infancy (ISRCTN64117538) (29, 30), or with DTaP combination vaccines (NTR4089) (19). The numbers of participants vary between 40 and 83 participants per time point.

Vaccination background

At 2, 3, 4, and 11 months of age, the participants received either a DTwP-IPV-Hib combination vaccine (Netherlands vaccine institute, Bilthoven, the Netherlands), or a DTaP-IPV-Hib (Infanrix-IPV-HibTM, GlaxoSmithKline (GSK), Rixensart, Belgium) combination vaccine. The children received a pre-school DTaP booster vaccination at 4 years of age (Infanrix-IPVTM, GSK), and a pre-adolescent Tdap booster vaccination at 9 years of age (Boostrix-IPVTM, GSK).

Serological analysis

Pertussis toxin (PT), filamentous hemagglutinin (FHA), pertactin (Prn) (the 3 aP vaccine antigens), diphtheria toxoid and tetanus toxin specific total IgG (IgGt) antibody concentrations were measured with the multiplex immunoassay as described earlier (31, 32). IgG subclass levels for the DTaP antigens were also determined with the fluorescent-bead-based multiplex immunoassay as described separately for PT, FHA and Prn (25) and for diphtheria toxoid and tetanus toxin (33). Since no IgG subclass reference sample is available for these vaccine antigens, each IgG subclass was expressed in mean fluorescent intensity (MFI) values. The sum of MFI values of all four IgG subclasses together was set at 100% and than each subclass was expressed as a proportion (in %) of the total. Using the IgGt concentration and the IgG subclass percentages, arbitrary IgG subclass concentrations were calculated. For the pertussis antigens, 5 arbitrary units (AU) per mL was used as cut-off for a seropositive response for each IgG subclass, in line with Hendrikx et al. (25), and

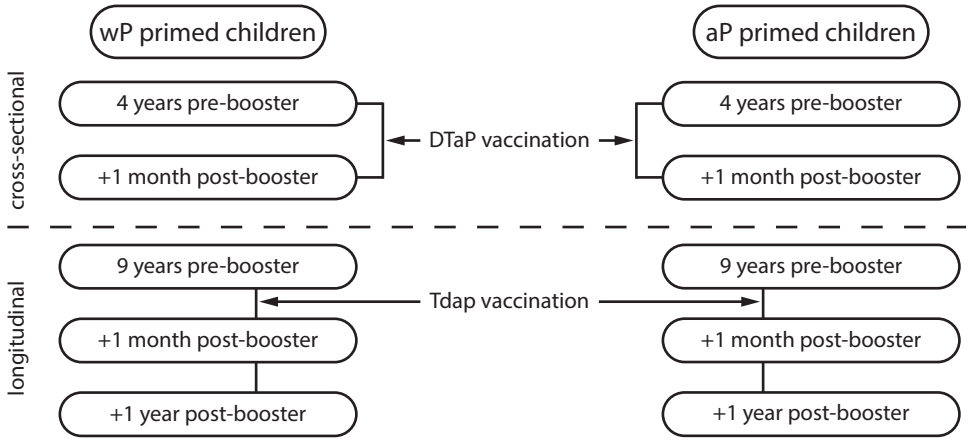

Figure 1. Overview of the study groups of the participants 4–9 years of age. Children were primed at the age of 2, 3, 4, and 11 months with either whole-cell pertussis (wP) (left panels) or acellular pertussis (aP) (right panels) combination vaccines according to the Dutch national immunisation program (NIP). Before and 1 month after the pre-school DTaP booster at age 4 years (received according to the Dutch NIP), blood samples were collected cross-sectionally (top panels) (14). Children 9 years of age, primed with wP vaccines (28, 29) or aP vaccines (NTR4089) received an additional Tdap booster vaccination and were sampled longitudinally before, 1 month and 1 year after the booster (bottom pannels).

the lower limit of quantitation was set at 0.1 AU/mL. For diphtheria and tetanus, an IgG concentration >0.01 IU/mL was used as a cut-off for protection, using the international standard for diphtheria and tetanus (34, 35). In line with this, we applied 0.01 AU/mL as a cut-off for the IgG subclasses as well. The lower limit of quantitation for diphtheria and tetanus was set at 0.001 AU/mL.

Statistical analysis

Percentages of the four IgG subclasses were expressed in means and IgGt and IgG subclass concentrations in geometric mean concentrations (GMC). To compare IgG subclass differences between wP- and aP-primed children at corresponding time points, the Mann-Whitney U test was applied. A p-value < .05 was considered statistically significant. Data were analysed using GraphPad Prism 7 (GraphPad Software, La Jolla, CA), and SPSS statistics 24 (IBM, Armonk, NY).

Results

Differences in IgG subclass proportions in the antibody response to PT, FHA, Prn, diphtheria and tetanus following wP- or aP-priming

The proportion of each IgG subclass in relation to the sum of the four IgG subclasses is depicted in **Figure 2**. Overall, IgG1 was the predominant subclass for both wP- and aP-primed children for all 5 vaccine antigens (PT, FHA, Prn, diphtheria and tetanus). Vaccine antigen-specific IgG4 was the next subclass that contributed most to IgGt followed by IgG2, while in general IgG3 contributed only minimally to the antigen-specific IgGt levels. After the pre-school booster at 4 years of age, the proportion of IgG1 increased for all vaccine antigens in both wP- and aP-primed children, except for FHA in aP-primed children. After the pre-adolescent booster at 9 years of age, IgG1 proportions remained more or less similar (Figure 2).

With respect to the IgG4 specific antibodies, the IgG4 proportion was significantly higher in aP-primed children compared with wP-primed children at all time points for all vaccine antigens, except for FHA and tetanus 1 year after the pre-adolescent booster (Figure 2).The IgG4 proportion of PT was higher in aP-primed children 9 years of age compared with 4 years of age (mean proportion of 6.5% and 3.6% respectively) before the booster vaccinations, but similar 1 month after the booster vaccinations (mean proportion in 4 and 9 year old children 13.6% and 12.5% respectively). The FHA-specific IgG4 proportion was similar in all age groups, whereas the IgG4 proportion of Prn was twice as high at age 9 years compared with 4 years in aP-primed children both before and after the booster vaccinations. In wP-primed children, the IgG4 proportion for the 3 pertussis vaccine antigens remained similar over time, with low IgG4 proportions upon repeated booster vaccinations for PT, FHA and Prn. For diphtheria and tetanus, two fold higher IgG4 proportions were observed at 9 years of age compared with 4 years of age, though with higher IgG4 levels in aP- versus wP-primed children. The IgG4 level for diphtheria was highest compared with the other vaccine antigens in both wP- and aP-primed children (mean diphtheria-specific IgG4 proportion ranging from 4.4–11.9% in wP- and 10.8–19.5% in aP-primed children) (Figure 2).

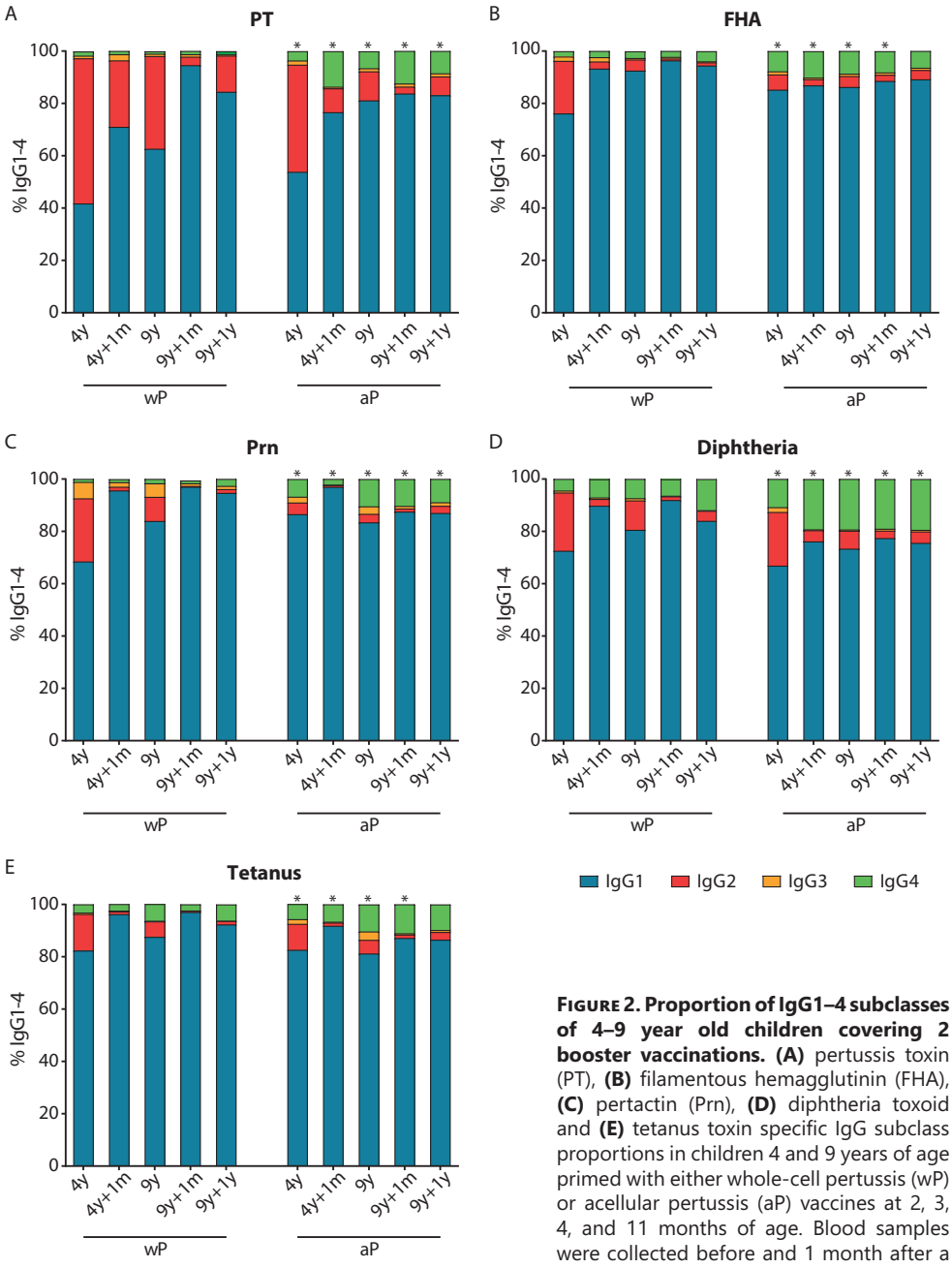

Figure 2. Proportion of IgG1–4 subclasses of 4–9 year old children covering 2 booster vaccinations. (A) pertussis toxin (PT), **(B)** filamentous hemagglutinin (FHA), **(C)** pertactin (Prn), **(D)** diphtheria toxoid and **(E)** tetanus toxin specific IgG subclass proportions in children 4 and 9 years of age primed with either whole-cell pertussis (wP) or acellular pertussis (aP) vaccines at 2, 3, 4, and 11 months of age. Blood samples were collected before and 1 month after a DTaP booster vaccination at age 4 years and before, 1 month and 1 year after a Tdap booster vaccination at age 9 years. Proportion IgG1 in blue, IgG2 in red, IgG3 in orange and IgG4 in green bars. Differences between wP- and aP-primed children were tested at each corresponding time point separately; * indicates a significant difference between wP- and aP-primed children in the percentage of IgG4 (p-value ≤.05). Differences between wP- and aP-primed children in the percentage of IgG1, IgG2, and IgG3 were not depicted. Note, data of PT, FHA and Prn for wP-primed children aged 4–9 years and aP-primed children aged 4 years were published previously (25).

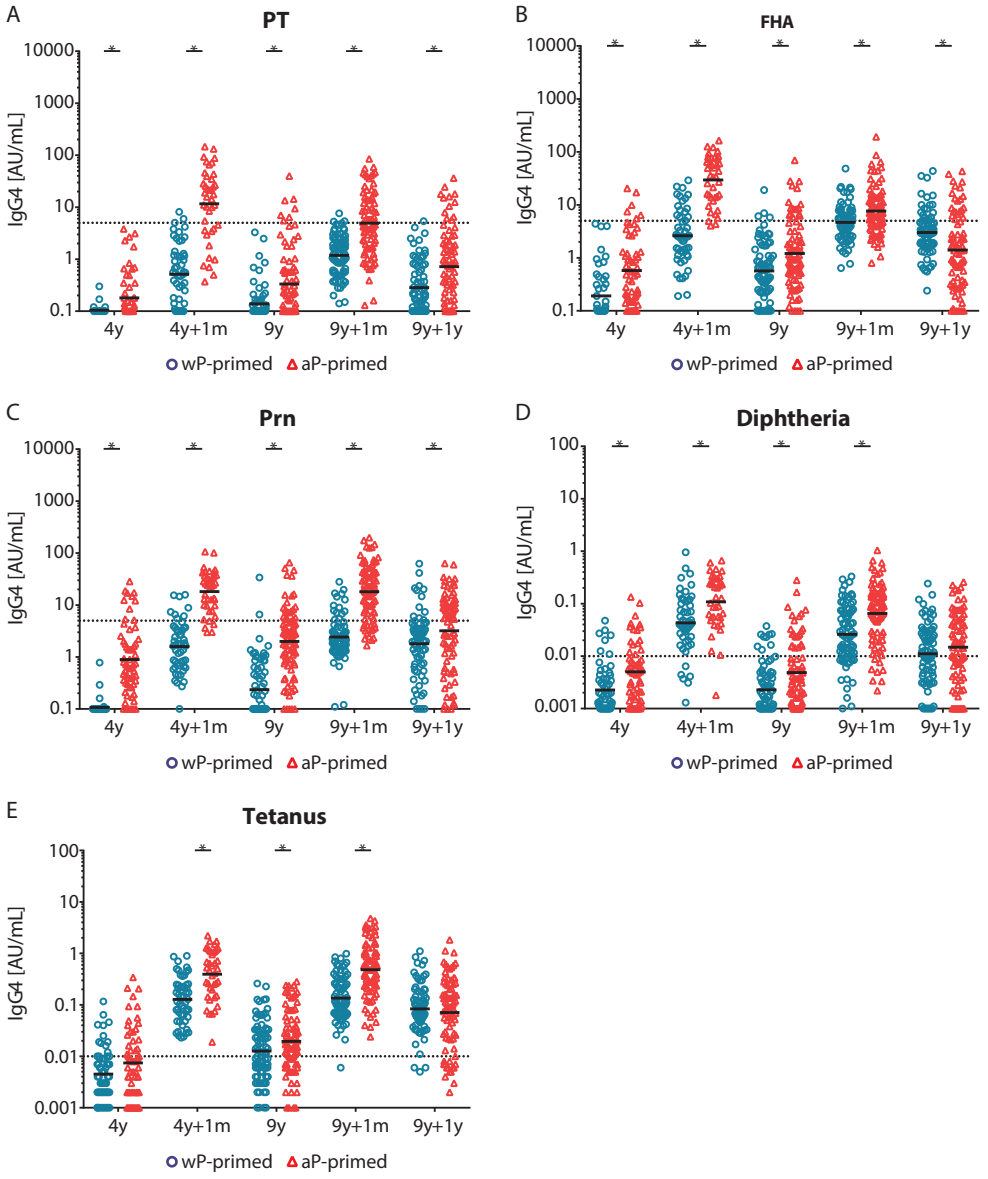

Figure 3. IgG4 subclass levels of 4–9 year old children covering 2 booster vaccinations. (A) pertussis toxin (PT), **(B)** filamentous hemagglutinin (FHA), **(C)** pertactin (Prn), **(D)** diphtheria toxoid and **(E)** tetanus toxin-specific IgG4 concentrations in arbitrary units (AU)/mL for whole-cell pertussis (wP) (blue circles) and acellular pertussis (aP) primed (red triangles) children 4 and 9 years of age. Samples were collected before and 1 month after the pre-school DTaP-booster vaccination and before, 1 month and 1 year after the pre-adolescent Tdap booster vaccination. Dotted lines indicate arbitrary cut-offs for seropositive concentrations which was 5 AU/mL for PT, FHA and Prn and 0.01 AU/mL for diphtheria and tetanus. * indicates a significant difference between wP- and aP-primed children (p-value ≤.05).

Differences in IgG subclass concentrations to PT, FHA, Prn, diphtheria and tetanus following wP- or aP-priming

Similar to the IgG4 proportions, the arbitrary IgG4 concentrations for all 5 vaccine antigens were higher in aP-primed children compared with wP-primed children. This difference reached significance for all 5 vaccine antigens at all time points, with the exception of tetanus-specific IgG4 concentrations at age 4 years before the booster and FHA-, diphtheria- and tetanus-specific IgG4 concentrations 1 year after the booster at age 9 years (**Figure 3**).

The pattern of the IgG1 concentrations depicted in supplementary Figure 1 was similar to that of the total IgG concentrations for the vaccine antigens (19). The aP-primed children had significantly higher PT-, FHA-, and Prn-specific IgG1 concentrations after the pre-school booster compared with wP-primed children. In contrast, after the pre-adolescent booster, the aP-primed children had significantly lower IgG1 concentrations for the vaccine antigens compared with wP-primed children.

Although the proportion of PT-specific IgG2 ranged from 3 to 55%, the IgG2 GMCs for PT were below 5 AU/mL at all time points for both groups of wP- and aP-primed children (supplementary Figure 2A), and the IgG3 GMCs for PT were even below 1 AU/mL (supplementary Figure 3A). Similar low concentrations were observed for FHA-, Prn-, diphtheria- and tetanus-specific IgG2 and IgG3 (supplementary Figures 2 and 3 respectively).

Differences in the prevalence of IgG subclass antibodies specific for PT, FHA, Prn, diphtheria and tetanus following wP- or aP-priming

An arbitrary cut-off value was used for the pertussis vaccine antigens of 5 AU/mL and for diphtheria and tetanus of 0.01 AU/mL to be able to discriminate between a seropositive and a seronegative response for each IgG subclass. The percentage of aP-primed children being IgG4 seropositive for the pertussis and diphtheria vaccine antigens was higher compared with wP-primed children at all time points (**Table 1**). For tetanus, almost all children regardless of wP- or aP-priming were seropositive 1 month after the booster vaccinations. Comparing age groups, more children at age 9 years were IgG4 seropositive for the vaccine antigens compared to 4 years, in both wP- and aP-primed groups. In contrast, 1 month after the booster vaccinations, more aP-primed children 4 years of age were IgG4 seropositive for all vaccine antigens compared with aP-primed children 9 years of age.

With respect to IgGt and IgG1, all participants were seropositive for vaccine antigen-specific IgGt and IgG1 after the booster vaccinations, except for a few wP-primed children for PT and Prn after the pre-school booster ate age 4 years (Table 1). One year after the pre-adolescent booster, the percentage of IgGt and IgG1 seropositive participants remained high for FHA, Prn, diphtheria and tetanus (90–95%), but had declined for PT-specific IgG1 (81–91%). IgG2 and IgG3 seropositivity for the DTaP vaccine antigens was low before and after both booster vaccinations (Table 1).

Table 1. Vaccine antigen-specific total IgG (IgGt) concentrations and the proportion of wP- and aP-primed participants with a seropositive response to IgGt, IgG1, IgG2, IgG3 and IgG4.

Antigen	Age group	n		IgGt GMC IU/mL		% IgGt positive		% IgG1 positive		% IgG2 positive		% IgG3 positive		% IgG4 positive	
		wP	aP	wP	aP	wP	aP	wP	aP	wP	aP	wP	aP	wP	aP
PT	4 years	51	61	4	7	43	69	6	31	35	33	0	0	0	0
	4 years + 1 month	52	40	52	164	96	100	87	100	54	43	6	0	6	70
	9 years	75	83	7	7	54	49	40	45	17	8	0	1	0	8
	9 years + 1 month	75	83	112	68	100	100	100	100	13	10	7	4	4	48
	9 years + 1 year	75	80	24	19	94	93	81	91	15	9	0	0	1	15
FHA	4 years	53	61	7	13	48	85	45	82	4	2	0	0	0	10
	4 years + 1 month	52	40	162	430	100	100	100	100	21	55	13	15	31	93
	9 years	75	83	34	27	94	99	92	99	5	8	1	1	5	20
	9 years + 1 month	75	83	282	158	100	100	100	100	7	22	7	5	47	64
	9 years + 1 year	75	80	106	60	100	100	100	100	7	13	1	1	25	26
Prn	4 years	53	61	3	23	26	95	15	89	4	3	2	3	0	16
	4 years + 1 month	52	40	186	1274	98	100	98	100	17	40	15	8	15	88
	9 years	75	83	13	36	73	96	69	95	5	7	4	1	3	25
	9 years + 1 month	75	83	343	307	100	100	100	100	4	20	19	11	19	82
	9 years + 1 year	75	80	93	99	97	100	96	100	4	13	5	6	16	53

Table 1. Continued

Antigen	Age group	n		IgGt GMC IU/mL		% IgGt positive		% IgG1 positive		% IgG2 positive		% IgG3 positive		% IgG4 positive	
		wP	aP	wP	aP	wP	aP	wP	aP	wP	aP	wP	aP	wP	aP
Diphtheria	4 years	61	61	0.1	0.1	100	95	97	92	41	43	0	5	11	26
	4 years + 1 month	52	40	1.3	0.8	100	100	100	100	52	50	8	8	88	98
	9 years	82	83	0.05	0.04	90	83	82	76	18	7	1	0	11	28
	9 years + 1 month	80	83	0.9	0.6	100	100	100	100	19	40	3	8	76	90
	9 years + 1 year	78	80	0.2	0.2	100	96	99	95	14	26	1	1	55	58
Tetanus	4 years	61	61	0.2	0.2	100	100	97	97	51	41	0	5	21	36
	4 years + 1 month	52	40	8.9	9.5	100	100	100	100	87	93	69	58	100	100
	9 years	82	83	0.5	0.4	100	100	100	100	35	35	0	22	54	69
	9 years + 1 month	80	83	9.2	7.4	100	100	100	100	74	93	20	70	99	100
	9 years + 1 year	78	80	2.1	2.0	100	100	100	100	42	86	1	51	96	83

Note: time points are before and after a pre-school DTaP booster vaccination in children aged 4 years and before and after an additional Tdap booster vaccination in children aged 9 years. Pertussis toxin (PT), filamentous hemagglutinin (FHA), and pertactin (Prn) seropositive cut-off was 5 AU/mL, for diphtheria and tetanus 0.01 AU/mL. Data of PT, FHA and Prn for wP-primed children 4 and 9 years of age and aP-primed children 4 years of age were published previously (25). Abbreviations: GMC: geometric mean concentration; IU: international units; wP: whole-cell pertussis; aP: acellular pertussis.

Discussion

In this study, we report that wP- and aP-priming in infancy determines the IgG subclass distribution against the 5 antigens in the DTaP combination vaccines in children 4–10 years of age before and after successive DTaP booster vaccinations. We found that after wP-priming in infancy, despite aP booster vaccinations at age 4 and 9 years, the IgG4 subclass proportion remained lower as compared to aP-priming in infancy (0.9–3.9% and 2.2–13.6% respectively for the pertussis vaccine antigens). This skewing towards IgG4 after aP-priming was observed not only for the pertussis antigens but also for the co-administered diphtheria and tetanus vaccine antigens.

Our results are in line with the study of Giammanco et al. who observed higher PT-specific IgG4 proportions in aP-primed infants 7 month of age as compared to wP-primed infants (36). They also showed a difference in the percentage of PT-specific IgG4 seropositive infants, as none of the wP-primed, and 37% of the aP-primed infants were seropositive for PT-specific IgG4. Accordingly, we found up to 70% of the aP-primed children to be seropositive for PT-specific IgG4, with a few wP-primed children who showed PT-specific IgG4 antibodies after the pre-school booster at age 4 years. The higher percentage of IgG4 found in our study is likely related to the age differences between the two studies. In addition, Zackrisson et al. reported PT-specific IgG4 responses in children 18–24 months of age who were primed with a monovalent PT vaccine (37).

Similar responses of tetanus-specific IgG4 antibodies have been described in the study of Kim et al. in infants primed with DTwP vaccines, compared with the tetanus-specific IgG4 levels found in our wP-primed children (38). Gruber et al. also reported an IgG4 up-regulation for diphtheria and tetanus in the absence of wP vaccine components in the combination vaccine, with diphtheria- and tetanus-specific IgG4 concentrations 4–5 times higher after DT- compared with DTwP-priming (39). Diphtheria- and tetanus-specific IgG subclass responses in DTaP-primed children have not been described before. Our findings indicate that 90–100% of the aP-primed children were IgG4 seropositive for diphtheria and tetanus after the pre-school and pre-adolescent booster vaccinations. Consequently, diphtheria- and tetanus-specific IgG4 proportions and arbitrary concentrations were significantly higher in aP-primed children compared with wP-primed children. This significant difference for diphtheria and tetanus was already noticed in children 4 years of age, before the pre-school DTaP booster vaccination, and similar as found for the pertussis vaccine antigens. Although aP-primed children showed higher IgG4 levels, all children from both groups had protective levels of IgG total and IgG1 for tetanus at all sampling time points and all except a few children for diphtheria as well.

One of the characteristics of wP combination vaccines is the presence of LPS and other bacterial components which are lacking in aP combination vaccines. These bacterial wP components trigger the innate immune system to induce a Th1 response against

the (co-administered) vaccine antigens (40). Consistent with these properties, we and others have demonstrated that pre-school children primed with DTwP vaccines elicit a more Th1-skewed response for the pertussis vaccine antigens after DTaP booster vaccination, but also upon clinical infection with *B. pertussis*. In contrast, DTaP-primed children showed a more mixed pertussis-specific Th1/Th2 profile (14, 18, 41). Another characteristic of wP-combination vaccines is the presence of a more diverse pertussis antigen pool, although the concentrations of the vaccine antigens included in the aP vaccines are lower in wP vaccines compared with aP vaccines (42). Repeated contact with high antigen concentrations, as is the case with the aP vaccines, can induce a more Th2-skewed immune response (43). Activated Th2 cells produce the cytokines IL-4 and IL-13, which stimulate B-cell class-switching to IgG4 (26). However, Th1 cells are more associated with protection against pertussis by their role in bacterial clearance than Th2 cells (44). Therefore, new adjuvants that skew the immune response towards a Th1 profile are desired.

IgG4 antibodies are associated with anti-inflammatory, tolerance inducing properties, and the upregulation of IgG4 is associated with less allergic symptoms in patients (45, 46). Unlike the other IgG isotypes, IgG4 is unable to activate the complement system (24). Consequently, an increase in pertussis-specific IgG4 antibodies may contribute to sub-optimal bactericidal activity. Although the IgG1 subclass was by far the most dominant, the proportion of IgG4 mounted to more than 20% in some aP-primed children. As a consequence, this could influence antibody-mediated protection against clinical pertussis. With higher vaccine antigen-specific IgG1/IgG4 ratios, wP-primed children may have a better protection against pertussis infection compared to aP-primed children, even after booster vaccinations. This is in line with epidemiological data indicating that adolescents, after aP vaccination in infancy, are more susceptible to pertussis compared with wP-primed adolescents, though wP-primed individuals become also susceptible over time (11).

Although recruitment periods differed for the 9 year old wP- and aP-primed children (2009 and 2013 respectively), no difference was observed in IgG levels to PT, as described previously (19). Exposure to *B. pertussis* was therefore most likely similar between the two groups and did not affect our results. Due to the lack of an international reference sample for the IgG subclasses, it is difficult to compare our results with other studies. However, all samples from both groups of wP- and aP-primed children were measured following the same procedure under strict quality requirements enabling the direct comparison of these 2 groups of children in IgG subclass responses after aP booster vaccinations.

In conclusion, our findings indicate that the switch from wP combination vaccines to aP combination vaccines in the first year of life in the national immunisation program, induced a permanently altered IgG subclass response with higher IgG4 levels against pertussis, diphtheria and tetanus vaccine antigens. New adjuvants included in the DTaP-priming vaccines for infants may direct towards the more preferred Th1 type

immune response which may contribute to better protection, particularly against clinical pertussis, over time.

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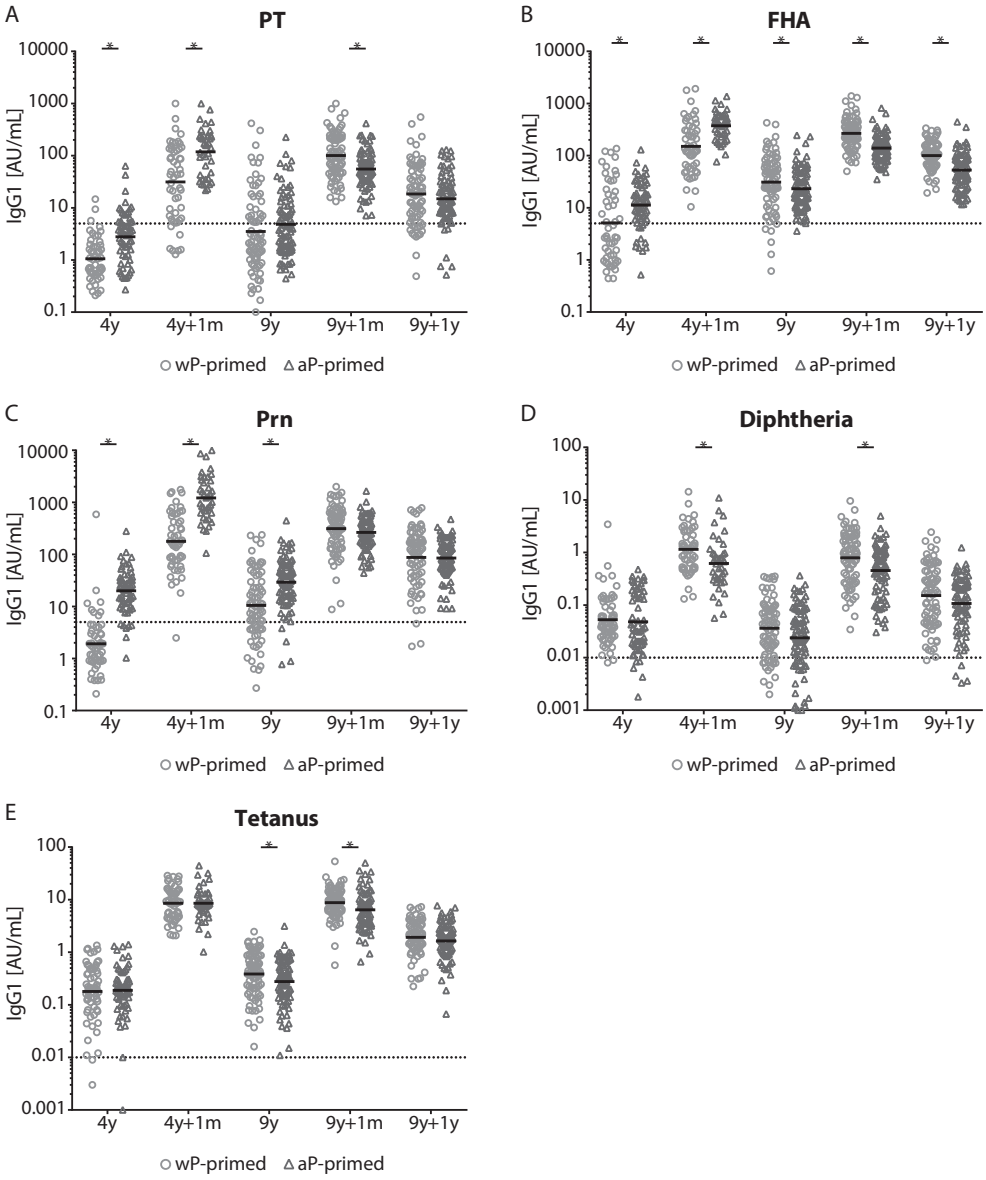
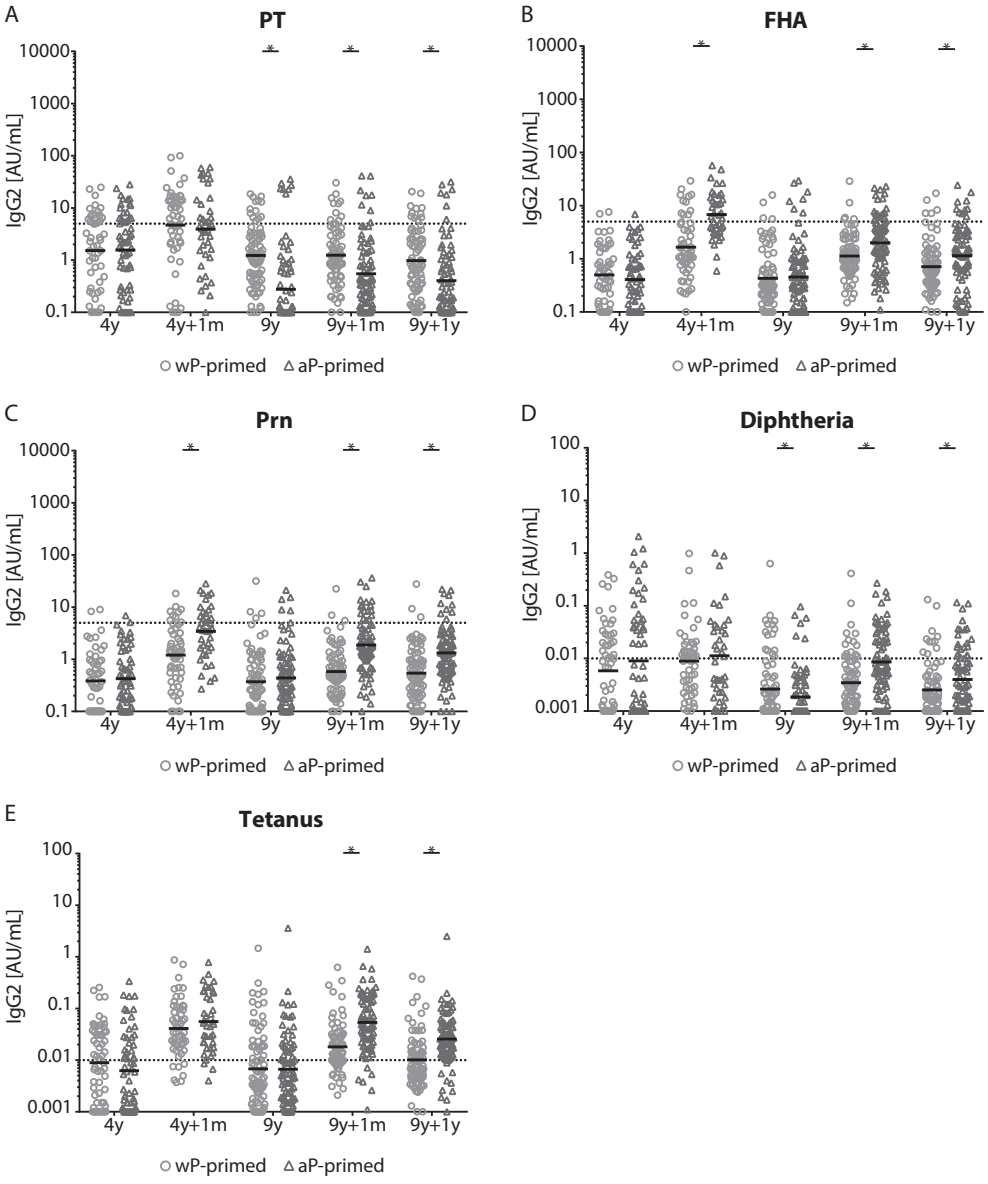
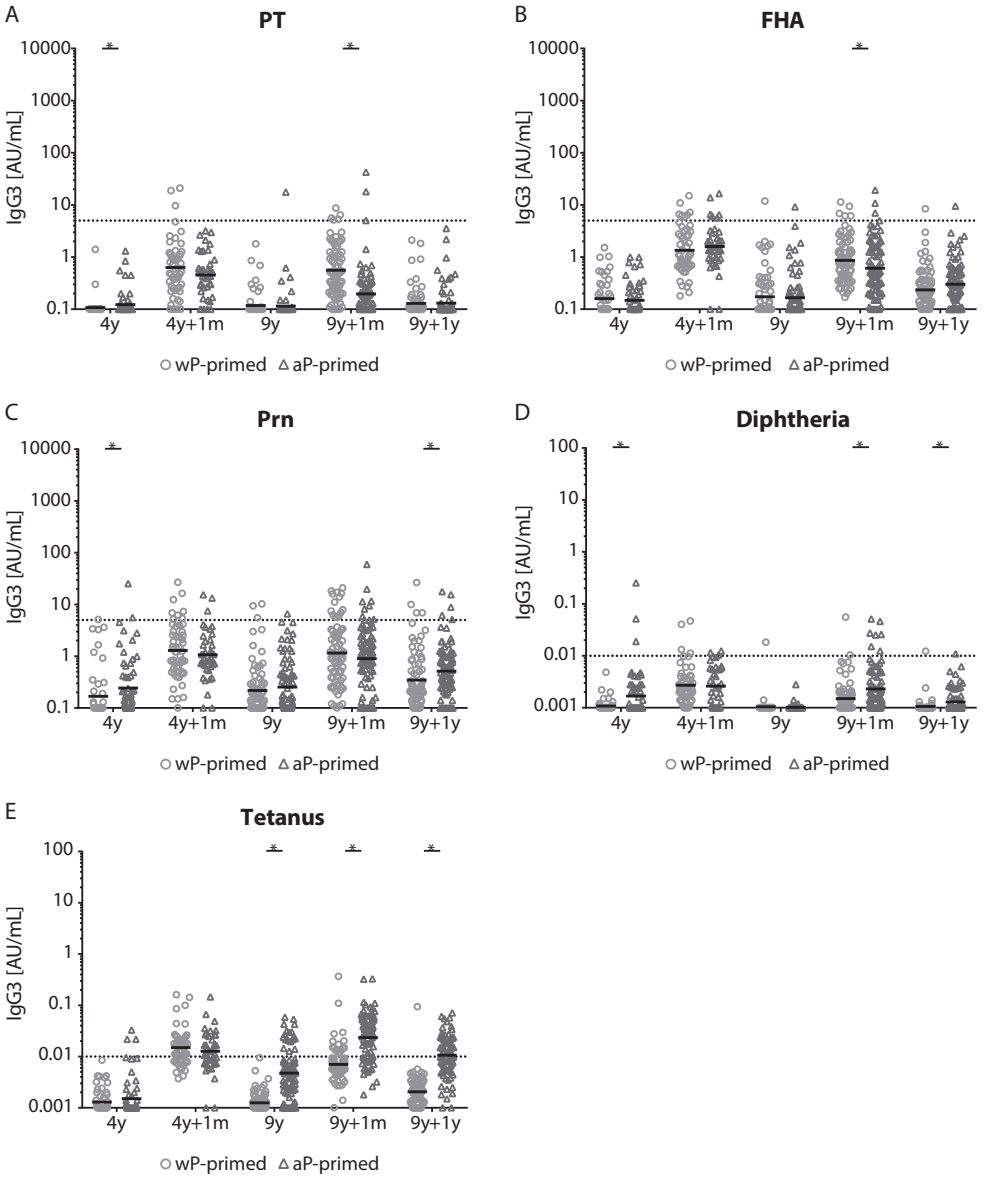
Supplementary data



Supplementary Figure 1. IgG1 subclass levels of 4–9 year old children covering 2 booster vaccinations. (A) pertussis toxin (PT), **(B)** filamentous hemagglutinin (FHA), **(C)** pertactin (Prn), **(D)** diphtheria toxoid and **(E)** tetanus toxin-specific IgG1 concentrations in arbitrary units (AU)/mL for whole-cell pertussis (wP) (light grey circles) and acellular pertussis (aP) primed (dark grey triangles) children 4 and 9 years of age. Samples were collected before and 1 month after the pre-school DTaP-booster vaccination and before, 1 month and 1 year after the pre-adolescent Tdap-booster vaccination. Dotted lines indicate arbitrary cut-offs for seropositive concentrations which was 5 AU/mL for PT, FHA and Prn and 0.01 AU/mL for diphtheria and tetanus. * indicates a significant difference between wP- and aP-primed children (p-value ≤.05).



Supplementary Figure 2. IgG2 subclass levels of 4–9 year old children covering 2 booster vaccinations. (A) pertussis toxin (PT), **(B)** filamentous hemagglutinin (FHA), **(C)** pertactin (Prn), **(D)** diphtheria toxoid and **(E)** tetanus toxin-specific IgG2 concentrations in arbitrary units (AU)/mL for whole-cell pertussis (wP) (light grey circles) and acellular pertussis (aP) primed (dark grey triangles) children 4 and 9 years of age. Samples were collected before and 1 month after the pre-school DTaP-booster vaccination and before, 1 month and 1 year after the pre-adolescent Tdap-booster vaccination. Dotted lines indicate arbitrary cut-offs for seropositive concentrations which was 5 AU/mL for PT, FHA and Prn and 0.01 AU/mL for diphtheria and tetanus. * indicates a significant difference between wP- and aP-primed children (p-value ≤.05).



Supplementary Figure 3. IgG3 subclass levels of 4–9 year old children covering 2 booster vaccinations. (A) pertussis toxin (PT), **(B)** filamentous hemagglutinin (FHA), **(C)** pertactin (Prn), **(D)** diphtheria toxoid and **(E)** tetanus toxin-specific IgG3 concentrations in arbitrary units (AU)/mL for whole-cell pertussis (wP) (light grey circles) and acellular pertussis (aP) primed (dark grey triangles) children 4 and 9 years of age. Samples were collected before and 1 month after the pre-school DTaP-booster vaccination and before, 1 month and 1 year after the pre-adolescent Tdap-booster vaccination. Dotted lines indicate arbitrary cut-offs for seropositive concentrations which was 5 AU/mL for PT, FHA and Prn and 0.01 AU/mL for diphtheria and tetanus. * indicates a significant difference between wP- and aP-primed children (p-value ≤.05).

Chapter 5

Elevated immune response among children 4 years of age with pronounced local adverse events after the fifth diphtheria, tetanus, acellular pertussis vaccination

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Abstract

Background

In the Netherlands, acellular pertussis vaccines replaced the more reactogenic whole-cell pertussis vaccines. This replacement in the primary immunisation schedule of infants coincided with a significant increase in pronounced local adverse events (AEs) in 4 years old children shortly after the administration of a fifth diphtheria, tetanus, acellular pertussis and inactivated polio (DTaP-IPV) vaccine. The objective of this study was to investigate possible differences in vaccine antigen-specific immune responses between children with and without a pronounced local AE after the fifth DTaP-IPV vaccination.

Methods

Blood was sampled in 2 groups of 4-year-olds: a case group reporting pronounced local swelling and/or erythema up to extensive limb swelling at the injection site (n = 30) and a control group (n = 30). PBMCs were stimulated with individual vaccine antigens. Plasma antigen-specific IgG, IgG subclass and total IgE concentrations and T-cell cytokine (interferon-gamma, interleukin (IL)-13, IL-17 and IL-10) production by stimulated PBMCs were determined by multiplex bead-based fluorescent multiplex immunoassays.

Results

In children with AEs, significantly higher total IgE and vaccine antigen-specific IgG and IgG4 responses as well as levels of the T-helper 2 (Th2) cytokine IL-13 were found after pertactin, tetanus and diphtheria stimulation compared with controls.

Conclusions

Children with pronounced local reactions show higher humoral and cellular immune responses. Acellular pertussis vaccines are known to skew toward more Th2 responses. The pronounced local AEs may be associated with more Th2-skewing after the fifth DTaP-IPV vaccination, but other biologic factors may also impact the occurrence of these pronounced local reactions.

Introduction

Whooping cough is re-emerging worldwide despite widespread adaptations of vaccination schemes. Since the early 1990s, whole-cell pertussis (wP) vaccines have been replaced by acellular pertussis (aP) vaccines (1-3). In 2005, the Dutch wP combination vaccine used in the primary vaccination series for infants at age 2, 3, 4 and 11 months, was replaced by an aP combination vaccine. Three years later, children who have been primed with aP vaccines in infancy received a fifth aP combination vaccine at 4 years of age. Since then, an increase in adverse events (AEs) shortly after the administration of the diphtheria, tetanus, acellular pertussis and inactivated polio (DTaP-IPV) preschool booster vaccine was observed. Significantly more pronounced local and systemic AEs were reported in aP-primed children compared with wP-primed children (4).

The underlying immunologic mechanisms for these heightened AEs in aP-primed children are not well understood. Previous studies revealed higher numbers of pertussis-specific T-helper 2 (Th2) cells indicative for Th2-skewing in aP-primed children compared with wP-primed children (5, 6). Profound Th2-skewing after aP vaccination was also shown in the baboon model when compared with wP vaccination (7). Besides pertussis-specific T-cell differences, significantly higher levels of pertussis-specific IgG, IgG4 and IgE antibodies were described in aP-primed children compared with wP-primed children (6, 8, 9). In general, IgG4, IgE and Th2-responses are associated with allergic inflammatory diseases such as asthma and atopic dermatitis (10, 11).

Pre-existing high tetanus-specific Th2 cytokine responses have been correlated with increased risk of local AEs (12) and pertussis toxin (PT)-specific IgE antibodies before and after DTaP booster vaccination were significantly higher in children with AEs compared with children without them (13). The differences in AEs between aP- and wP-priming suggest that the occurrence of AEs in aP-primed children could be related to pertussis-specific humoral and cellular immune responses. Few studies have reported on immune responses in children with AEs after a DTaP booster vaccination at pre-school age. We therefore performed a study in 4 years old children with and without a pronounced local AE. Blood samples were collected shortly after a fifth DTaP-IPV vaccination to investigate a possible association between immune responses and AEs. Vaccine antigen-specific humoral and cellular immune responses were compared between the 2 groups.

Methods

Study design and participants

AEs are requested to be reported to the Dutch Pharmacovigilance Centre (Lareb). Parents of 4 years old children who spontaneously reported local swelling and/or erythema of ≥5 cm at the injection site within 48 hours after the booster vaccination were invited to participate in the study by Lareb (case group). If parents were willing to participate, the study nurse made an appointment for a home visit 10 days (± 3 days) after the booster vaccination. A blood sample (8 mL) and a questionnaire including a few questions regarding atopic background of the children were collected. Parents of 4-year-olds without pronounced AEs (<1 cm) were invited to participate by an information letter (control group). The same study procedures after the booster vaccination applied for the control group. All children were vaccinated according to the Dutch national immunisation programme. Children were excluded in case of other vaccinations within a month before DTaP-IPV booster vaccination and in case of comorbidity or fever ≥38°C at the day of blood sampling. This phase IV case-control study was approved by the Medical research Ethics Committees United (MEC-U, Nieuwegein, the Netherlands). Written informed consent from both parents and/or legal representatives was obtained.

Vaccination background

At 4 years of age, children received a paediatric combination vaccine (DTaP-IPV) (Infanrix-IPV, GlaxoSmithKline Biologicals (GSK), Rixensart, Belgium). In infancy, children had received 4 paediatric DTaP-IPV combination vaccines at 2, 3, 4 and 11 months of age (Infanrix-IPV+Hib, Infanrix-hexa (both GSK) or Pediacel (Sanofi Pasteur, Lille, France)).

Blood samples

Peripheral blood was collected in vacutainer cell preparation tubes containing sodium citrate (BD Biosciences, San Diego, CA) and peripheral blood mononuclear cells (PBMCs) were isolated within 18 hours, washed, counted and stored at −135°C as described earlier (14) and plasmas were stored at −20°C.

Serological analysis

Plasma IgG antibody concentrations against diphtheria toxoid, tetanus toxin, and the 3 pertussis vaccine antigens namely PT, filamentous hemagglutinin (FHA) and pertactin (Prn) (DTaP antigens) were determined using the fluorescent-bead-based multiplex immunoassay (MIA) as described (15, 16). The in-house pertussis reference sample was calibrated to the World Health Organization International Standard (pertussis antiserum 1st international standard, 06/140, national institute for biologic standards and control, Potters Bar, UK) to express IgG concentrations in international units per millilitre (IU/mL). As a control, plasma IgG antibody levels against mumps, measles and rubella (MMR) were quantified using MIA as described (17).

The 4 IgG subclasses against DTaP vaccine antigens were measured as reported (9). Blanc mean fluorescent intensity (MFI) values were subtracted from all samples. Individual IgG subclass levels were first expressed as MFI values, and subsequently, each IgG subclass was expressed as a percentage of the sum of MFI of all 4 IgG subclasses together. Samples without a detectable response were given a value of 1 MFI.

DTaP antigen-specific IgE was determined according to the IgG MIA described above, by using mouse anti-human IgE-PE (eBioscience, San Diego, CA) as secondary antibody and expressed as MFI. For quantification of total IgE, Bio-Plex Pro Human IgE Isotyping Assay (Bio-Rad Laboratories, Hercules, CA) was used according to manufacturers' instructions.

T-cell stimulation

PBMCs were stimulated according to Schure et al. (6). In short, 3.0 × 105 viable cells per well were cultured in AIM-V medium (Gibco Invitrogen, Grand Island, NY) containing 5% heat-inactivated human AB serum (Harlan Laboratories, Leicestershire, UK) and stimulated with 2 µg/mL heat-inactivated PT or FHA (Kaketsuken, Kumamoto, Japan), 4 µg/mL recombinant Prn (18), 6.67 Lf/mL tetanus toxoid (Netherlands Vaccine Institute (NVI), Bilthoven, The Netherlands), 10 Lf/mL diphtheria toxoid (NVI), or 4.4 µg/ml lectin (LC) (Pokeweed mitogen, Sigma-Aldrich, MO) as a positive control. Non-stimulated cells served as negative controls. Cells were incubated for 5 days at 37°C with 5% CO2 in 96-well round-bottom culture plates, supernatants were collected and stored at −80°C until cytokine analysis.

Cytokine analysis

Cytokines interleukin (IL)-10, IL-13, IL-17 and interferon-gamma (IFN-γ) were quantified with MIA as described (19, 20). Cytokine concentration of non-stimulated cells were subtracted from all samples. Values below the detection limit were set at half of the lowest quantifiable concentration for each cytokine.

Statistical analysis

Geometric mean concentrations (GMCs) and corresponding 95% confidence intervals (CIs) of vaccine antigen-specific antibody and cytokine responses were calculated. Percentages of IgG subclasses were described as mean with standard deviations. Normal distribution of GMCs and mean percentages of log transformed data was checked before analysis. Differences between case and control groups were tested with Mann-Whitney U test. Differences in the ratio of IFN-γ/IL-13 within the groups were tested with Wilcoxon signed rank test. GMCs and corresponding 95% CIs were calculated using GraphPad Prism 6 (GraphPad Software, La Jolla, CA), and statistical analysis was performed using SPSS statistics 22 (IBM, Armonk, NY). A p-value of ≤.05 was considered statistically significant. Because of changes in the immunisation programme regarding vaccine manufacturer, participants have been divided in subgroups based on primary vaccines for subanalysis (**Table 1**).

Table 1. Characteristics of the case and control-groups.

Group	Number	Male no (%)	Age mean years (±SD)	Window mean days (±SD)	Priming vaccines[$#]		
					Infanrix[a]	Pediacel[b]	Mix[c]
Case	30	12 (40)	3.9 (0.2)	10.4 (2.0)*	7	11	12
Control	30	16 (53)	3.8 (0.1)	9.0 (1.8)	4	22	4

[$] Significant difference between case and control-group (p-value ≤.05).
[#] All priming vaccines received according to dutch national immunisation programme at 2, 3, 4, and 11 months of age.
[a] Number of children who received Infanrix-IPV-Hib (hexa); DTaP vaccine content: 25 µg PT, 25 µg FHA, 8 µg Prn, ≥30 IU Dd, ≥40 IU Td.
[b] Number of children who received Pediacel; DTaP vaccine content: 20 µg PT, 20 µg FHA, 3 µg Prn, 5 µg Fimbriae type 2 and type 3, ≥30 IU Dd, ≥40 IU Td.
[c] Number of children who received a combination of Infanrix-IPV-Hib (hexa) and Pediacel.

A linear mixed model analysis was performed to explore possible differences between the 2 study groups regarding the response to all vaccine antigens together per participant. This model consists of 3 parts. First, the response variable, which are the IgG4 MFI values, IFN-γ or IL-13 cytokine concentrations. Second, the so-called fixed effect variables (the explanatory variables), here a variable indicating whether a participant belongs to the case or control group (our variable of interest), and a variable indicating one of the 5 DTaP vaccine antigens, because vaccine responses will differ between the antigens. Third, the so-called random effect variable, here the participant ID, which is included in the model as a random intercept.

Results

Study population

Between March 2012 and September 2013, 63 children were enrolled in the study. During the inclusion period, 290 eligible children with pronounced local AEs were reported to Lareb and were invited to participate, of which 10% accepted to join the study. From 60 children, that is, 30 cases and 30 controls, sufficient plasma was available and from 59 enough PBMCs. Of the cases, 16 children showed inflammation of the whole upper limb and adjacent body parts (extensive limb swelling), and 14 children had injection site inflammation with at least 2 inflammation symptoms (swelling, redness, heat and/or pain) (see supplementary Figure 1 for an example of an AE). There were no differences in mean age and gender between the 2 groups (**Table 1**). A small but significant difference existed in the sampling window between the case and the control groups (mean days 10.4 ± 2.0 vs. 9.0 ± 1.8, respectively). Therefore, children were also divided into subgroups excluding those sampled at day 13 (7 children in the case group, none in the controls), which resulted in a similar sampling window between these subgroups. Because subgroup analysis excluding children sampled at day 13 resulted in similar findings, humoral and cellular immune responses were described for the entire study population.

Table 2. IgG antibody concentrations for the DTaP and MMR vaccine antigens around day 10 post DTaP-IPV pre-school booster vaccination at age 4 years.

	GMC IgG IU/mL (95% CI)		
Antigen	**Case group**	**Control group**	**p-value***
PT	76.9 (51.6–114.5)	63.4 (43.7–92.1)	.487
FHA	266.6 (199.7–356)	173.3 (119.1–252.1)	.067
Prn	590.5 (384.2–907.5)	358.6 (202.3–635.5)	.231
Dd	7.1 (5.6–9.1)	3.8 (2.5–5.6)	**.010**
TT	9.5 (7.0–12.8)	7.1 (5.1–10.0)	.574
Measles	1.8 (1.3–2.4)	1.7 (1.2–2.2)	.745
Mumps[#]	166.2 (115.4–239.3)	116.9 (80.7–169.5)	.690
Rubella	76.3 (55.0–105.7)	80.2 (59.8–107.7)	.145

Abbreviations: PT: pertussis toxin; FHA: filamentous hemagglutinin, Prn: pertactin, Dd: diphtheria toxoid; TT: tetanus toxin; GMC: geometric mean concentration; CI: confidence interval; MMR: measles, mumps, rubella; IU: international unit; RU: rijksinstituut voor volksgezondheid en milieu (RIVM) units.
[#] IgG concentration for mumps presented in RU/mL.
* significant different when p-value ≤.05 (bold value).

Humoral immune responses

The GMC of non-antigen-specific total IgE (supplementary Figure 2) of the case group (284.2 ng/mL, 95% CI 138.2–584.4) was significantly higher than that of the controls (109.8 ng/mL, 95% CI 50.6–238.4; p-value = .029). Antigen-specific IgE levels were also determined but were below the detection limit for the majority of children (data not shown).

Overall, the GMCs of DTaP antigen-specific IgG responses upon the pre-school booster vaccination tended to be higher in the case group compared with the controls, reaching significance only for diphtheria-specific IgG (p-value = .01) (**Table 2** and **Figure 1)**. As a control, IgG antibodies to the MMR vaccine antigens, which all children had received at 14 months of age, were measured. No differences in MMR-IgG GMCs between the case and control groups were observed (Table 2 and supplementary Figure 3).

Subsequently, IgG subclass responses showed that DTaP antigen-specific IgG2 and IgG4 MFI values were significantly higher in the case group compared with the controls. In concordance with total vaccine antigen-specific IgG, IgG1 MFI values were only significantly different between the 2 groups for diphtheria (**Table 3**). No differences in IgG3 MFI values were found between the groups. Notably, within the case group and the controls, the antigen-specific MFI values for the IgG subclasses varied considerably between the individuals. In line with results of a previous study, the sum of the MFI values of the IgG subclasses correlated well with the MFI values of antigen-specific total IgG (9).

The predominant IgG subclass for all 5 vaccine antigens in both groups was IgG1, with mean values ranging from 86.2% in the case group up to 95.4% in the controls

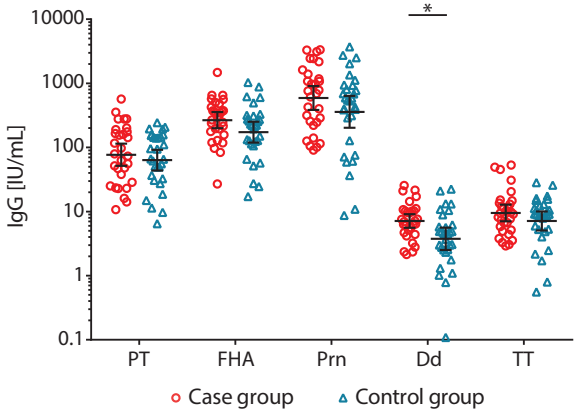

Figure 1. IgG concentrations in children 4 years of age around day 10 after the pre-school DTaP booster vaccination. Pertussis toxin (PT), filamentous hemagglutinin (FHA), pertactin (Prn), diphtheria toxoid (Dd) and tetanus toxin (TT) specific IgG levels (IU/mL) in plasma of children in the case group (red circles) and the control group (blue triangles). Note, black lines indicate geometric mean concentration with 95% confidence interval, * = p-value <.05.

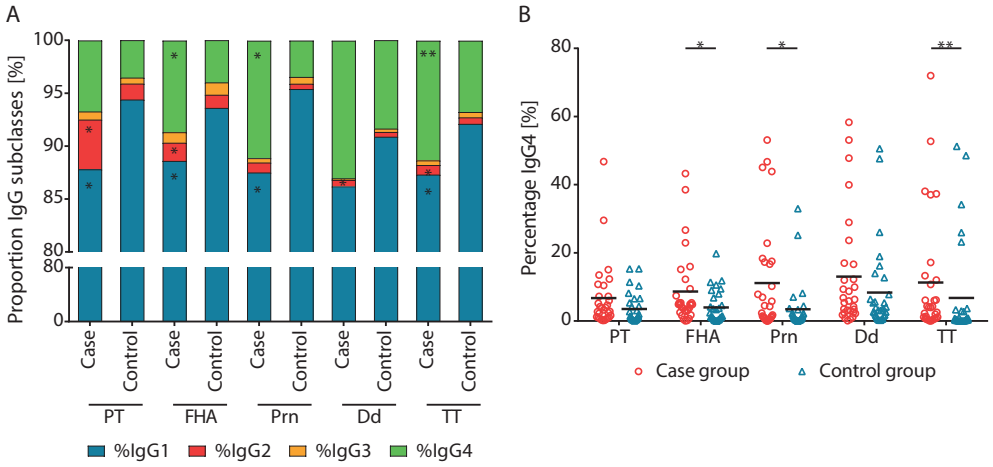

Figure 2. Mean IgG subclasses proportions in children 4 years of age around day 10 after the pre-school DTaP booster vaccination. (A) Distribution of IgG subclasses against pertussis toxin (PT), filamentous hemagglutinin (FHA), pertactin (Prn), diphtheria toxoid (Dd) and tetanus toxin (TT) for IgG1 (blue), IgG2 (red), IgG3 (yellow) and IgG4 (green). **(B)** PT, FHA, Prn, Dd and TT- specific IgG4 percentages for the case group (red circles) and the control group (blue triangles). Note * = p-value <.05 and ** = p-value <.01.

Table 3. DTaP vaccine antigen-specific IgG-subclasses.

		Geometric mean MFI x10^5 (95% CI)[#]			Mean percentage (± SD)[$]		
Subclass	**Antigen**	**Case group**	**Control group**	**p-value***	**Case group**	**Control group**	**p-value***
IgG1	PT	8.7 (5.7–13.2)	7.5 (5.1–10.9)	.559	87.8 (± 14.9)	94.4 (± 5.9)	**.022**
	FHA	58.6 (44.2–77.5)	39.8 (27.4–57.7)	.132	88.6 (± 11.1)	93.6 (± 5.3)	**.041**
	Prn	235.1 (160.2–344.9)	164.8 (96.6–281.2)	.469	87.5 (± 16.6)	95.4 (± 7.9)	**.029**
	Dd	246.4 (194.7–311.9)	139.1 (93.0–208.1)	**.021**	86.2 (± 16.4)	90.9 (± 12.8)	.135
	TT	183.3 (133.1–252.4)	151.9 (107.8–213.9)	.525	87.3 (± 18.2)	92.1 (± 15.3)	**.015**
IgG2	PT	0.12 (0.07–0.21)	0.04 (0.02–0.06)	**.010**	4.7 (± 9.9)	1.5 (± 2.6)	**.023**
	FHA	0.76 (0.52–1.13)	0.29 (0.16–0.55)	**.011**	1.7 (± 1.7)	1.2 (± 1.8)	**.025**
	Prn	0.81 (0.53–1.22)	0.23 (0.14–0.38)	**.001**	0.9 (± 1.6)	0.5 (± 0.8)	.084
	Dd	1.10 (0.82–1.47)	0.45 (0.32–0.65)	**<.001**	0.6 (± 0.8)	0.5 (± 0.5)	.329
	TT	0.75 (0.49–1.15)	0.24 (0.14–0.42)	**.001**	0.9 (± 1.3)	0.6 (± 1.1)	**.040**
IgG3	PT	0.03 (0.02–0.06)	0.02 (0.01–0.04)	.574	0.8 (± 1.6)	0.6 (± 0.9)	.941
	FHA	0.36 (0.23–0.57)	0.32 (0.22–0.47)	.941	1.0 (± 1.4)	1.2 (± 1.7)	.086
	Prn	0.33 (0.13–0.82)	0.20 (0.07–0.61)	.679	0.4 (± 0.8)	0.6 (± 1.1)	.941
	Dd	0.27 (0.19–0.38)	0.25 (0.17–0.36)	.701	0.2 (± 0.2)	0.3 (± 0.4)	**.046**
	TT	0.48 (0.32–0.73)	0.49 (0.35–0.69)	.888	0.5 (± 0.6)	0.5 (± 0.5)	.515
IgG4	PT	0.3 (0.2–0.6)	0.1 (0.1–0.2)	**.016**	6.7 (± 9.8)	3.5 (± 4.5)	.081
	FHA	2.7 (1.5–4.8)	0.6 (0.3–1.3)	**.002**	8.7 (± 10.8)	4.0 (± 4.8)	**.018**
	Prn	7.5 (3.3–16.7)	1.5 (0.7–3.2)	**.004**	11.1 (± 15.9)	3.5 (± 7.3)	**.024**
	Dd	17.0 (9.4–30.7)	5.2 (3.3–8.3)	**.002**	13.0 (± 16.4)	8.4 (± 12.7)	.128
	TT	6.8 (3.6–12.8)	1.2 (0.5–2.7)	**.001**	11.3 (± 17.9)	6.8 (± 14.4)	**.003**

Abbreviations: PT: pertussis toxin; FHA: filamentous hemagglutinin; Prn: pertactin; Dd: diphtheria toxoid; TT: tetanus toxin.

[#] Geometric means of mean fluorescent intensity (MFI) with 95% confidence intervals (95% CI). MFI values were corrected for measurement dilution.

[$] Individual MFI values of each IgG-subclass was expressed as a percentage of the sum of MFI of all four IgG-subclasses together and depicted as mean percentages with standard deviation (SD).

* Significant different when p-value ≤.05 (bold values).

(**Figure 2A** and **Table 3**). After IgG1, IgG4 contributed most to the IgG levels, up to 13%. IgG2 and IgG3 hardly contributed to the antigen-specific IgG levels, respectively ranging from 0.5% to 4.7% and 0.2% to 1.2%. The contribution of IgG1 to total IgG was significantly lower in the case group compared with the controls for the pertussis antigens PT, FHA and Prn and for tetanus (Table 3). In particular, the relative contribution of IgG4 specific for FHA, Prn and tetanus was significantly higher in the case group compared with the controls. Also, most of the IgG2 contributions were significantly higher in the case group, but these percentages were (very) low (Table 3). Next to the overall antibody values, several children in both groups showed high proportions of antigen-specific IgG4, varying from around 20% up to even 72% (**Figure 2B**). More specific, 17 of 30 children (57%) in the case group and 7 of 29 (23%) in the controls showed a proportion of IgG4 of ≥10% for 1 or more antigen. Interestingly, 3 children in the case group and 2 in the control group showed ≥10% IgG4 antibodies for all 5 vaccine antigens. Overall, children with extensive limb swelling tended to show higher humoral responses than children with injection site inflammation, reaching significance for PT- and Prn-specific IgG4.

The response to all 5 vaccine antigens together was analysed with a linear mixed model for IgG4 MFI values. Comparing the case and the control groups, the combined IgG4 response to the 5 DTaP antigens resulted in a GMC ratio of 4.06 (95% CI 1.84–8.86; p-value <.001). This corroborates the difference in IgG4 response between the 2 groups even further.

Because a difference was observed between the case and the control groups in vaccines during the primary series, a subanalysis was performed including just the participants primed with Pediacel vaccines (Table 1). Overall, the subanalysis resulted in similar differences in humoral immune responses between the case and the control group compared with the analysis of the entire study population, although for some parameters significance was lost because of low numbers in these subgroups (data not shown).

Cellular vaccine antigen-specific cytokine responses

T-helper cytokines IFN-γ (Th1), IL-13 (Th2), IL-17 (Th17) and IL-10 (T-regulatory) were evaluated after antigen stimulation. IFN-γ was higher in the case group compared with the controls for FHA, Prn and diphtheria, although not significant (supplementary Figure 4). Furthermore, no difference was found between the 2 groups for the combined results of the DTaP antigens using the linear mixed model. IL-13 values after PBMC stimulations with Prn, diphtheria and tetanus, as well as IL-10 values after stimulation with Prn, were significantly higher in the case group compared with the controls. IL-17 responses after the PBMC stimulation of all vaccine antigens were not significantly different between the 2 groups.

Variation between individuals within the groups proved high for all different cytokine concentrations, up to a 1000-fold. While some children did not have detectable IFN-γ

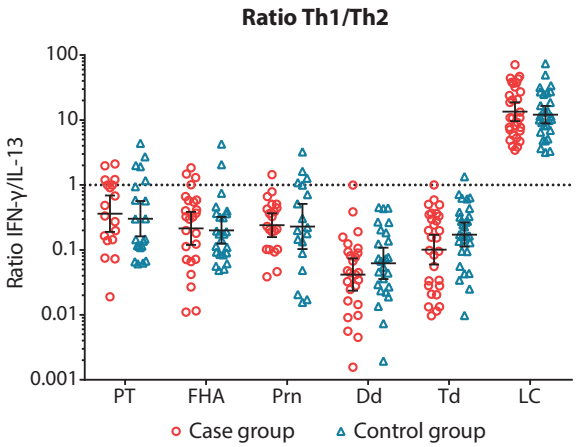

Figure 3. Th1/Th2 ratio in children 4 years of age around day 10 after the pre-school DTaP booster vaccination. The IFN-γ (Th1)/ IL-13 (Th2) ratio in supernatants of pertussis toxin (PT), filamentous hemagglutinin (FHA), pertactin (Prn), diphtheria toxoid (Dd) and tetanus toxin (TT) stimulated PBMCs of children in the case group (red circles) and the control group (blue triangles). Note, black lines indicates geometric mean IFN-γ/IL-13 ratio with 95% confidence interval and dotted lines indicates an IFN-γ/IL-13 ratio of 1.

or IL-13 responses, high values of them were produced by PBMCs stimulated with LC, indicating that all children were able to induce Th1 and Th2 responses. Furthermore, all children showed IL-10 and IL-17 responses to at least one of the antigens. After LC stimulation, no significant differences between the 2 groups were observed for any of the cytokines supporting that differences between groups were antigen-specific (see supplementary Figure 4).

Comparing IFN-γ and IL-13 concentrations within the 2 groups, IFN-γ was significantly lower than IL-13 for all 5 DTaP vaccine antigens (all p-values <.01). This resulted in IFN-γ/IL-13 ratios <1 for all antigens in both groups, which is indicative for Th2-skewing (**Figure 3**). This low IFN-γ/IL-13 ratio was most pronounced for diphtheria (ratio of 0.06 for the case group and 0.04 for the controls) but without differences between the 2 groups. In contrast, a-specific LC stimulation induced significantly more IFN-γ than IL-13 (with IFN-γ/IL-13 ratio of 13.5 for the case group and 12.1 for the controls), showing that the Th2-skewing was specific for the antigens in the pre-school DTaP booster vaccination. Similar results were found in the subgroups based on vaccination background showing significance just for IL-13 levels after tetanus stimulation.

Discussion

This study showed elevated immune responses to the DTaP vaccine antigens in children with a pronounced local AE within 48 hours upon a fifth DTaP-IPV vaccination at 4 years of age compared with children without an AE (controls), though with high individual variation. Children with AEs showed significantly higher values of total IgE, diphtheria-specific IgG, IgG4 for all DTaP antigens and IL-13 for Prn, diphtheria and tetanus. Th2-skewing was observed for all DTaP vaccine antigens in both groups.

Two previous studies found no differences in total antigen-specific IgG responses between children with and without AEs after vaccination (12, 21). In contrast to our study, only tetanus-specific IgG was analysed (12) or the allocation of subgroups was based on AEs after the primary vaccinations rather than the occurrence of AEs after the booster vaccination (21). However, the significantly higher diphtheria-IgG levels in our study were in agreement with results of Edelman et al. (13) This similarity was remarkable since their study was conducted in 2 years old children sampled 1 month after their fourth DTaP vaccination.

The most remarkable finding in our study was the high values of all vaccine antigen-specific IgG4 responses in the case group. In general, protein antigens predominantly elicit an IgG1 subclass response, with less IgG3 and IgG4 and rarely IgG2 (22-24). Surprisingly, we found high proportions of DTaP antigen-specific IgG4, with a prevalence of even 11% tetanus-specific IgG4 antibodies in the case group. This is in contrast to previous studies, where a very low prevalence of tetanus-specific IgG4 was reported (23, 25). With regard to pertussis-specific IgG subclass responses, so far only 3 studies were conducted in aP-primed children (9, 26, 27). The results of these studies were consistent with our study showing higher IgG4 proportions after repeated exposure to vaccine antigens (23, 25). IgG4 responses are associated with chronic antigenic stimulations (28), suggesting that the high amounts of purified antigens in the DTaP vaccines given within a relatively short period (at 2, 3, 4 and 11 months of age) initiate this response. Interestingly, a sixth DTaP booster vaccination in pre-adolescents also results in at least similar high IgG4 production (29). Because class switching to IgG4 is dependent on the Th2 cytokines IL-4 and IL-13, similar to IgE class switching, IgG4 is often associated with Th2-skewed responses (22). IgG4 antibodies are suggested to have anti-inflammatory properties as the appearance of IgG4 seems to be related to symptom reduction in allergic patients (22, 30). This might suggest that an AE following DTaP vaccination is associated with higher total IgE and IL-13 levels and that IgG4 may not contribute to clinically observed AEs in these children. The determination of IgG4 responses could be relatively easily implemented in more vaccine immunogenicity studies to be able to evaluate Th2-skewing of vaccine responses.

Studies investigating atopic diseases have reported significantly lower Th1/Th2 ratios in supernatants of LC stimulated PBMCs in atopic children compared with non-atopic

children (11). In contrast, we observed a high Th1/Th2 ratio in both groups upon LC stimulation suggesting that the higher IgE values 10 days post vaccination may be related to vaccine antigens and not to the already established Th2-skewing in the case group. However, it is also reported that plasma Th2 cytokine values in 4–6 years old aP-primed children were higher before and 35 days after aP booster vaccination in atopic children compared with non-atopic children, all without AEs (31). We did not find more atopic children in our case group based on questionnaires, but the numbers of children in our groups were small. However, the higher total IgE values in children with an AE suggests a more atopic background. Atopic children could be at increased risk for AEs after a fifth DTaP vaccination but this needs to be further studied (10, 11, 28, 32). Interestingly, swelling of the injection site is also seen after repeated administration of influenza vaccines in children and adults (33).

Recently, Scheifele et al. reported a doubled incidence of erythema at the injection site in children with detectable diphtheria-specific cell-mediated immunity before a DTaP booster vaccination, compared with children without diphtheria-specific cell-mediated immunity (34). This suggests that pre-existing high levels of vaccine antigen-specific Th2 cells and the Th2-skewed immune response to DTaP vaccination influence the susceptibility to local AEs post-booster vaccination. We found significantly higher values of the Th2 cytokine IL-13 for Prn, diphtheria and tetanus in the case group, which is in line with others showing this just for tetanus (12). The high Th2 responses to the vaccine antigens in aP-primed children might be due to the absence of bacterial components in the aP combination vaccines, which were present in wP vaccines. The lack of these components could be the cause of a more Th2-skewed response, not just specific for the pertussis antigens but also for the diphtheria and tetanus antigens (35, 36). Our findings of Th2-skewing were vaccine antigen-specific since a-specific stimulation with LC induced Th1-dominated responses as expected, and the IFN-γ/IL-13 ratio below 1 was only found for the vaccine antigens. Although human studies are scarce, we and others previously reported mixed Th1/Th2 profiles in aP-primed children, with a tendency to Th2-skewing (5, 37).

Immune responses in relation to AEs have been studied for one or some of the DTaP vaccine antigens, mostly 1 month post-booster vaccination in 4–6-year-olds (12, 34). A strength of our study was the ability to investigate a broad range of vaccine antigen-specific humoral and cellular immune responses in relation to AEs already 10 days after the pre-school DTaP booster vaccination, when immune responses are already at its peak (6, 8, 38). This short time interval between vaccination and sampling enabled us to establish differences between groups that would otherwise have remained unobserved. Although there was a significant difference in sampling window between the 2 groups, a subanalysis was performed excluding children sampled on day 13, which resulted in a similar sampling window between the 2 groups. Moreover, results of the subanalysis regarding differences in immune responses between the 2 groups were comparable with the analysis performed on all children. Furthermore, de Voer

et al. demonstrated that meningococcal serogroup C–specific IgG concentrations were similar 10 and 17 days after meningococcal serogroup C conjugate booster vaccination (39), indicating that variation in sampling interval from 10 to 13 days post vaccination may not be critical as the IgG values seem not to increase any further.

One of the limitations of this study was that pre-vaccination samples were not included. However, the occurrence of pronounced AEs is too low and does not allow the enrolment of many children for longitudinal sampling and also resulted in small group numbers in this study. Despite the small groups, we do see interesting differences between children with and without pronounced AEs. Another limitation is the different priming with 3 aP combination vaccines. However, we reanalysed all findings in subgroups with participants only vaccinated with Infanrix or Pediacel combinations. Although numbers were low and the power limited, similar differences as described were observed in these subgroups. This indicates that the differences observed between the case and the control groups in humoral immune responses after the pre-school booster vaccination were most likely not driven by a difference in priming vaccines.

As a control, MMR antibody levels, induced after MMR vaccination at 14 months of age, were determined to explore if the antibody values for non-DTaP vaccine antigens between both groups were similar. From a population-based serosurveillance study performed in 2006/2007, it was clear that the decay of MMR antibody values was only minimal between 14 months and 4 years. We found no differences in MMR antibody values between the 2 groups indicating that other vaccines induced comparable immune responses in both groups (40).

In summary, it was previously suggested that the AEs after the pre-school DTaP booster vaccination were most likely due to the repeated administration of the aP vaccine antigens, because the increase in AEs was mainly seen after introduction of aP combination vaccines in the primary vaccination schedule. We now demonstrated high immune responses in children with pronounced AEs not only to the pertussis vaccine antigens but also to diphtheria and tetanus, and Th2-skewing for all antigens in all participants. The variation in the individual immune responses of children with an AE indicates that these events are associated with an accumulation of high Th2-skewed responses in combination with an additional biologic factor. This needs to be evaluated in further studies. On the long run, new DTaP vaccines that elicit a more Th1-skewed response might reduce the risk of these rare local, but pronounced, reactions.

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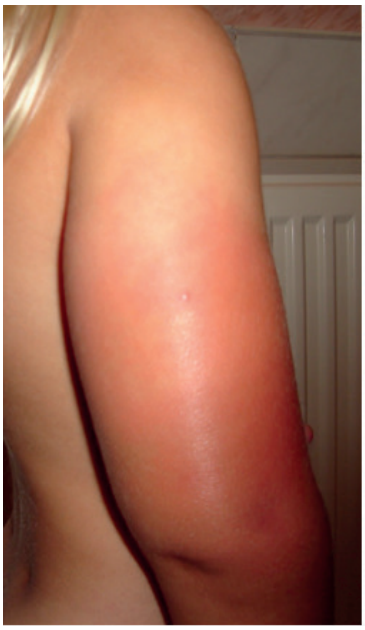
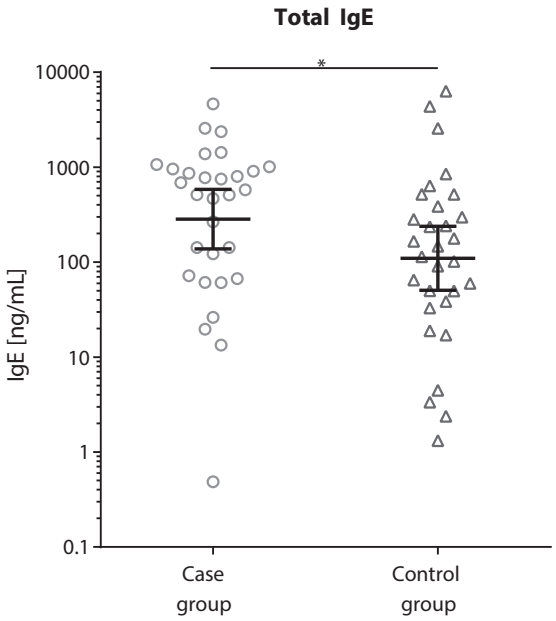
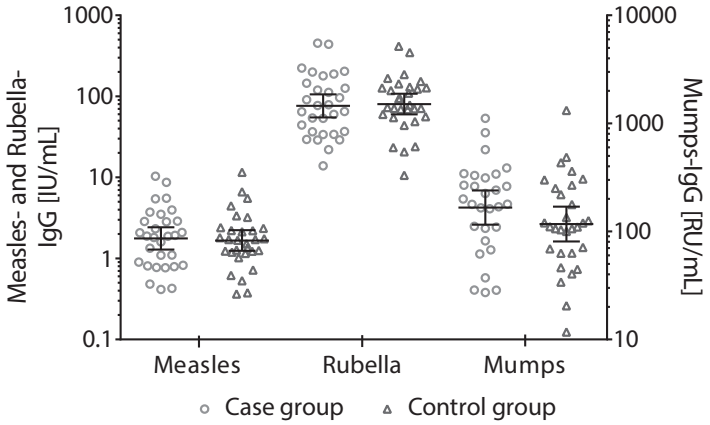
Supplementary data



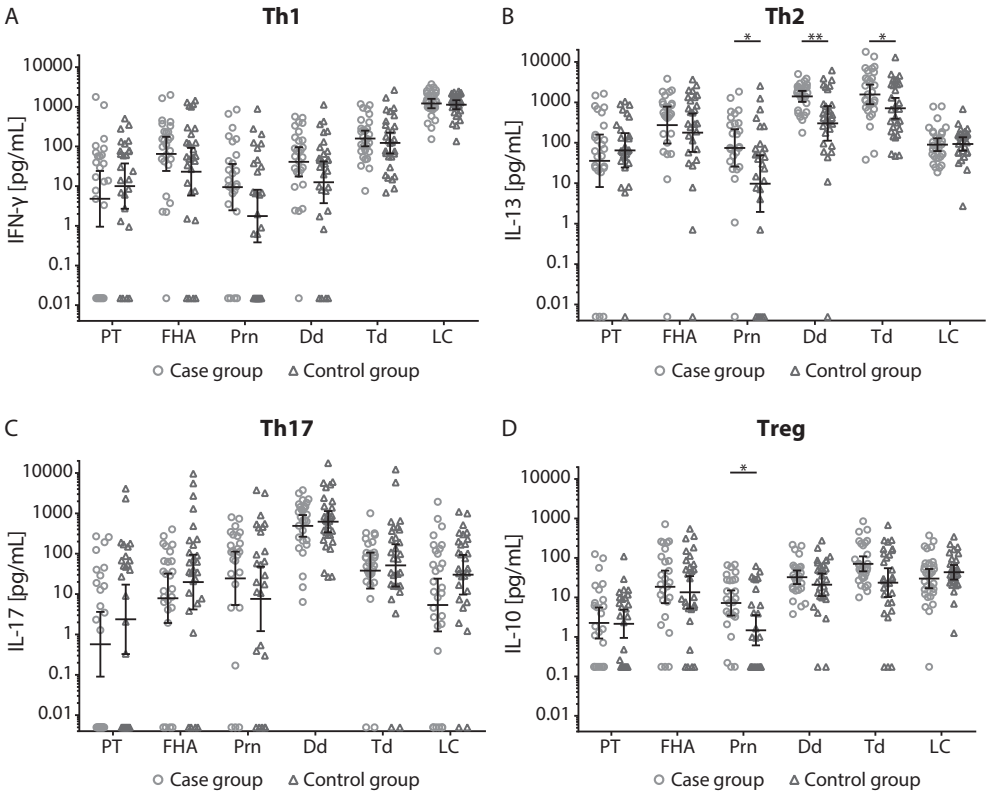
Supplementary Figure 1. Example of an adverse event after the fifth paediatric DTaP-IPV vaccination at four years of age (David et al., 2010, Nederlands Tijdschrift voor Geneeskunde).



Supplementary Figure 2. Total IgE antibody levels in children 4 years of age around day 10 after the pre-school DTaP booster vaccination. Total IgE geometric mean concentration (ng/mL) with 95% confidence interval for the case group (light grey circles) and the control group (dark grey triangles). * = p-value ≤.05



Supplementary Figure 3. MMR IgG antibody levels in children 4 years of age around day 10 after the pre-school DTaP booster vaccination. Measles, Mumps and Rubella (MMR) specific IgG levels (IU/ml for measles and rubella (left Y-axis), RU/ml for mumps (right Y-axis)) for the case group (light grey circles) and the control group (dark grey triangles) in children four years of age. Note, black lines indicate geometric mean concentrations with 95% confidence intervals.



Supplementary Figure 4. Cytokine levels of stimulated PBMCs in children 4 years of age around day 10 after the pre-school DTaP booster vaccination. (A) Th1 (IFN-γ), **(B)** Th2 (IL-13), **(C)** Th17 (IL-17) and **(D)** Treg (IL-10) in supernatants of stimulated PBMCs of the case-group (light grey circles) and the control-group (dark grey triangles). Stimulated with: pertussis toxin (PT), filamentous hemagglutinin (FHA), pertactin (Prn), diphtheria toxoid (Dd), tetanus toxoid (Td), and pokeweed mitogen lectin (LC). N = 25–30. Note, black lines indicate geometric mean concentrations with 95% confidence intervals, * = p-value ≤.05; ** = p-value ≤.01.

Chapter 6

Robust humoral and cellular immune responses to pertussis in adults after a first acellular booster vaccination

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Submitted

Abstract

Background

To reduce the pertussis disease burden, nowadays several countries recommend acellular pertussis (aP) booster vaccinations for adults. We aimed to evaluate the immunogenicity of a first adult aP booster vaccination at childbearing age.

Methods

In 2014, healthy adults aged 25–29 years (n = 105), vaccinated during infancy with 4 doses of whole-cell pertussis (wP) vaccine, received a Tdap (tetanus, diphtheria and aP) booster vaccination. Blood samples were collected longitudinally pre-booster, 2 and 4 weeks and 1 and 2 years post-booster. Tdap vaccine antigen-specific antibody levels and memory B- and T-cell responses were determined at all time points. Antibody persistence was calculated using a bi-exponential decay model.

Results

Upon booster vaccination, the IgG levels specific to all Tdap vaccine antigens were significantly increased. After an initial rapid decline in the first year, PT-IgG antibody decay was limited (15%) in the second year post-booster. The duration of a median level of PT-IgG ≥20 IU/mL was estimated to be approximately 9 years. Vaccine antigen-specific memory B- and T-cell numbers increased and remained at high levels although a significant decline was observed after 4 weeks post-booster. However, Th1, Th2 and Th17 cytokine production remained above baseline for 2 years.

Conclusions

The Tdap booster vaccination in wP-primed Dutch adults induced robust long-term humoral and cellular immune responses to pertussis antigens. Furthermore, PT-IgG levels are predicted to remain above the presumed protective cut-off for at least 9 years which deserves further attention in evaluating the current recommendation to revaccinate women during every new pregnancy.

Introduction

The incidence of clinical pertussis cases strongly declined after the introduction of whole-cell pertussis (wP) vaccines in infant national immunisation programmes (NIP) in the 1940s and 1950s (1). Despite a consistently high vaccination coverage, an increase in numbers of pertussis cases is observed in many countries and in all age groups (2-6). Consequently, in addition to pre-school acellular pertussis (aP) booster vaccinations, several countries have implemented aP booster vaccinations for adolescents and adults (7-9). Furthermore, to protect unvaccinated infants against pertussis, pregnant woman are advised to receive maternal aP booster vaccination in more than 25 countries (10-12).

Waning immunity after vaccination and natural infection as well as the switch from wP to aP vaccines in the primary infant vaccination series are thought to have contributed to the pertussis resurgence (13-15). After an aP vaccination in adults, low pertussis antibody levels and in particular low anti-pertussis toxin (PT) antibodies, that are considered the most protective against clinical *B. pertussis* symptoms, have been observed already within 1 year (16, 17). These studies were however conducted in a period with a presumed lower circulation of *B. pertussis* (late 1990s) in comparison to the last decade (18, 19).

Nowadays, increased circulation of *B. pertussis* might allow for more natural boosting of the immune system, and affect antibody kinetics as well as cellular immunity after an adult aP booster vaccination. We aimed to evaluate the long-term immunogenicity of a first adult aP booster vaccination at childbearing age. A bi-exponential antibody decay model was used to predict the duration of antibody persistence to PT after vaccination (20, 21). This study provides valuable information for the improvement of adult and maternal pertussis vaccination programmes.

Methods

Study design and participants

In this phase IV, longitudinal intervention study, healthy Dutch adults 25–29 years of age were recruited to receive a tetanus, diphtheria and acellular pertussis (Tdap) booster vaccination. Exclusion criteria were pregnancy at the start of the study; present severe disease or medical treatment that might interfere with study results; an adverse event after previous vaccinations; other pertussis vaccinations than those given according to the Dutch NIP; diphtheria and/or tetanus vaccination in the last 5 years; plasma products received in the last 6 months; any vaccination in the last month and/or antibiotic use or fever (≥38 ºC) in the 2 weeks before study enrolment. Written informed consent was obtained at the start of the study. The study was approved by the Medical Ethics Review Committee North Holland (METC-NH, Alkmaar, the Netherlands) and registered at the European clinical trials database

(2013-005355-32) and the Dutch trial register (www.trialregister.nl; NTR4494).

Vaccination background

All participants had received the Dutch diphtheria, tetanus, whole-cell pertussis and inactivated poliovirus (DTwP-IPV) combination vaccine (National institute for Public Health, Bilthoven, the Netherlands) according to the then NIP at 3, 4, 5 and 11 months of age. In this study, the participants received a Tdap booster vaccine (Boostrix™, GlaxoSmithKline, Rixensart, Belgium). The vaccine contained 8 µg PT and filamentous hemagglutinin (FHA), 2.5 µg pertactin (Prn), ≥2 IU diphtheria toxoid (Dd) and ≥20 IU tetanus toxoid (Td).

Blood samples

Serum samples were collected just before, 14 days (± 2 days), 28 days (± 2 days), 1 year (± 2 weeks) and 2 years (± 2 weeks) after the Tdap booster vaccination. Sera were stored at –20ºC until analysis. From a randomly selected subset of 60 participants, additional blood was sampled in vacutainer cell preparation tubes containing sodium citrate (Becton Dickinson (BD) Biosciences, San Jose, CA). PMBCs were isolated within 16 hours, and stored at –135ºC as described previously (22).

Serological analysis

PT-, FHA- and Prn-specific IgG and IgA and Dd- and tetanus toxin (TT)-specific IgG antibody concentrations were quantified using the fluorescent-bead-based multiplex immunoassay (MIA) as described previously (23-25). To express pertussis-IgG and IgA concentrations in international units (IU) per mL, the WHO international standard (pertussis antiserum 1st international standard, 06/140, NIBSC) was used. A PT-IgG concentration of 20 IU/mL was used as an arbitrary cut-off for protection (26) and 50 IU/mL to indicate an infection with pertussis in the preceding years (18, 24). An IgA concentration ≥1 IU/mL was used as seropositive.

From 42 longitudinal samples, the PT- and Prn-IgG avidity was determined using the MIA with minor modifications (27), using 1.5 M (for PT) and 2.5 M (for Prn) ammonium thiocyanate (NH_4SCN). The geometric mean avidity index (GMAI) was expressed as the percentage of antibodies that remained bound to PT- or Prn-conjugated beads after NH_4SCN treatment in comparison to untreated (PBS) samples.

Flow cytometry

The absolute numbers of circulating B-cells and B-cell subsets were determined in 60 paired samples before and 2 weeks after the booster vaccination with a lyse-no-wash protocol using TruCOUNT tubes (BD Biosciences). The fluochrome-conjugated antibodies CD19(J3-119)-PE-Cy7 (Beckman Coulter, Fullerton, CA), CD27(M-T271)-BV421, IgD(IA6-2)-FITC (both from Biolegend, San Diego, CA), and CD38(HB7)-APC-H7 (BD Biosciences) were used. Samples were measured using a LSRFortessa flow cytometer (BD Biosciences). The B-cell population in PBMCs before and after

culture was determined using CD19-PerCPCy5.5, (BD Biosciences) and samples were measured on a FacsCanto flow cytometer (BD Biosciences). Data were analysed using FACSDiva™ v8 (BD Biosciences) and FlowJo v10 (FlowJo company, Ashland, OR) with a gating strategy as described previously (28).

Antigen-specific B- and T-cell responses

From 30 longitudinal samples, vaccine antigen-specific B- and T-cell responses were determined. For B-cell responses, PBMCs were polyclonally stimulated for 5 days after which the number of specific IgG memory B-cells/10^5 $CD19^+$ cells were determined in PT-, FHA-, Prn- and Td-specific ELISpot assays (22). Per participant, samples were determined simultaneously. Lower limit of quantification was 0.5 spots/10^5 $CD19^+$ cells.

For T-cell responses, PBMCs were stimulated for 5 days with PT (heat inactivated), FHA, Prn, Dd or Td after which supernatants were collected and stored at –80ºC (29). Unstimulated and pokeweed mitogen stimulated cells served as negative and positive controls respectively. The cytokines interferon-gamma (IFN-γ) (Th1), interleukin-13 (IL-13) (Th2), IL-17 (Th17), and IL-10 (Treg) were quantified in the supernatants using an in-house MIA developed according to de Jager et al. (30, 31) and calibrated against the Bio-Plex cytokine assay kit (Bio-Rad Laboratories, Hercules, CA).

Statistical analysis

Geometric mean concentrations with corresponding 95% confidence intervals were calculated for vaccine antigen-specific IgG, IgA and cytokine concentrations. Numbers of vaccine antigen-specific memory B-cells are reported as geometric mean values/10^5 $CD19^+$ cells. The mean percent reduction of the IgG concentrations was calculated between the different time points after vaccination. The kinetics of IgG antibody levels was determined with a bi-exponential decay model as described earlier (32).

Normal distribution of (log-transformed) data was confirmed prior to each analysis. Differences between time points were tested with paired sample t-tests (normal distributed data) or with Wilcoxon Signed Ranks tests (not normally distributed data). Correlations between variables were determined with Spearman correlations and linear regression analysis. A p-value <.05 was considered statistically significant. Data were analysed using GraphPad Prism v7 (GraphPad Software, La Jolla, CA) and SPSS statistics v24 (IBM, Armonk, NY).

Results

Study baseline characteristics

At the start of the study in April 2014, 105 participants received a Tdap booster vaccination (**Figure 1**). Two years after the booster, 90.6% (96/106) of the participants completed all study visits and ≥100 blood samples had been collected at every time point. Mean age at the start of the study was 27.6 ± 1.4 years and 34% was male (36/106).

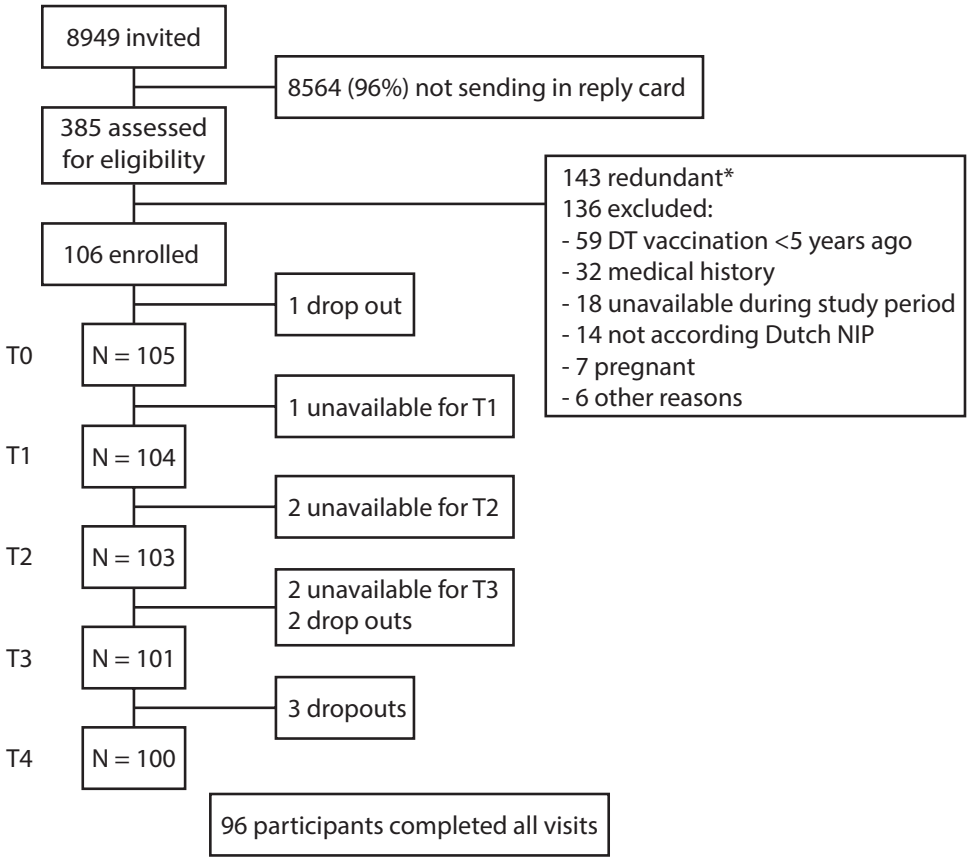

Figure 1. Flow-chart of study enrolment. Schematic overview of the recruitment, enrolment and the follow-up of the study participants. At the start of the study, Dutch participants 25 to 29 years of age received a tetanus, diphtheria and acellular pertussis booster vaccination (Tdap). Blood samples were collected before (T0), 2 weeks (T1), 4 weeks (T2), 1 year (T3), and 2 years (T4) after the booster vaccination. * 143 potential participants were excluded because the target for inclusion was achieved.

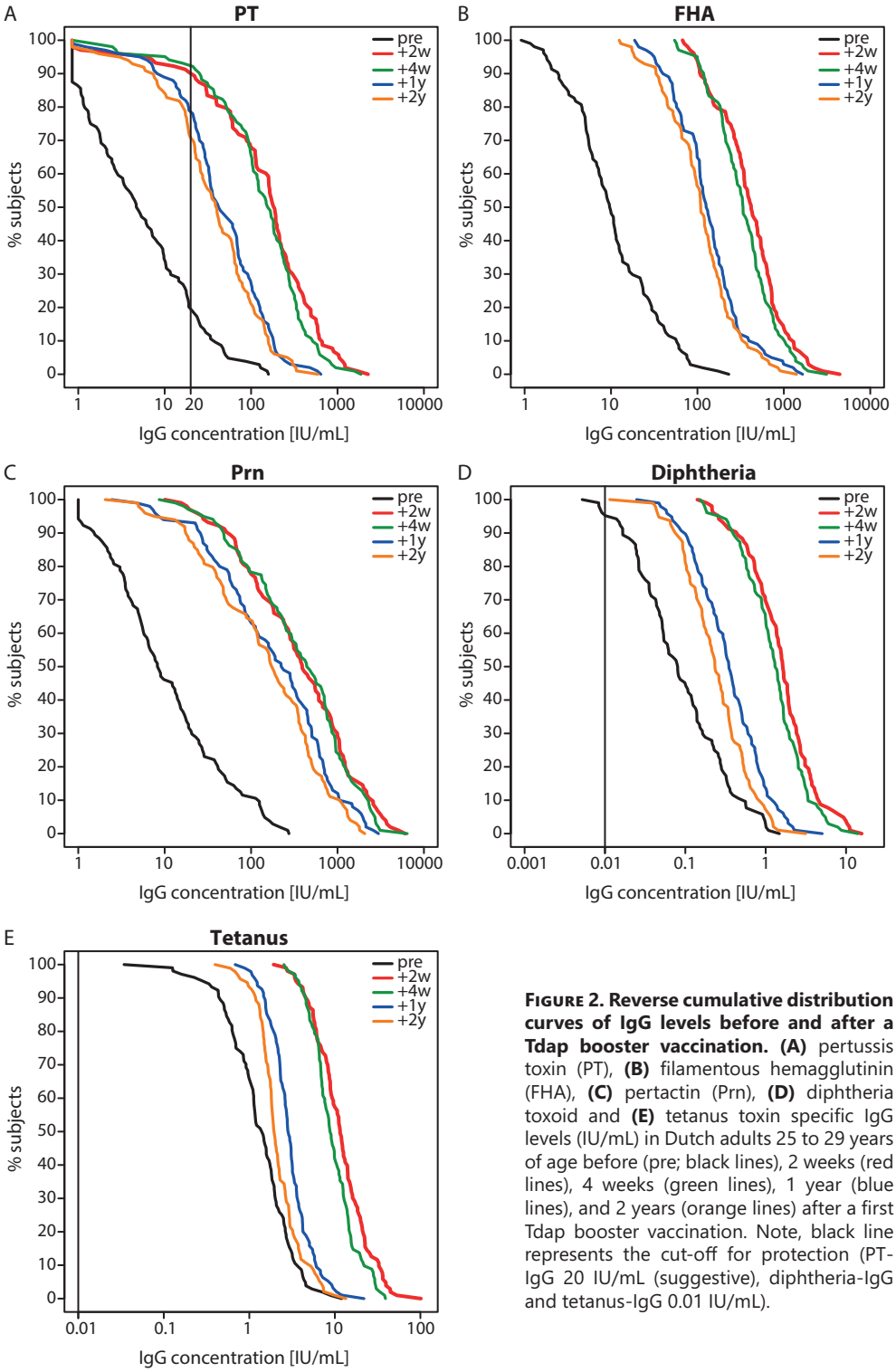

Figure 2. Reverse cumulative distribution curves of IgG levels before and after a Tdap booster vaccination. (A) pertussis toxin (PT), **(B)** filamentous hemagglutinin (FHA), **(C)** pertactin (Prn), **(D)** diphtheria toxoid and **(E)** tetanus toxin specific IgG levels (IU/mL) in Dutch adults 25 to 29 years of age before (pre; black lines), 2 weeks (red lines), 4 weeks (green lines), 1 year (blue lines), and 2 years (orange lines) after a first Tdap booster vaccination. Note, black line represents the cut-off for protection (PT-IgG 20 IU/mL (suggestive), diphtheria-IgG and tetanus-IgG 0.01 IU/mL).

Table 1. IgG geometric mean concentrations (IU/mL) with 95% confidence intervals following a first adult Tdap booster vaccination and percent reduction[#] of IgG antibody levels.

	GMC (95% CI)					Reduction (%)[#]	
Antigen	**Pre**	**+2 weeks**	**+4 weeks**	**+1 year**	**+2 years**	**4w-1y**	**1y-2y**
PT	5.4 (4.1-7.2)	130 (95-178)	123 (93-161)	43 (33-55)	35 (27-45)	58	15
FHA	10.6 (8.4-13.2)	404 (340-480)	339 (288-399)	132 (110-158)	108 (90-131)	53	17
Prn	10.5 (7.8-14.0)	360 (270-481)	357 (269-473)	180 (132-247)	142 (104-194)	41	20
Diphtheria	0.09 (0.07-0.11)	1.5 (1.3-1.9)	1.3 (1.1-1.5)	0.34 (0.28-0.42)	0.24 (0.20-0.29)	66	25
Tetanus	1.3 (1.1-1.5)	11.6 (10.1-13.4)	9.4 (8.4-10.6)	3.0 (2.6-3.3)	2.2 (1.9-2.4)	59	24

Abbreviations: GMC: geometric mean concentrations; CI: confidence intervals; PT: pertussis toxin; FHA: filamentous hemagglutinin; Prn: pertactin.
[#] Samples were excluded if no increase in antibody concentration was observed 2 weeks post-booster compared with pre-booster, or when an increase after 4 weeks post-booster was observed.

IgG and IgA antibody kinetics after the Tdap booster vaccination

Before the Tdap booster vaccination, IgG levels against PT, FHA, and Prn were low (**Figure 2** and **Table 1**), although 7% (7/105) of the participants had a PT-IgG level ≥50 IU/mL. One and 2 years post-booster, this percentage increased to respectively 78% (79/101) and 71% (71/100) of the participants. Following the Tdap booster vaccination the IgG levels increased for all vaccine antigens and remained higher at all time points compared with pre-booster levels (p-values <.01) (Figure 2 and Table 1). Surprisingly, 8% (8/105) of the participants did not show a PT-IgG level ≥20 IU/mL at any of the time points. The IgG levels for diphtheria and tetanus were above the protective level pre-booster for most individuals and IgG levels increased upon the booster but started to decline already after 2 weeks post-booster and progressed to decline significantly after 1 and 2 years post-booster (p-values <.01) (Figure 2). The reduction in vaccine antigen-specific IgG varied between 41–66% in the first year but was more limited (15–25%) during the second year post-booster (Table 1). Overall, the PT-IgG levels were similar between males and females, but 7/8 participants who did not have arbitrarily protective PT-IgG levels in the first month post-booster were females. At 2 years post-booster, more females showed PT-IgG levels under the protective cut-off (21/66 females, 32%) compared with males (8/34 meals, 24%).

According to the bi-exponential model, the median PT-IgG level was predicted to remain above 20 IU/mL for approximately 9 years (**Figure 3**). Median duration of protection against diphtheria and tetanus (both >.01 IU/mL (33, 34)) was predicted to approximately last 16 and 93 years respectively post-booster.

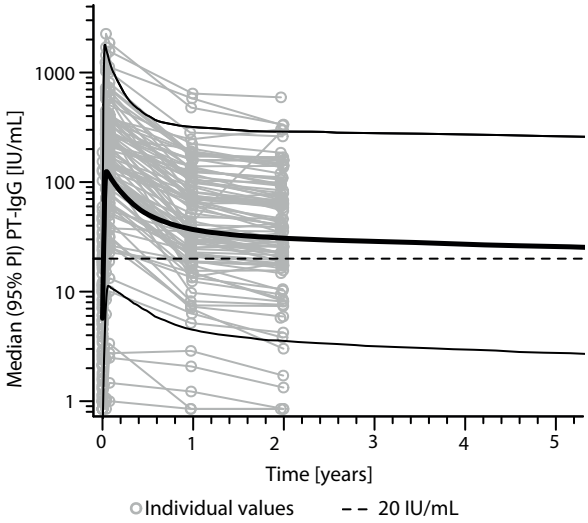

Figure 3. Predicted levels of pertussis toxin (PT) specific IgG levels (IU/mL) after a first adult Tdap booster vaccination in Dutch adults 25 to 29 years of age using a bi-exponential model. Note, solid bold line represents the median, solid lines the 95% predicted interval (95% PI) and dotted line a PT-IgG concentration of 20 IU/mL.

The avidity (GMAI) of the PT-IgG antibodies was significantly higher 2 and 4 weeks post-booster compared with pre-booster (p-values <.01), while no differences were observed in the GMAI of Prn-IgG antibodies (supplementary Figure 1).

Seropositive PT-IgA levels were observed in 85% (89/105) of the participants pre-booster, and in 99% (103/104) of the participants 2 weeks post-booster (**Figure 4A**). Two and 4 weeks post-booster, the IgA levels for all three pertussis antigens had increased but declined subsequently within the first year post-booster (p-values <.001) (**Figure 4**).

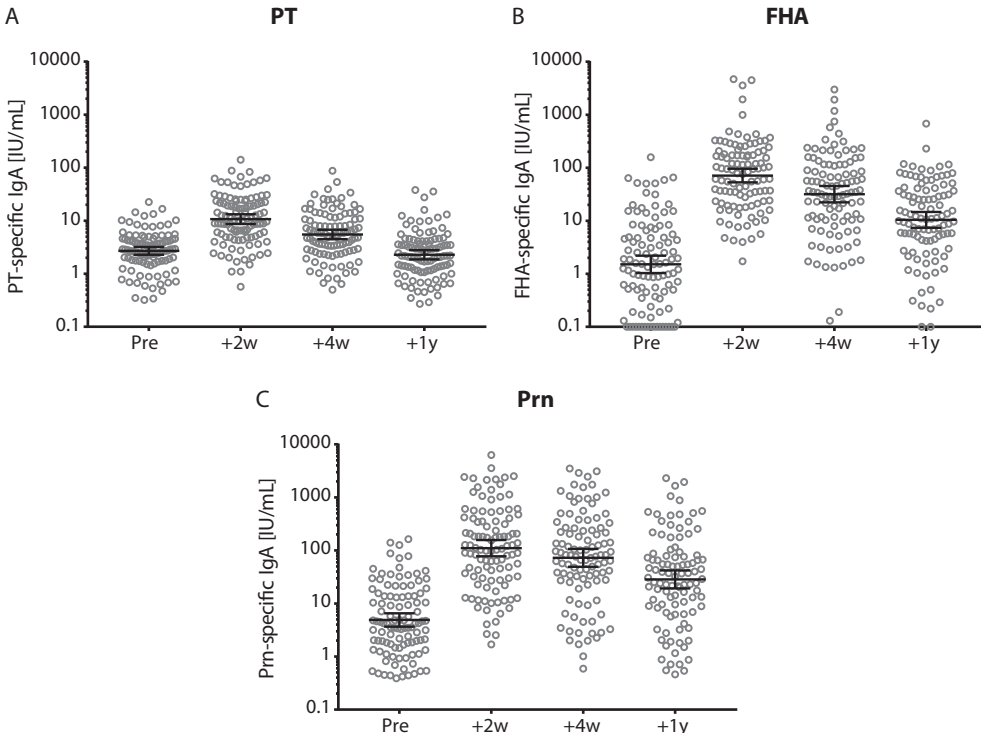

Figure 4. IgA antibody levels before and after a Tdap booster vaccination. (A) pertussis toxin (PT), **(B)** filamentous hemagglutinin (FHA) and **(C)** pertactin (Prn) specific IgA levels (IU/mL) in Dutch adults 25 to 29 years of age before (pre), 2 weeks, 4 weeks and 1 year after a first Tdap booster vaccination. Note, black lines represents the geometric mean concentration with 95% confidence interval. Each time point was significantly different compared with other time points.

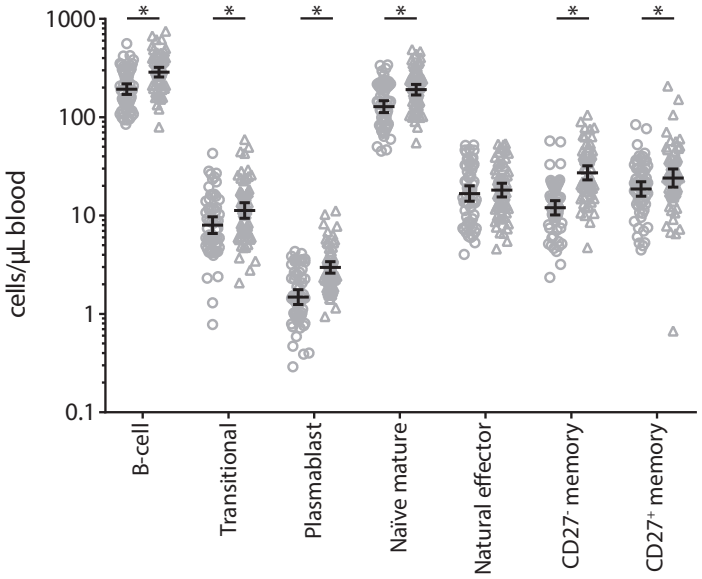

Figure 5. Absolute numbers of B-cell and B-cell subsets before (circles) and 2 weeks after (triangles) a first Tdap booster vaccination in Dutch adults 25 to 29 years of age. Gating of populations (all SSC^{low}): B-cell: $CD45^+CD19^+$; Translational: $CD45^+CD27^-CD38^+$; Plasmablast: $CD45^+CD27^+CD38^+$; Naïve mature: $CD45^+CD27^-IgD^+CD38^{dim}$; Natural effector: $CD45^+CD27^+IgD^+CD38^{dim}$; $CD27^-$ memory: $CD45^+CD27^-IgD^-CD38^{dim}$; $CD27^+$ memory: $CD45^+CD27^+IgD^-CD38^{dim}$. Note, black solid line represents the geometric mean numbers with 95% confidence intervals, * p-value <.05.

B-cell responses after the Tdap booster vaccination

At 2 weeks post-booster, the absolute numbers of circulating B-cells and plasma-, naïve-, and memory-B-cell subsets had increased significantly compared with pre-booster numbers, except for natural effector B-cell numbers (**Figure 5**). Pre-booster, detectable numbers of vaccine antigen-specific circulating memory B-cells were observed in just a few participants (**Figure 6**). Following Tdap, the numbers of the vaccine antigen-specific memory B-cells/10^5 $CD19^+$ cells had increased significantly at all time points compared with pre-booster values, except for PT and Prn 2 years post-booster, but numbers had declined significantly between 4 weeks and 1 year post-booster (Figure 6). A correlation was observed between the numbers of specific memory B-cells at 2 weeks versus the specific IgG levels at 1 year and 2 years post-booster for PT and Prn (r = 0.64 and 0.58 for PT and r = 0.65 and 0.66 for Prn, respectively; p-values <.01) (supplementary Figure 2).

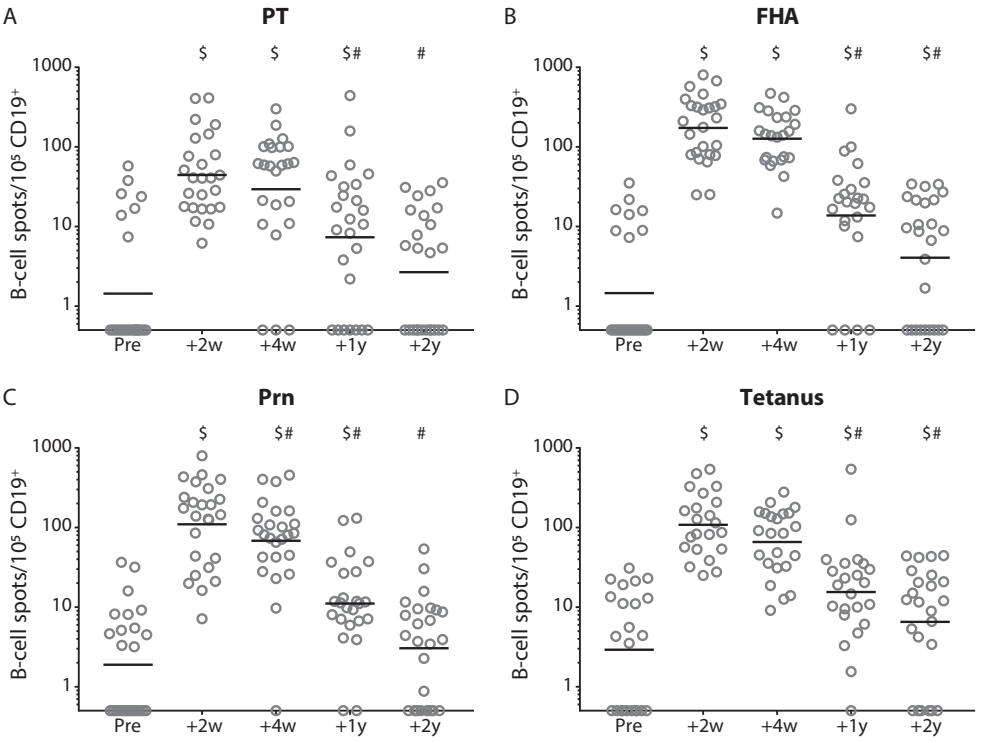

Figure 6. Numbers of memory B-cells before and after a Tdap booster vaccination. Numbers of **(A)** pertussis toxin (PT), **(B)** filamentous hemagglutinin (FHA), **(C)** pertactin (Prn), and **(D)** tetanus toxoid specific IgG producing memory B-cells/10^5 $CD19^+$ cells in Dutch adults 25–29 years of age before (pre), 2 weeks, 4 weeks, 1 year, and 2 years after a first Tdap booster. Note, black solid line indicates geometric mean number, $ indicates significant increase compared with pre-booster and # indicates significant decrease compared with previous time point(s) post-booster (p-values <.05).

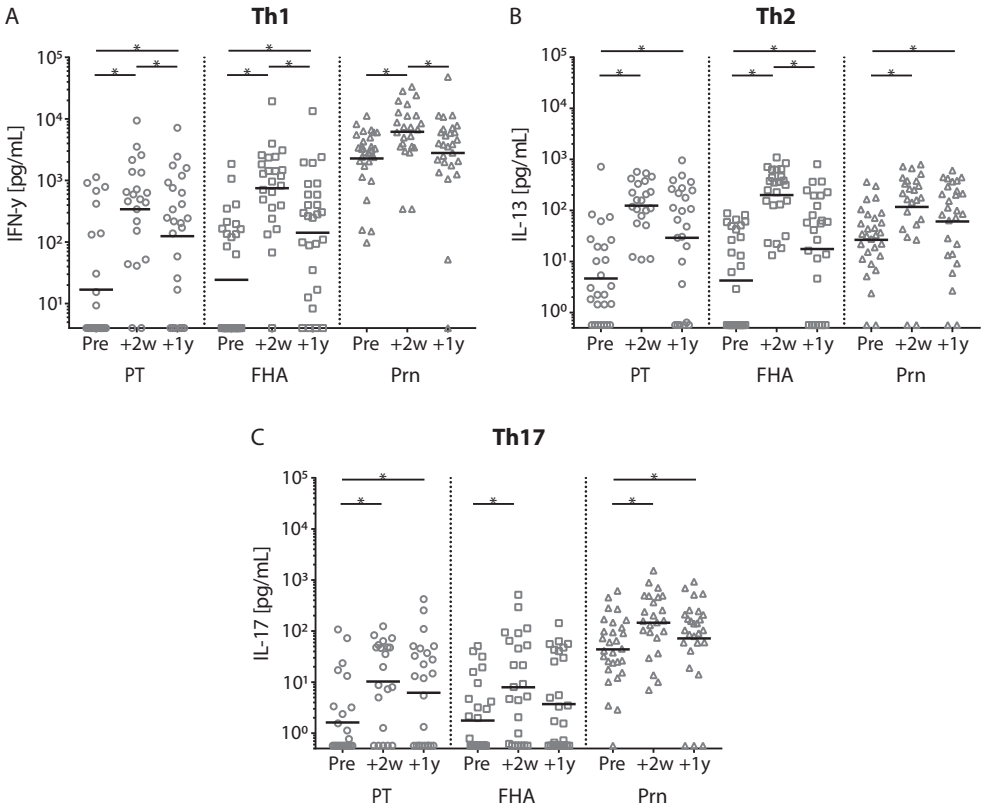

Figure 7. Cytokine levels of stimulated T-cells before and after a Tdap booster vaccination. (A) IFN-γ, **(B)** IL-13, and **(C)** IL-17 cytokine concentrations (pg/mL) in the supernatants of T-cells stimulated with heat inactivated pertussis toxin (PT) (circles), filamentous hemagglutinin (FHA) (squares), or pertactin (Prn) (triangles) in Dutch adults 25 to 29 years of age before, 2 weeks and 1 year after a first Tdap booster vaccination. Note, black lines represents the geometric mean concentration; * = p-value <.05.

T-cell cytokine responses after the adult Tdap booster vaccination

In general, the production of IFN-γ, IL-13 and IL-17 cytokines increased significantly at all time points post-booster vaccination compared with pre-booster values (**Figure 7** and supplementary Figure 3). Similar levels were observed at 2 and 4 weeks and again at 1 and 2 years following Tdap (data 4 weeks and 2 years post-booster not shown). Pertussis-specific IFN-γ production and FHA-specific IL-13 production decreased significantly between 2 weeks and 1 year post-booster (Figure 7). Only low pertussis-specific IL-10 production was observed both pre- and post-booster (data not shown).

Discussion

In this study, we demonstrated that systemic IgG levels against the pertussis vaccine antigens PT, FHA and Prn persisted at higher levels for at least 2 years after a first adult aP booster vaccination in young Dutch adults 25 to 29 years of age, who had been primed in infancy with whole-cell pertussis vaccine. A limited antibody decay was observed during the second year after the booster with antibody levels against PT above 20 IU/mL in at least 70% of the participants and an estimated median duration of protection for about 9 years. The numbers of PT- and Prn-specific B-cells observed at 2 weeks post-booster correlated with the corresponding IgG antibody levels after 1 and 2 years. Furthermore, the booster enhanced Th1, Th2 and Th17 cytokine production for at least 2 years.

Using the same vaccine antigen dose, the adult Tdap booster vaccination in this study induced 1.5–4 times higher PT-IgG levels compared with previous studies (16, 35, 36), which were conducted between 1997 and 2002 in a period with presumed lower circulation of *B. pertussis* than nowadays (18, 19). The participants of the present study were therefore most likely more frequently exposed to *B. pertussis*. This is underlined by the fact that the majority of our participants showed pre-booster PT-IgA levels, that further increased post-booster. IgA responses are not induced by aP vaccines in infants (25), but exposure to *B. pertussis* induces systemic pertussis-specific IgA-producing memory B-cells in children and adults (25, 37, 38). Therefore, the presence of IgA antibodies pre-booster and their rise after the booster may result from the activation of pre-existing pertussis-specific memory B-cells induced by previous *B. pertussis* contact in life, indicative of enhanced *B. pertussis* circulation in the present days. Together, the high pertussis circulation, compared to a population that is not or only minimally boosted with pertussis infections, alters pertussis immunity with enhanced antibody levels and cellular immunity upon a booster vaccination.

Using a bi-exponential antibody decay model with the arbitrarily defined level of protection of ≥20 IU/mL (26), the median duration of PT-IgG levels in our study was estimated to last up to 9 years after the Tdap booster. We need to point out that the young adults in our study have been primed with wP vaccines during infancy, as is the case for the majority of the current adult Dutch population for many years.

In this study, the highest pertussis-specific IgG levels were seen at day 14 post-booster followed by a slight decrease at day 28. In line with our results, Halperin et al. reported increased IgG levels from day 7 with a peak around day 14 (39), and Kirkland et al. reported comparable IgG levels at 2 and 4 weeks, both after a Tdap booster (40), though using lower vaccine antigen concentrations compared with our study (39, 40). Other pertussis booster studies measured serological responses from 4 weeks post-booster onwards (16, 41-43). Since peak IgG levels will be missed with sample collections from 4 weeks onwards, vaccine antibody responses could be measured already around day 14 post-booster.

The higher IgG responses upon Tdap vaccination due to presumed recall of memory B-cells induced by a previous *B. pertussis* infection is reflected by the high numbers of pertussis-specific IgG memory B-cells after the booster, and the significantly higher absolute numbers of recirculating B-cell subsets. In line with this, Hendrikx et al. found similar numbers of PT-specific memory B-cells in pre-adolescents after a second aP booster vaccination (44). In contrast, post-booster numbers of FHA- and Prn-specific memory B-cells were higher in adults, probably a result of more natural boosting compared with pre-adolescents. Although the homing of the pertussis-specific memory B-cells normally occurs quickly, waning circulating IgG levels during the second year was limited, probably by the presence of long-lived plasma cells. The induction of memory B-cells and long-lived plasma cells by the adult booster vaccination might contribute to long-term protection against pertussis.

In agreement with other studies, the adult booster resulted in increased levels of Th1, Th2, and Th17 cytokines (45, 46). However, Huygen et al. did not find increased Th1 levels upon the same vaccination in pregnant women or their age-matched controls (42). Also, a pre-adolescent Tdap booster in children 9 years of age did not enhance T-cell responses (47), which we explained at the time by the high pre-booster levels already induced by a booster vaccination 5 years earlier. So far, just one study investigated the influence of pertussis priming vaccines on adult T-cell responses after a Tdap booster vaccination (46). That study showed a general Th2 dominated immune response after an aP booster in adults primed with aP vaccines during infancy, while wP-primed adults showed a Th1 dominated response (46). Since Th1 cells are essential for bacterial clearance and associated with protection (48) the increased pertussis-specific Th1 levels observed in our wP-primed adults after the booster, could possibly confer protection against pertussis, while this may be less in aP-primed adults with a Th2 dominated response.

The increase in the number of pertussis related deaths in infants during the epidemic of 2010 in California (11) and that of 2012 in the UK (12), led to the implementation of maternal pertussis booster vaccinations (10). Maternal aP vaccinations are very effective in preventing pertussis in infants in the time window from birth until their first routine pertussis immunisation (49, 50). In several countries pregnant women are advised to be vaccinated during every pregnancy (42, 51). The persistence of high PT-IgG antibody levels reported here could indicate that the repeated administration of a Tdap booster vaccination might not be necessary for the majority of pregnant women. IgG antibody kinetics after an aP booster vaccination in pregnant women should be studied in more detail. Also, attention must be paid to potential non-responders to aP vaccinations since these comprise 10% (7/70) of our female study participants.

The switch from wP to aP vaccines during infancy in 2005, will bring the first Dutch aP-primed cohort reaching the age of 18 years in 2023. However, other countries have already used aP vaccines in the infant immunisation programme for more than

2 decades. Since protection against clinical pertussis wanes faster after priming with aP vaccines compared with wP vaccines (52), women at childbearing age primed with aP vaccines, may experience the consequences of less longer persistence of pertussis-specific antibodies after booster vaccination. Therefore, the effectiveness of adult and maternal Tdap vaccinations and antibody persistence deserves further study, accounting for previous vaccinations and current *B. pertussis* exposure in the population.

To conclude, we showed a robust immune response and persistence of high pertussis IgG antibody levels after a Tdap booster in Dutch adults 25 to 29 years of age. These adults have been primed with wP vaccines during infancy and might benefit from the booster vaccine by an elevated immune response to pertussis. Maternal aP vaccination is currently the best strategy to protect newborns from pertussis. Long-term follow-up of antibody levels in women vaccinated with aP during pregnancy could elucidate the necessity to vaccinate during every pregnancy. In addition, Tdap booster responses in growing cohorts of aP-primed individuals reaching childbearing age should be further investigated.

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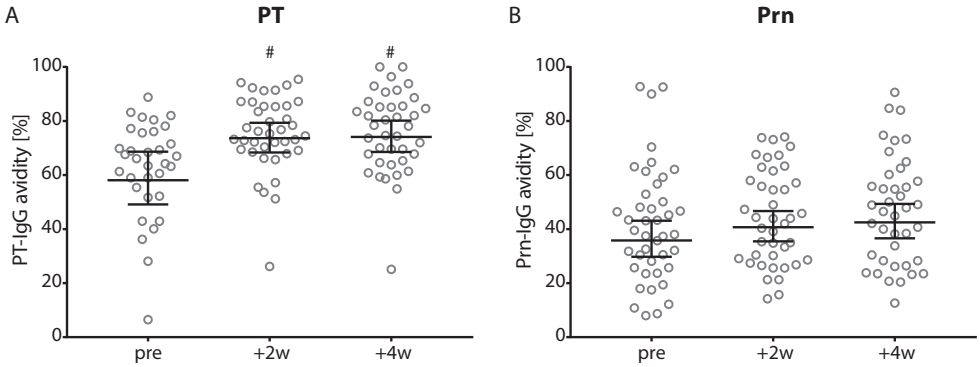
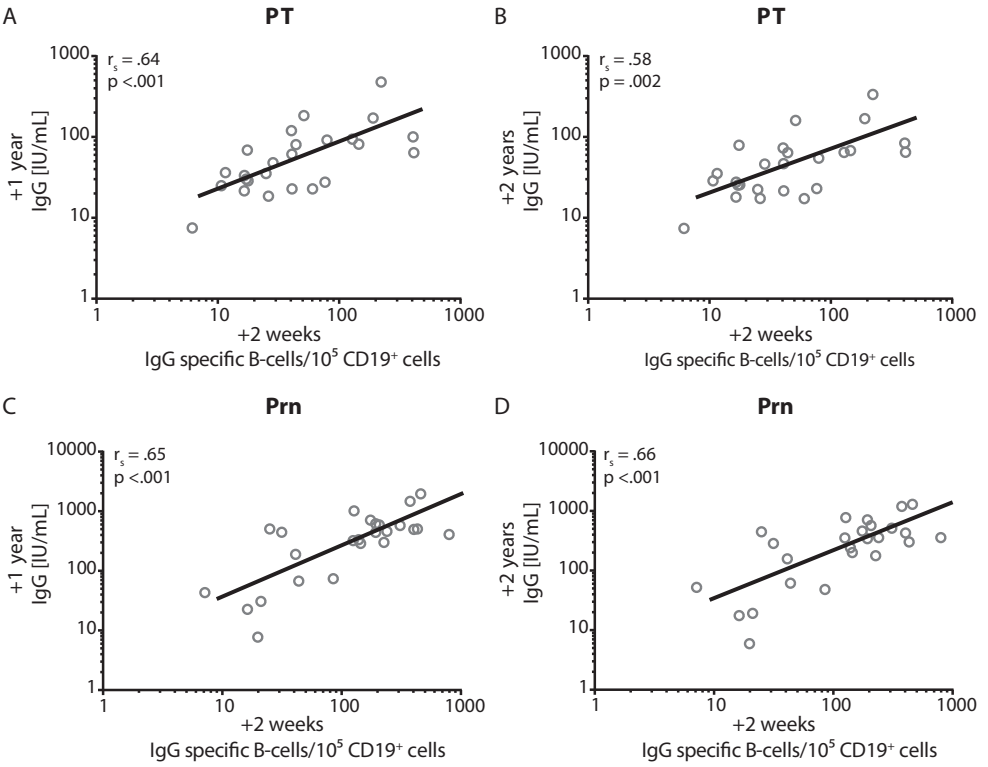
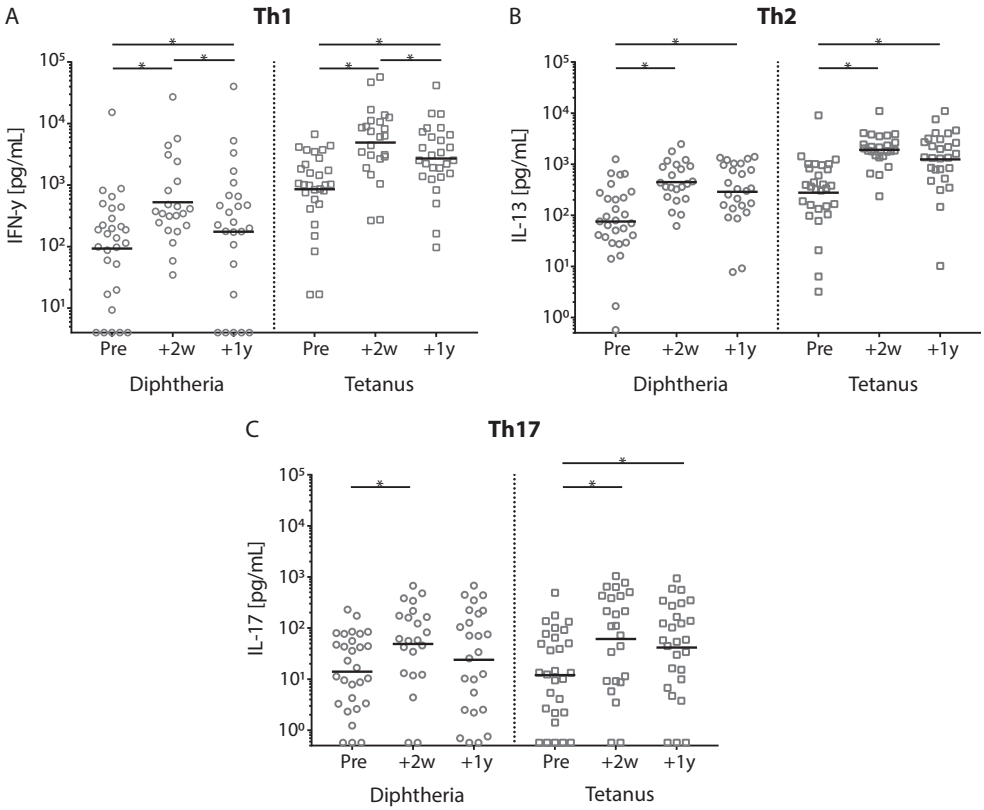
Supplementary data



Supplementary Figure 1. Avidity of PT- and Prn-IgG antibodies. (A) pertussis toxin (PT) and **(B)** pertactin (Prn) avidity index in Dutch adults 25 to 29 years of age before (pre), 2 weeks and 4 weeks after a first Tdap booster vaccination. Note, black lines represents the geometric mean percentage with 95% confidence interval, # p-value <.01 compared with pre-booster levels.



Supplementary Figure 2. Correlation between IgG and memory B-cells. Spearman's correlation coefficient (r_s) between the numbers of IgG specific memory B-cells/10^5 $CD19^+$ cells (x-axis) at 2 weeks post-booster and the specific IgG levels in IU/mL (y-axis) at 1 year post-booster **(A, C)** and at 2 years post-booster **(B, D)** for pertussis toxin (PT; **A, B**) and for pertactin (Prn; **C, D**). p = p-value.



Supplementary Figure 3. Cytokine levels of stimulated T-cells before and after a Tdap booster vaccination. (A) IFN-γ, **(B)** IL-13, and **(C)** IL-17 cytokine concentrations (pg/mL) in the supernatants of T-cells stimulated with diphtheria toxoid (circles) or tetanus toxoid (squares) in Dutch adults 25 to 29 years of age before, 2 weeks and 1 year after a first Tdap booster vaccination. Note, black lines represents the geometric mean concentration; * = p-value <.05.

Chapter 7

General discussion

Despite intensive pertussis vaccination programmes already implemented since the 1940s and 1950s, *Bordetella pertussis* continues to circulate and causes progressively more clinical disease over the last decades (1-3). Clinical pertussis is most severe in infants resulting in on average 150 infant hospitalisations each year in the Netherlands, with a peak in incidence rates once in every 2 to 3 years. During the major pertussis outbreak of 2011–2012, more than 250 infants with pertussis were hospitalised, and 3 infants too young to be (completely) protected by vaccination died as a consequence of pertussis disease (4). Clinical pertussis is also frequently reported in children, adolescents and adults, although the severity of disease in those vaccinated persons is lower compared with infants (5-7). Protection against pertussis, induced by whole-cell pertussis (wP) and acellular pertussis (aP) vaccines administered during infancy, wanes rapidly. Clinical pertussis is already reported within 3 years after the primary immunisation series (8). aP booster vaccinations at pre-school age, implemented in many national immunisation programmes (NIP), prolong the duration of protection against pertussis disease until approximately young adolescent age, with emerging disease from age 8–9 years onwards in the Netherlands (8, 9). In an increasing number of countries, this shift in enhanced incidence of pertussis in adolescence resulted in the recommendation of aP booster vaccinations for adolescents and adults (10-12). The long-term immunological effects of aP booster vaccinations in adolescents and young adults are not well known. This final chapter discusses and evaluates the main findings of the clinical studies presented in this thesis.

Summary of results

In **Chapter 2** we assessed the *B. pertussis* acquisition rate in Dutch adolescents of 10, 12, and 15 years of age during a large pertussis outbreak in the Netherlands. We demonstrated that 10% of the study participants became infected with *B. pertussis* during the 2012 epidemic, indicating a large reservoir of *B. pertussis* in this age group. Importantly, we highlighted that using the current PT-IgG cut-off ≥50 IU/mL as a marker of a pertussis infection in serosurveillance studies, may lead to an overestimation of infected individuals due to a longer persistence of antibody levels after infection than expected.

In **Chapter 3** we observed that an additional aP booster vaccination at 9 years of age increased pertussis-specific IgG antibody levels and numbers of memory B-cells, which were higher in children primed with wP vaccines compared with aP-primed children. Moreover, the production of cytokines by pertussis-specific T-cells did not increase upon the extra booster vaccination. Surprisingly, Th1 cytokine levels, considered to play an important role in long-term protection against pertussis, were higher in wP-primed children while the Th2 cytokine levels were similar between the 2 groups and resulted in a Th2-skewed immune response in aP-primed children. These results were consistent with the findings in **Chapter 4**, indicating that higher proportions of IgG4 antibodies to PT, FHA and Prn are found after aP-priming compared with wP-priming. The type of pertussis priming vaccines administered at infant age affects the immunological imprinting and subsequently the protection against pertussis until at least adolescence, explaining the better protection against clinical pertussis found in adolescents primed with wP vaccines.

In **Chapter 5** we described the immune response in children primed with aP vaccines who showed an adverse event following the pre-school aP booster vaccination. Children with an adverse event had higher pertussis-specific IgG, IgG4 and Th2 cytokine levels as well as increased total IgE levels compared to age-matched aP-primed controls without adverse events. The Th2-skewed immune response was observed in both groups of children, and is most likely caused by the four high dose vaccinations administered in the first year of life. Pre-booster high Th2-skewed cytokine levels together with a yet unknown additional biological factor most likely cause the exceptional adverse events after a fifth aP vaccination.

In **Chapter 6** we evaluated the immune response to a first aP booster vaccination in young adults primed with wP vaccines in the first year of life. The immunogenicity of this first booster proved robust resulting in pertussis-specific IgG levels, numbers of memory B-cells and Th1, Th2, and Th17 cytokine levels that remained higher for up to 2 years compared with pre-booster levels, and will most likely confer protection against pertussis in these adults. Furthermore, the induction of IgA antibodies after a parenteral aP booster vaccination indicates a previous encounter with *B. pertussis*.

General discussion

Pertussis epidemiology and (sub-) clinical infections in adolescents

In the past two decades, outbreaks of pertussis have been reported all over the world, including the USA (11), the UK (13), Australia (14) and Argentina (15). In the Netherlands, cyclic outbreaks of pertussis have been observed every 2–3 years since 1996 (8). Although pertussis is a notifiable disease in the Netherlands (16), there is a discrepancy between the numbers of reported symptomatic pertussis cases and that of pertussis infections. To estimate the incidence of pertussis infections in the population, antibody levels against pertussis toxin (PT), only expressed by *B. pertussis*, are used in serosurveillance studies (17). In the Dutch population, the proportion of participants with a seropositive PT-IgG level (≥50 IU/mL) increased from 4% in 1995–1996 to 9% in 2006–2007 as measured in two serosurveillance studies (18, 19). To prevent the influence of pertussis vaccine induced antibodies in these analyses, children younger than 9 years of age were excluded. The percentage of PT-IgG seropositive participants was found to be 100 times higher than the number of reported cases during the same period (20). In the survey of 2006–2007, only 25% of the seropositive participants indicated that they had experienced coughing episodes which lasted for more than 2 weeks in the year before sampling. The sampling of a third surveillance study conducted in the Netherlands (2016–2017) has just been finished, and results about the current PT-IgG seroprevalence in the Dutch population are expected in 2018. Surveillance studies in other countries also noticed higher numbers of pertussis infections compared to the reported pertussis incidence (reviewed in 17). In line with these studies, we observed a comparable PT-IgG seropositivity rate of 9% in blood samples from adolescents 10, 12, and 15 years of age collected in October 2011, just before the largest outbreak in the Netherlands occurred since the introduction of the pertussis vaccinations (**Chapter 2**). Longitudinal follow-up samples from these adolescents collected in October 2012 showed that 10% of the participants who initially were seronegative (PT-IgG level <50 IU/mL), were likely to have become infected with *B. pertussis* during that outbreak. In 2012, the calculated *B. pertussis* acquisition rate was 97 per 1,000 person-years (which means around 1:10) in adolescents 10–15 years of age, which was more than 40 times higher than the reported incidence of clinical pertussis in this age group. During the 2 year follow-up, both the number of notified clinical pertussis cases and the *B. pertussis* infection rates dropped markedly in the study population. Adolescents tend to mix especially with people of the same age (21). Their social behaviour together with the easy transmission of *B. pertussis* via exposure through close contact and respiratory droplets (22) likely contributed to the high acquisition rate of *B. pertussis*, next to waning vaccine induced immunity.

Due to a low antibody decay between the sampling time points, the majority of the adolescents that presented with anti PT-IgG levels above 50 IU/mL, remained seropositive for over 1 year, and almost half of them even for at least 3 years.

Therefore, using the current presumed indicator for recent infection for a time window of one year (PT-IgG level ≥50 IU/mL) should now be used with caution, since this results in an overestimation of acquisition of *B. pertussis* infections in cross-sectional studies. Longitudinal serosurveillance studies are a better tool to monitor *B. pertussis* exposure in the population. The occurrence of coughing symptoms, together with pertussis-specific antibody levels may provide more accurate predictors for the susceptibility to infection and to symptomatic disease in the population. We already found that of the 28 individuals who developed a rise in PT-IgG levels, due to a presumed *B. pertussis* infection after 2011, 26/28 had PT-IgG levels <10 IU/mL in the preceding sampling time point. This suggests protection against (sub-) clinical pertussis already in the presence of PT-IgG antibody levels around 10 IU/mL, though exposure of course may vary. Till now, an arbitrary PT-IgG level of 20 IU/mL is used as an indicative marker for protection against pertussis (23).

Similar to the pertussis incidence in adolescents, the reported pertussis cases in adults and elderly proved to be higher during the outbreak of 2012 as compared with other outbreaks. To reduce the disease burden of pertussis, adaptations in the current national immunisation programmes might contribute to a decrease in *B. pertussis* circulation in the population. In order to target the appropriate age groups for booster vaccinations, duration of vaccine induced protection against pertussis acquisition should also be determined in young and older adults as well as elderly who may have considerable but an often under diagnosed burden of pertussis and complications.

The longitudinally followed adolescents of 10, 12, and 15 years of age that were studied in Chapter 2 have been primed with wP vaccines in the first year of life. However, at present, the cohort of Dutch adolescents aged 13 years is primed with aP vaccines following to the replacement of wP by aP vaccines in the infant vaccination schedule in 2005. The pertussis baboon model demonstrated that aP-priming of baboons prevented clinical pertussis symptoms and leucocytosis, but did not preclude *B. pertussis* infection, colonisation and transmission to other baboons (24). Moreover, recent epidemiological studies have shown that aP-primed adolescents are at higher risk of contracting clinical pertussis symptoms compared with wP-primed adolescents (7). This indicates that the present aP-primed adolescents could form an even larger reservoir of *B. pertussis* than now observed in wP-primed adolescents. Establishing pertussis acquisition rates in a population with growing cohorts of aP vaccinated individuals seems crucial to monitor the reservoir of *B. pertussis* after the switch of wP- to aP-priming vaccines.

Pre-adolescent pertussis immune profiles

An increasing number of countries has implemented (pre-) adolescent aP booster vaccinations in their NIPs (10-12). However, the immunological response to these booster vaccinations have not been extensively investigated. We determined and compared long-term humoral and cellular immune responses in Dutch children between 4 to 10 years of age following two successive aP booster vaccinations at age 4 and 9 years. These children had received either wP or aP combination vaccines in infancy (**Chapters 3** and **4**). Vaccine induced humoral and cellular immunity was enhanced in children primed with aP vaccines compared with wP-primed children up to 2 years after the pre-school booster vaccination administered at 4 years of age (25-27). This was consistent with previous studies showing higher antibody responses and cellular immunity against the vaccine antigens in infants primed with aP vaccines compared with wP-primed infants (28, 29). In contrast, the additional booster vaccination administered at 9 years of age induced lower IgG antibody responses to pertussis antigens and less memory B-cells in aP-primed compared with wP-primed children, although these differences had disappeared one year post-booster. However, in both wP- and aP-primed children, that received aP booster vaccinations at age 4 and 9 years, antibody levels waned rapidly, suggesting a suboptimal induction of long-lived plasma cells and memory B-cells able to maintain persistently high pertussis-specific antibodies after booster vaccinations (**Chapter 3**). The fast waning of especially PT-IgG antibodies, which are considered to be important in protection against disease (30-34), indicates a limited duration of antibody mediated protection against clinical pertussis also after an additional aP booster vaccination in pre-adolescent children.

Of importance, we demonstrated the presence of lower levels of Th1 cytokines in aP-primed children 9 years of age compared with wP-primed children, while the Th2 cytokine levels were similar between the two groups. We observed that the immune response against all Tdap vaccine antigens in aP-primed pre-adolescents at 9 years of age was more Th2-skewed, whereas previously a more mixed Th1/Th2 cell response was reported in children up to 6 years of age who were primed with aP vaccines (28, 35, 36). In agreement with earlier studies, the wP-primed pre-adolescents had a more Th1 dominated immune response (28, 35, 36). The different Th1/Th2 ratios seemed already established after priming in infancy, since the additional aP booster vaccinations did not influence the already established Th1/Th2 ratios. Also, high T-cell cytokine production was already present before the aP booster vaccination at age 9 years and did not further increase in either group of children. This was in contrast with previous observations after the pre-school DTaP (diphtheria, tetanus and aP) booster vaccination at age 4 years where the aP booster induced high levels of T-cell cytokines in wP-primed children, which further increased during time after the booster (37). In contrast, T-cell cytokine levels were still high in aP-primed children 4 years of age, 3 years after the infant vaccinations, and did not further increase upon the pre-school booster vaccination (25). This suggests that once a certain high level

of pertussis-specific T-cell memory is present, only a very minimal response to a subsequent antigen encounter is induced. In this respect, it is important to note the presence of more terminally differentiated CD4+ T-cells, not able to respond properly to additional boosters, in children 4 years of age primed with aP vaccines in comparison with wP-primed children (38, 39).

Long-term protection against clinical pertussis is thought to be conferred by pertussis-specific Th1 cells, while the role of Th2 cells to direct protection against pertussis remains unclear (40-42). Our cellular immunity responses in pre-adolescents confirms epidemiological data of an increased susceptibility to clinical pertussis after priming with aP vaccines during infancy with Th2 dominating over Th1 cytokines (7, 9, 43, 44). Moreover, the immune response stimulated by the vaccinations administered at infant age, so-called immunological imprinting, appears to determine long-term cellular immune profiles at least until adolescent age that cannot be altered by subsequent aP booster vaccinations in time. The difference in immune profiles is most likely explained by the presence of extra bacterial components in wP vaccines that act as adjuvants and induce a more Th1 type immune response against the vaccine antigens (45). As the aP vaccines are composed of some purified pertussis proteins, they do not contain LPS and other cellular pertussis components that may act as adjuvants and repeated high aP vaccine antigen dose administered 4 times at infant age may also contribute to the Th2-skewed immune responses.

Th17 cells are associated with protection against mucosal extracellular pathogens (46), and animal models showed an important role for Th17 cells in the clearance of *B. pertussis* (47, 48), which is important for transmission and herd protection. In contrast to the baboon model, we observed only low levels of Th17 cytokines in both wP- and aP-primed groups of children at age 4 to 10 years. To elucidate the role of Th17 cells in protection and bacterial clearance after a *B. pertussis* infection, studies using a human challenge model, as currently performed in the UK within the framework of the IMI Periscope project (49), will be performed.

In **Chapter 4** we evaluated the IgG response induced by the priming and booster vaccinations in more detail. Consistent with the Th2-skewed immune response, the children primed with aP vaccines during infancy had 2 to 10 fold higher proportions of IgG4 antibodies to pertussis vaccine antigens, as well as to the co-administered diphtheria and tetanus toxoids, compared with wP-primed children. This is in agreement with other reports showing an upregulation of IgG4 to diphtheria and tetanus toxoids in the absence of wP vaccine antigens in the primary vaccinations (50), and higher IgG4 levels against PT in aP-primed compared with wP-primed individuals (51, 52). Furthermore, the two successive aP booster vaccinations did not significantly influence the distribution of the IgG subclasses in both groups of children. Unlike the other IgG subclasses, IgG4 is unable to activate the complement system (32). This could result in a reduced bactericidal activity in individuals with high IgG4 levels. The high IgG1 levels against diphtheria and tetanus were sufficient

to protect against disease, but pertussis-specific antibodies decreased rapidly and could lead to a reduced protection.

Optimally, pertussis vaccines should generate robust Th1-type immune responses to establish long-term protection against clinical pertussis, and preferably also good Th17 induction. It appears to be essential to induce this Th1 immune response profile already with the primary series of infant vaccinations. Even the composition of the very first pertussis vaccine dose seems crucial for the immune response profile, because lower numbers of clinical pertussis were observed in adolescents who had received one wP vaccine as the first vaccine, followed by a mixed wP/aP schedule, compared with adolescents who have received an aP vaccine as a first dose (44). This corroborates the idea that the type of immune response is determined by the immune-stimulatory components included in wP vaccines upon the first antigen encounter.

Adverse events following the pre-school aP booster vaccination

Adverse events (AEs) after aP priming may also relate to the more skewed Th2 immune response after priming in infancy with aP containing vaccines. When the first aP-primed cohort of children reached the age of 4 years by the end of 2008, the numbers of reported AEs following the pre-school aP booster, the fifth aP vaccination, increased compared with those in the period of wP-priming (53). Local and systemic AEs were up to 4 times more frequently reported in aP-primed children compared with wP-primed children (54, 55). We found higher humoral and cellular immune responses to the pertussis vaccine antigens in children with pronounced local AEs compared to age-matched controls. This was also noticed for the co-administered diphtheria and tetanus toxoids. Also IgG4 and IL-13 concentrations as markers of the Th2-skewed response to the vaccine antigens, as well as total serum IgE levels, were higher in the presence of an AE. However, the Th2-skewed immune response was also observed in the majority of other aP-primed children without AEs after the pre-school aP booster, and antibody and cytokine levels varied largely in children with and without AEs, and did not necessarily correlate with each other (**Chapter 5**). Nevertheless, our results were consistent with other studies that also reported on higher IgG levels to diphtheria (56) and higher levels of Th2 cytokines to tetanus (57) in children with AEs compared to controls. We already described overall high T-cell cytokine levels to the pertussis antigens in children 4 years of age after previous aP vaccinations (36).

Repeated contact with a high antigen dose such as the aP vaccines administered at 2, 3, 4, and 11 months of age, stimulates a Th2 type immunity with the presence of Th2 cytokines IL-4 and IL-13 directing B-cell class-switching towards the production of IgE and IgG4 (58). The clinical relevance of the presence of higher IgE levels in the occurrence of AEs remains unclear. IgE levels to PT have been associated with AEs after the pre-school booster vaccination (56) and a correlation between IgG4 and

IgE levels was reported in children 4 years of age (59). We, in contrast, also observed high IgG4 levels but only very low IgE values to the vaccine antigens in both children with and without reported AEs. IgE mediated allergic responses (type I) usually develop directly after antigen contact (60, 61). Instead, the AEs observed in relation to the pre-school aP booster vaccination develop over 48 hours after vaccination and antihistamine treatment was not effective in treatment of these AEs (53). Most likely, the AEs are a type IV allergic response (delayed-type hypersensitivity reaction), driven by pre-existing vaccine antigen-specific memory T-cells (62, 63).

Our current hypothesis is that the AEs after the high dose aP booster vaccination administered at 4 years of age, predominantly occur in children who possess an already Th2-skewed vaccine immunity, possibly related to an atopic background. But we cannot exclude the potential role of other, still unknown, biological factors. Combination vaccines with a reduced dose of diphtheria and pertussis vaccine antigens (Tdap) used as a pre-school booster vaccination appeared to lower AE reporting compared with DTaP recipients (62, 64, 65) and points to the potential role of the high antigen dose in the occurrence of AEs. A Tdap booster vaccine replaced the pre-school DTaP booster vaccine in the beginning of 2017 in the Netherlands. Whether this vaccine replacement will result in a reduction of AEs remains to be seen, but of high interest to monitor and evaluate in the upcoming years.

Pertussis specific immunity in adults after a first aP booster vaccination

The increased incidence of clinical pertussis in adults led to the recommendation of adult aP booster vaccinations in many countries (11, 12, 66-69). The induction of IgG antibodies elicited by an adult aP booster vaccination has been studied in the late 1990s (70-72). However, during the past 20 years, the (sub-) clinical incidence of adult pertussis has increased (13, 14, 19). Consequently, additional boosting of the immune system by natural exposure could influence the response to an aP booster vaccination. Indeed, in **Chapter 6** we found that at present in times of enhanced pertussis circulation, anti-pertussis IgG levels are up to 4 times higher upon a first adult Tdap booster vaccination in 2014 in comparison with data reported in the late 1990s (70-72). In line with exposure to *B. pertussis*, we observed systemic PT-IgA levels following the adult aP booster vaccination. After infant aP immunisation, or pre-school aP booster vaccination, no IgA induction was observed (73). This is highly suggestive for *B. pertussis* contact and induction of IgA immunity upon natural exposure. IgA is the main mucosal antibody isotype and after a *B. pertussis* infection, systemic IgA memory B-cells and IgA antibodies are induced (73-75). This might suggest that an adult Tdap booster vaccination would also increase protective mucosal IgA levels, in case a previous *B. pertussis* infection had stimulated pertussis-specific IgA memory B-cell development.

The adult aP booster vaccination induced specific memory B-cells, which are considered to be of importance in the protection against pertussis disease (76, 77).

The estimated duration of PT-IgG antibodies above 20 IU/mL for approximately 9 years indicates the induction of long-lived plasma cells that maintain these systemic antibody levels. Although this was the first aP booster vaccination in these adults, numbers of pertussis-specific memory B-cells were at least equal or even higher than those after an additional aP booster vaccination in pre-adolescents (77), which is also likely to result from natural boosting during life. Furthermore, we reported a Th1 dominated immunity to the pertussis vaccine antigens, and Th1, Th2 and Th17 cytokines levels remained enhanced up till at least 2 years after the adult aP booster vaccination.

Besides the direct protection for vaccinated individuals, adult booster vaccinations have essential secondary benefits. Parents are an important source of *B. pertussis* infections in infants, and the aP booster may reduce transmission by parents and promote protection against pertussis in unvaccinated infants and children (78-80). Furthermore, maternal aP booster vaccination administered during pregnancy was shown to be highly efficacious in preventing pertussis disease in infants until their routine immunisation, even with low antibody levels at 2 months of age (<15 IU/mL) (81-84). The long-term persistence of PT-IgG antibody levels described in Chapter 6 indicates that an aP booster vaccination administered during the first pregnancy might be sufficient for subsequent pregnancies as well, though this remains to be studied.

The adults who participated in the VIKING study (Chapter 6) received wP vaccines in the first year of life. As discussed earlier, aP-priming vaccines induces a different immunological imprinting compared with wP-priming vaccines, which affects also subsequent aP booster responses and susceptibility to clinical pertussis at least until adolescent age. Adults primed with aP vaccines may respond differently to an aP booster vaccination than wP-primed adults. Therefore, it is important to determine pertussis immune responses in aP-primed adults, since a change in immunity and susceptibility could reduce the effectiveness of adult/ maternal aP booster vaccinations in the prevention of pertussis in infants.

Recommendations and future perspectives

National pertussis immunisation programmes primarily aim to protect infants as well as young children against clinical pertussis. aP booster vaccinations later in life aim to reduce pertussis disease burden of children, adolescents and adults and transmission in order to provide herd protection. Possible vaccination strategies to reduce the pertussis disease burden are discussed.

Recommendations for wP-primed individuals

Clinical pertussis is reported in all age groups of wP-primed individuals. We found that a first aP booster vaccination in young adults induced anti-pertussis IgG antibodies that unexpectedly persist for a long period. The duration of these high antibody levels needs to be confirmed by follow-up of the participants. But our VIKING-study in young adults shows that it is of interest to vaccinate adults against pertussis, especially around the childbearing age of 20–30 years of age, to increase protection against pertussis and infection and to reduce *B. pertussis* circulation. Implementation of such a vaccine should be accompanied by proper public information and monitoring, otherwise vaccine uptake in adults is expected to be low, as is already observed in other countries (85). To establish pertussis acquisition rates, longitudinal serosurveillance studies are important, especially after aP booster vaccinations. This will provide insight in the long-term efficacy of aP boosters and offers guidance to when and how often aP booster vaccinations are desired for adolescents and/or adults.

Recommendations for aP-primed individuals

The part of the population that has been primed with aP vaccines continues to increase since wP vaccines have been replaced by aP vaccines in all high income countries. Unfortunately, the immunological imprinting of aP-priming vaccines administered at infant age results in even more short-lived immune responses and subsequently in a higher susceptibility to clinical pertussis in older children and adolescents. Should we start vaccinating (pre-) adolescents against pertussis? Booster vaccinations in Finnish adolescents and military recruits reduced the pertussis incidence in the population, probably by the establishment of herd immunity (86, 87). However, repeated booster vaccinations in (pre-) adolescents will shift the peak incidence of pertussis towards childbearing age groups and parents, increasing the risk of a *B. pertussis* infection in adults with infants. It is therefore questionable whether additional aP booster vaccinations for (pre-) adolescents should be introduced in the Dutch NIP without young adult booster vaccinations as well.

Recommendations for the current infant aP vaccination programme

In the first year of life, high dose aP vaccinations are administered within a short time window in the current schedule of the Dutch NIP. In view of the high response, but also the high cytokine levels potentially associated with adverse events, less doses

and longer intervals with spread of aP vaccinations over a longer period would seem appropriate. Infants are already partially protected against severe pertussis after the first one or two vaccinations (88). Also, a previous study evaluating the immunogenicity of 4 different pneumococcal infant vaccination schedules demonstrated superior pneumococcal-specific IgG levels by a vaccination schedule administering vaccines at 2, 4, and 6 months of age compared to the other schedules (89). Only a small difference in pneumococcal immunogenicity was observed comparing the 2, 4 and 6 months schedule with a 3 + 5 months schedule. Furthermore, several studies showed a low PT-IgG seroprevalence and pertussis incidence in aP-primed children up to 6 years of age (7, 90-93), which implies that the pre-school booster vaccination currently administered at 4 years of age could be postponed with 1 or 2 years. This may also result in lower numbers of AEs reported after a fifth aP vaccination. We propose that an immunisation schedule of aP vaccinations administered at 3, 5 and 12–18 months of age combined with a low dose aP booster vaccination at 5–6 years of age would be preferable. Of course, the long-term immunological effects of such a vaccination schedule should be investigated.

Recommendations to protect newborns against clinical pertussis

The current best strategy to protect the vulnerable infants until their routine pertussis immunisation, is by vaccinating pregnant women (94). We demonstrated persisting high levels of PT-IgG antibodies after a first adult aP booster vaccination, which suggests that it could be sufficient to vaccinate only during the first pregnancy (Chapter 6). However, approximately 5–8% (Chapter 6 and (95)) of our study participants had (very) low PT-IgG levels post-booster vaccination. Screening of PT-IgG antibody levels in pregnant women could provide an indication whether a (second) aP booster vaccination is needed. Important to note is that maternal IgG antibodies interfere with the infant immune response to priming vaccines and could lower the effectiveness of infant vaccination (96, 97). This might mean that repeated booster vaccinations during every pregnancy, as recommended in some countries (98, 99), could even further interfere with the infant immune response to the primary vaccinations and requires further studies.

The response to an adult/ maternal aP booster vaccination in individuals primed with aP vaccines is currently not known and should also be studied to ensure that maternal vaccinations in these aP-primed women are still highly effective in preventing pertussis in infants. To understand the long-term immunological effects of maternal vaccinations in infants, infant immune responses after priming and subsequent booster vaccinations should be closely monitored to optimise vaccination schedules after maternal pertussis vaccinations. Postponing the first infant vaccination with 2–5 weeks was modelled to already improve infant vaccination responses (97). In the Netherlands, a study investigating vaccine induced immunity in infants born from vaccinated mothers, with a delayed infant vaccination schedule (3, 5 and 11 months of age) is currently conducted and of high relevance for future vaccination strategies.

Other possible vaccination strategies

Optimally, pertussis priming vaccines should induce a Th1 dominated immune response to confer long-term protection against clinical pertussis. wP vaccines are more resembling this immune response compared with aP vaccines. Previous wP vaccines administered at infant age were abandoned due to the frequent occurrence of AEs, in particular persistent crying, after the primary vaccination series in the first year of life. However, the immunological imprinting by the very first encounter of the pertussis vaccine antigens is very important. We should introduce new Th1/Th17 inducing adjuvants (100) or we may also consider the use of wP vaccines as a first priming vaccination. Of interest, in models, the effectiveness of a first wP-priming vaccine followed by aP vaccines was found to reduce clinical pertussis up to 96% in infants compared with an exclusive aP vaccine programme, although with inevitably some increase in AEs (101). It would therefore be very interesting to investigate long-term immune responses to pertussis in individuals who received a first wP vaccine dose followed by aP vaccines in the primary immunisation series. In the Netherlands, the switch from wP to aP vaccines occurred at January 1st, 2005. Cohorts of individuals with approximately the same age, receiving different wP and aP vaccination schedules can be identified through the Dutch vaccination register "Praeventis". Such a study would be feasible and can give very relevant information to optimise the infant pertussis vaccination series with regard to long-term protection.

Other vaccination strategies could be:

- A genetically modified PT could be used in aP vaccines instead of the currently chemically detoxified PT. Genetic modification conserves natural epitopes and vaccine induced antibodies will be more able to neutralise PT expressed by *B. pertussis* during an infection (102, 103);
- Inclusion of more antigens to aP vaccines to induce a broader immune response to pertussis (104, 105);
- Changing the route of transmission to improve mucosal immunity, such as the life-attenuated BPZE1 vaccine by intranasal administration (106);
- Reducing the antigen content in aP vaccines to inhibit or lower the Th2-sweking of the immune response.

Concluding remarks

Unfortunately, pertussis has persisted as a frequently occurring disease despite high vaccination coverage and remains an important challenge for the (scientific) community. Short-term vaccination strategies aim to prevent pertussis in the most vulnerable newborn infants, which is best achievable via maternal pertussis immunisations. wP vaccines are used in most low income countries, and in Europe still available in Poland (107). wP vaccination may provide better immune priming, thereby making a mid-term vaccination strategy possible: use wP vaccines as

a first priming vaccine followed by aP vaccines. Long-term vaccination strategies should include the development of new vaccines, which often take one or more decade(s) to become available on the market. Results presented in this thesis show the importance of investigating humoral and cellular immune responses in pertussis vaccinated and pertussis infected children and adults to evaluate pertussis immunity in the population.

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Appendix

Nederlandse samenvatting

Kinkhoest

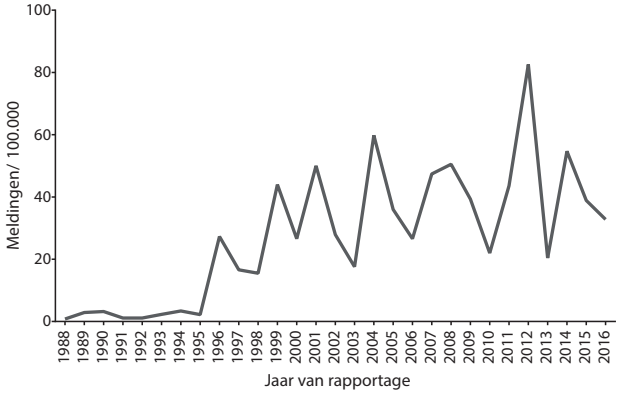
De bacterie *Bordetella pertussis* veroorzaakt kinkhoest, een ernstige en zeer besmettelijke infectieziekte van de bovenste luchtwegen. Na een incubatieperiode van ongeveer 1 week begint kinkhoest als een milde verkoudheid. Vervolgens beginnen de hoestaanvallen die steeds heftiger worden. Een typische kinkhoestaanval bestaat uit 5-10 ernstige en krampachtige hoestepisodes, waarna een krachtige en soms piepende inademing volgt. Complicaties, die met name bij baby's en jonge kinderen voorkomen, zijn braken na een hoestaanval, lange adempauzes, longontsteking of middenoorontsteking en soms kan kinkhoest leiden tot sterfte. Na een aantal weken tot maanden nemen de hoestaanvallen geleidelijk aan af en worden ze minder intens. Kinkhoest is met name zeer ernstig voor pasgeboren baby's en kinderen die niet of onvolledig zijn gevaccineerd tegen kinkhoest. Ook oudere kinderen, adolescenten en volwassenen kunnen een typische kinkhoestinfectie doormaken, hoewel de meerderheid vanwege eerdere vaccinatie of eerdere infectie milde of helemaal geen symptomen zal vertonen.

Kinkhoest vaccins

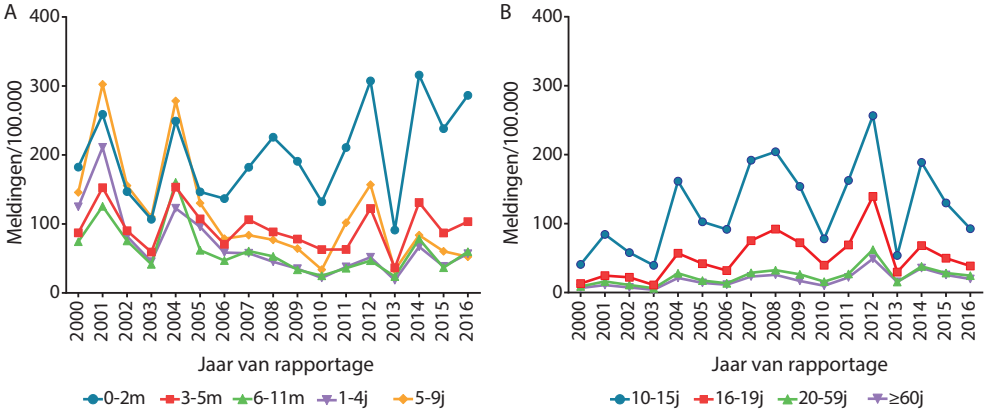
Voor de introductie van kinkhoestvaccins was kinkhoest wereldwijd een belangrijke oorzaak van morbiditeit en mortaliteit bij kinderen. In 1957 is in Nederland een hele bacterie (whole-cell) vaccin tegen kinkhoest ingevoerd in het Rijksvaccinatieprogramma. Sindsdien worden baby's in hun eerste levensjaar 4 keer gevaccineerd tegen kinkhoest. Dit leidde tot een drastische daling van het aantal ziekte- en sterftegevallen door kinkhoest. Deze whole-cell vaccins, bestaande uit geïnactiveerde *B. pertussis* bacteriën, gaven relatief veel bijwerkingen, zoals enige uren aanhoudend huilen, koorts, lokale zwelling en pijn. Een nieuw ontwikkeld acellulair vaccin, dat uit enkele gezuiverde eiwitten (antigenen) bestaat, geeft minder bijwerkingen. Dit vaccin wordt ook als herhalingsvaccinatie (boostervaccinatie) gebruikt bij kinderen en volwassenen. In 2001 is in Nederland een acellulaire boostervaccinatie toegevoegd aan het Rijksvaccinatieprogramma voor 4-jarige kinderen, voordat ze naar school gaan. Daarnaast is in 2005 het whole-cell vaccin bij zuigelingen vervangen door het acellulaire vaccin toegediend op de leeftijd van 2, 3, 4, en 11 maanden.

Kinkhoest epidemiologie

Ondanks de hoge vaccinatiegraad in Nederland (>95% van de baby's wordt gevaccineerd tegen kinkhoest) neemt het aantal kinkhoestmeldingen sinds 1996 in Nederland langzaam toe en worden er bovendien elke 2-3 jaar uitbraken gemeld (**Figuur 1**). De grootste kinkhoestepidemie in Nederland sinds de introductie van de kinkhoestvaccins was in 2012. Tijdens die uitbraak zijn er meer dan 13.000



Figuur 1. Kinkhoest meldingen in Nederland. De jaarlijkse incidentie (alle leeftijden) van gemelde kinkhoest gevallen in Nederland per 100.000 inwoners van 1988 tot 2016.



Figuur 2. Kinkhoest meldingen in Nederland per leeftijdsgroep. De jaarlijkse incidentie van gemelde kinkhoest gevallen in Nederland per 100.000 **(A)** voor kinderen tot en met 9 jaar oud, en **(B)** voor adolescenten en volwassenen.

kinkhoestgevallen gemeld en zijn 3 zuigelingen, die nog te jong waren om (volledig) beschermd te zijn door vaccinaties, overleden. Behalve bij zuigelingen neemt de laatste jaren het aantal gevallen van kinkhoest (de incidentie) vooral in adolescenten en jongvolwassenen toe (**Figuur 2**). Ook in veel andere landen met een hoge vaccinatiegraad, zoals o.a. Amerika, Engeland en Australië, neemt het aantal kinkhoestmeldingen toe. Enkele oorzaken voor de stijging zijn een verbeterde diagnostiek en een verhoogde surveillance, maar ook de snelle afname van de afweer (immuniteit) tegen kinkhoest binnen enkele jaren na infectie of vaccinatie, de verandering van whole-cell naar acellulaire vaccins en veranderingen in de kinkhoestbacterie zelf in de tijd.

Immuniteit tegen kinkhoest

Het immuunsysteem beschermt ons tegen ziekteverwekkers (pathogenen), zoals bacteriën en virussen, en bestaat uit twee onderdelen, het niet-specifieke aangeboren (innate) immuunsysteem en het specifieke aangeleerde (adaptieve) immuunsysteem. Bij een kinkhoestinfectie zal als eerste het innate immuunsysteem proberen de infectie snel te bestrijden. Vervolgens wordt het adaptieve immuunsysteem geactiveerd om het innate systeem te helpen. Het adaptieve immuunsysteem richt zich specifiek tegen een klein stukje eiwit (antigeen) van een pathogeen en is erg effectief in het opruimen van pathogenen. Maar het duurt even voor het adaptieve systeem volledig in werking is. Een andere eigenschap van het adaptieve afweersysteem is de opbouw van geheugencellen na contact met een pathogeen. Zo zorgen de vaccinaties tegen kinkhoest ervoor dat er geheugencellen worden aangemaakt specifiek tegen de kinkhoestbacterie. Deze geheugencellen kunnen bij een infectie versneld reageren en voorkomen dat er ziekte optreedt. Er zijn grofweg twee type immuuncellen van het adaptieve systeem te onderscheiden, de B-cellen en de T-cellen. B-cellen produceren afweerstoffen die bescherming bieden tegen het binnendringen van pathogenen en die toxines van de bacterie neutraliseren. Daarnaast zijn helper T-cellen (Th) belangrijk voor de ondersteuning van B-cellen, en zijn cytotoxische T-cellen belangrijk in het verwijderen van de kinkhoestbacterie. Th-cellen zijn ook onder te verdelen in veel verschillende types (subsets) afhankelijk van de signaalstoffen die ze produceren. In dit proefschrift hebben we de Th-cel subsets bestudeerd die worden geactiveerd door kinkhoestvaccinatie (subsets Th1, Th2, Th17 en Treg).

Doel en onderzoeksvragen van dit proefschrift

Het feit dat het aantal kinkhoestmeldingen gestaag toeneemt in alle leeftijdsgroepen en daarmee ook het aantal kinkhoest gerelateerde ziekenhuisopnames van niet-gevaccineerde zuigelingen, benadrukt het belang van verbeterde kinkhoestvaccins. Aangezien de ontwikkeling van nieuwe vaccins vele jaren duurt, kan aanpassing van het huidige vaccinatieprogramma voor kinkhoest bijdragen aan het verminderen van de huidige ziektelast door kinkhoest. In meerdere landen worden acellulaire kinkhoest boostervaccinaties aanbevolen voor adolescenten, volwassenen en zwangere vrouwen. Echter, hoe lang de vaccinaties bij deze oudere leeftijdsgroepen bescherming bieden is momenteel niet volledig bekend.

In dit proefschrift hebben we de lange-termijn immuunreactie onderzocht na één of meerdere boostervaccinaties. Dit hebben we gedaan aan de hand van vier onderzoeksvragen:

1. *Wat is de (sub-) klinische kinkhoestincidentie bij adolescenten tijdens een kinkhoest epidemie in Nederland? Is de concentratie van afweerstoffen die momenteel gebruikt wordt om een recente kinkhoestinfectie vast te stellen (cut-off waarde) onderscheidend genoeg?*
2. *Wat is de immuunreactie op een boostervaccinatie tegen kinkhoest op 9-jarige leeftijd? Verschilt deze tussen kinderen die in het eerste levensjaar gevaccineerd zijn geweest met het whole-cell of met het acellulaire kinkhoestvaccin?*
3. *Hebben kinderen met een heftige (lokale) bijwerking rond de injectieplaats na de booster vaccinatie op 4-jarige leeftijd een andere immuunreactie op de vaccinatie in vergelijking met kinderen zonder deze bijwerking?*
4. *Wat is de immuunreactie op een eerste boostervaccinatie bij volwassenen en hoe lang blijven de afweerstoffen aanwezig in de circulatie (het bloed) na de boostervaccinatie?*

De (sub-) klinische kinkhoestincidentie in adolescenten

In Hoofdstuk 2 hebben we onderzocht hoeveel adolescenten een (sub-) klinische kinkhoestinfectie hebben doorgemaakt tijdens de kinkhoestepidemie van 2012 in Nederland op basis van afweerstoffen tegen kinkhoest in het bloed. Van ruim 200 adolescenten zijn bloedmonsters verzameld in oktober 2011, oktober 2012 en in oktober 2014 en is de concentratie van afweerstoffen specifiek tegen het pertussis toxine bepaald. Bij een concentratie hoger dan 50 IU/mL wordt aangenomen dat deze persoon in het afgelopen jaar een kinkhoestinfectie heeft doorgemaakt. Op basis van dit onderzoek zou maar liefst 1 op de 10 deelnemers tijdens de epidemie van 2012 een kinkhoestinfectie hebben doorgemaakt. Dit is ruim 40 keer hoger dan het aantal officieel gemelde kinkhoestgevallen bij adolescenten van deze leeftijd. Het is dus heel aannemelijk dat infecties met *B. pertussis* in deze leeftijdsgroep vele malen hoger is dan het aantal gemelde kinkhoestgevallen veronderstelt. Adolescenten zijn een bron voor het verspreiden van infecties naar zuigelingen. Om goed inzicht te krijgen in de totale circulatie van de kinkhoestbacterie, is het wenselijk dit onderzoek ook bij andere leeftijdsgroepen te verrichten zodat zicht wordt gekregen bij wie een extra boostervaccinatie tegen kinkhoest gewenst is. Ook is het de vraag wat de optimale leeftijd voor herhalingsvaccinatie(s) zou kunnen zijn om de circulatie van de kinkhoestbacterie in de bevolking te verminderen.

We hebben in Hoofdstuk 2 ook gevonden dat 22 deelnemers al voor de aanvang van de 2012 epidemie recent een kinkhoestinfectie hadden doorgemaakt. Bij 17 van deze 22 deelnemers was de concentratie van de afweerstoffen een jaar later nog steeds boven de grenswaarde, waaruit blijkt dat de daling van de concentratie van de afweerstoffen veel langzamer verloopt dan vooraf gedacht. Dit leert ons dat de arbitraire grens van 50 IU/mL die nu wordt toegepast als indicatie voor een kinkhoestinfectie in het voorafgaande jaar, voorzichtig moet worden toegepast omdat deze tot een overschatting kan leiden van het aantal (sub-) klinische kinkhoestinfecties.

Immuniteit tegen kinkhoest bij pre-adolescenten

Het type vaccin wat als eerste vaccinatie wordt gebruikt, whole-cell of acellulair, is belangrijk voor de opbouw van de afweer later in het leven. Epidemiologische studies laten zien dat de bescherming tegen kinkhoest sneller afneemt na boostervaccinaties bij kinderen en adolescenten wanneer zij in het eerste levensjaar het acellulaire kinkhoestvaccin hebben gekregen in vergelijking met kinderen die whole-cell kinkhoestvaccinaties hebben gekregen. Het whole-cell vaccin leidt tot meer kinkhoest antigeen-specifieke afweerstoffen (van het type IgG1) en Th1-cellen, die belangrijk zijn voor de bescherming tegen kinkhoest, terwijl het acellulaire vaccin meer Th2-cellen induceert die waarschijnlijk minder goed bijdragen aan de bescherming. Deze Th2 cellen zorgen er onder andere ook voor dat B-cellen meer afweerstoffen van het type IgG4 aanmaken in plaats van IgG1. Naast een verminderde bescherming, worden IgG4 afweerstoffen en Th2 cellen bovendien geassocieerd met allergische reacties. In Hoofdstuk 3 en Hoofdstuk 4 beschrijven we de immuunreactie na een boostervaccinatie op zowel 4-jarige als 9-jarige leeftijd en vergelijken we deze reactie tussen kinderen die in hun eerste levensjaar whole-cell dan wel acellulaire kinkhoestvaccinaties hebben gekregen. Direct voor de boostervaccinatie op 4-jarige leeftijd, en 1 maand en 2 jaar (op 6-jarige leeftijd) na deze boostervaccinatie is een bloedmonster afgenomen van verschillende groepen kinderen. Daarnaast is onderzoek gedaan bij kinderen die een extra kinkhoest boostervaccinatie hebben gekregen op 9-jarige leeftijd. Bij deze kinderen is bloed afgenomen direct voor de boostervaccinatie en 1 maand en 1 jaar na de vaccinatie bij dezelfde kinderen. Hierdoor kunnen we de immuunreactie volgen per individu. De concentratie afweerstoffen, het aantal geheugen B-cellen en het aantal T-cellen gericht tegen kinkhoest vaccinantigenen blijkt hoger te zijn bij de 4–6 jarige kinderen die het acellulaire vaccin in het eerste levensjaar hebben gekregen dan bij de 4–6 jarige kinderen die het whole-cell vaccin hebben gekregen. Deze verschillen zijn op 9-jarige leeftijd voorafgaand aan de herhalingsvaccinatie grotendeels verdwenen. Maar opvallend is dat na de extra acellulaire boostervaccinatie op 9-jarige leeftijd de kinderen die het whole-cell vaccin hebben gehad in het eerste levensjaar een betere immuunreactie vertonen tegen kinkhoest dan kinderen die het acellulaire vaccin hebben gehad. Deze kinderen hebben namelijk minder IgG1 afweerstoffen en minder Th1 dan Th2 cellen (een Th2 dominante immuunreactie) vergeleken met de whole-cell gevaccineerde kinderen die meer IgG1 afweerstoffen en meer Th1 dan Th2 cellen hebben aangemaakt. Daarnaast hebben de acellulair gevaccineerde 4–9 jarige kinderen hogere concentraties IgG4 dan de whole-cell gevaccineerde kinderen. Deze immunologische data ondersteunen hiermee de epidemiologische studies, die aangeven dat adolescenten die in hun eerste levensjaar zijn gevaccineerd met acellulaire kinkhoestvaccins een grotere kans hebben om op latere leeftijd kinkhoest te krijgen dan kinderen die als zuigeling zijn gevaccineerd met whole-cell vaccins.

Het type vaccin dat wordt gebruikt voor de eerste stimulatie (priming) van het immuunsysteem in het eerste levensjaar zorgt voor een "immunologische inprenting"

die in ieder geval tot op adolescente leeftijd aanwezig blijft. Deze verschillen worden waarschijnlijk veroorzaakt door de extra bacteriële componenten die aanwezig zijn in whole-cell vaccins. Deze bacteriële componenten stimuleren als "adjuvans" het immuunsysteem om een Th1 dominante reactie te induceren. Daarnaast zorgt herhaald contact met een hoge dosis antigeen (zoals bij de acellulaire vaccins) ook voor een meer Th2 dominante immuunreactie. Omdat lange-termijn bescherming tegen kinkhoest vooral is gekoppeld aan de aanwezigheid van Th1 cellen, is het belangrijk voor de bescherming tegen kinkhoest dat de primaire serie van vaccinaties in het eerste levensjaar een Th1 dominante immuunreactie induceert. Wellicht dat in de toekomst het gebruik van ander adjuvantia in de acellulaire kinkhoestvaccins toegepast wordt om de gewenste Th1 immuunreactie te induceren.

Bijwerkingen na de boostervaccinatie op 4-jarige leeftijd

Een Th2 dominante immuunreactie kan ook invloed hebben op bijwerkingen na vaccinaties. Sinds 1 januari 2005 worden alle zuigelingen in Nederland met het acellulaire kinkhoestvaccin gevaccineerd. Het aantal gemelde bijwerkingen na de 5^{e} acellulaire vaccinatie op 4-jarige leeftijd nam in Nederland toe aan het eind van 2008. Lokale en systemische bijwerkingen na deze kinkhoestvaccinatie op 4-jarige leeftijd werden tot 4 keer vaker gemeld bij kinderen die het acellulair vaccin als zuigeling hadden gekregen ten opzichte van whole-cell gevaccineerde kinderen. In Hoofdstuk 5 hebben we de immuunreactie van kinderen met een heftige lokale bijwerking kort na de boostervaccinatie op 4-jarige leeftijd onderzocht en vergeleken met de immuunreactie van kinderen zonder een bijwerking (de controlegroep). De kinderen met een heftige bijwerking toonden hogere concentraties afweerstoffen en een grotere Th2 reactie ten opzichte van de controlegroep. Daarnaast vonden we dat de concentratie IgE afweerstoffen, een type afweerstof dat net als IgG4 wordt geassocieerd met een allergische reactie, hoger was bij kinderen met een bijwerking. De Th2 dominante immuunreactie die we zagen bij zowel de kinderen met een heftige bijwerking als in de controle groep, wordt waarschijnlijk veroorzaakt door de hoge antigeen concentratie in de acellulaire vaccins die in een relatief korte periode 5 keer wordt toegediend (2, 3, 4 en 11 maanden oud en een boostervaccinatie op 4 jaar). Vlak voor deze boostervaccinatie op 4-jarige leeftijd is de cellulaire immuunreactie tegen kinkhoest nog erg hoog. Dit kan zeker bijdragen aan de kans op bijwerkingen na de booster vaccinatie. Sinds januari 2017 wordt voor de 5^{e} acellulaire vaccinatie gebruik gemaakt van een boostervaccin met een lagere antigeen concentratie. Hopelijk zal dit leiden tot een vermindering van het aantal kinderen met bijwerkingen en dit zal goed gevolgd en geëvalueerd worden in de komende jaren.

Kinkhoest immuniteit in volwassenen na een eerste acellulaire boostervaccinatie

In Hoofdstuk 6 hebben we de immuunreactie onderzocht op een eerste acellulaire kinkhoestvaccinatie bij volwassenen in de leeftijd van 25-29 jaar. Deze volwassenen zijn in het eerste levensjaar gevaccineerd geweest met een whole-cell kinkhoestvaccin

en hebben verder geen boostervaccinaties gehad. Bij deze volwassenen vinden we dat de concentratie aan afweerstoffen en de reacties van de Th1 cellen 2 jaar na de boostervaccinatie nog steeds hoger zijn dan vlak voor de vaccinatie. Met behulp van wiskundige modellering wordt gezien dat het niveau van de afweerstoffen tegen pertussis toxine ongeveer 9 jaar boven de verwachte beschermingsgrens zal blijven bij de helft van de deelnemers. Een boostervaccinatie bij deze volwassenen zorgt dus waarschijnlijk voor een langdurige immuniteit tegen kinkhoest. Het vaccineren van volwassenen, vooral in de leeftijdsgroep van 20–30 jaar, kan bijdragen aan het verminderen van de circulatie van de kinkhoestbacterie waardoor ook overdracht van de kinkhoestbacterie naar baby's en kinderen wordt verminderd.

Naar aanleiding van een verhoging in kinkhoest gerelateerde sterfte van pasgeborenen tijdens de kinkhoestepidemie in California in 2010 en in het Verenigd Koninkrijk in 2012, wordt in die landen aan zwangere vrouwen geadviseerd zich te laten vaccineren tegen kinkhoest tijdens elke zwangerschap. Inmiddels zijn er wereldwijd al meer dan 25 landen die een zogenoemde maternale kinkhoestvaccinatie aanbevelen. Maternale afweerstoffen worden tijdens de zwangerschap naar de baby overgebracht waardoor de baby in de eerste maanden na de geboorte beschermd is tegen kinkhoest. We hebben aangetoond dat bij volwassenen de afweerstoffen na een vaccinatie veel langer aanwezig blijven dan vooraf gedacht. Het is daarom wellicht voldoende om alleen tijdens de eerste zwangerschap te vaccineren tegen kinkhoest en niet standaard elke zwangerschap, ook als de volgende zwangerschap al snel daarna volgt. Dit moet echter nog nader onderzocht worden. Ook is het de vraag of volwassenen die als zuigeling een acellulair vaccin hebben gekregen op dezelfde wijze zullen reageren op een boostervaccinatie. In Nederland zijn de personen die niet meer gevaccineerd zijn geweest met het whole-cell vaccin maar alleen het acellulaire vaccin hebben gekregen nu, in 2018, 13 jaar.

Aanbevelingen

Een boostervaccinatie op 9-jarige leeftijd zal de immuniteit tegen kinkhoest voor een relatief korte periode verhogen. Het invoeren van een boostervaccinatie op (pre-) adolescente leeftijd is daarom het overwegen waard, als er ook een (jong-) volwassen boostervaccinatie wordt overwogen om te voorkomen dat de piekincidentie van kinkhoest doorschuift naar de volwassenen met kleine kinderen of die kinderen gaan krijgen. Het bepalen van de (sub-) klinische kinkhoestincidentie in de Nederlandse bevolking, zoals gedaan in Hoofdstuk 2, kan helpen bij de beslissing wat de beste leeftijdsgroep(en) is/zijn voor een boostervaccinatie om de circulatie van *B. pertussis* te verminderen.

Er zijn verschillende opties om de lange-termijn bescherming tegen kinkhoest te verbeteren. Het aanpassen van het vaccinatieschema in het eerste levensjaar (nu op 2, 3, 4, en 11 maanden), zoals het uitstellen van de eerste vaccinatie met 1 maand

en/ of het achterwege laten van de tweede vaccinatie uit de primaire serie van 3 vaccinaties, kan er wellicht voor zorgen dat er een minder sterke Th2 immuunreactie zal worden geïnduceerd, met behoud van bescherming tegen kinkhoest. Ook kan overwogen worden om de boostervaccinatie op 4-jarige leeftijd met 1 of 2 jaar uit te stellen aangezien de cellulaire immuniteit tegen kinkhoest op 4 jaar nog voldoende aanwezig is. Een nieuwe optie zou het gebruik van het whole-cell vaccin als allereerste vaccinatie voor zuigelingen kunnen zijn, gevolgd door acellulaire vaccins. Dit zou al een positief effect kunnen hebben op de inductie van een meer Th1 type immuunreactie, dat wellicht voor bescherming tegen kinkhoest zorgt tot adolescentie. In plaats van een whole-cell vaccin kan overwogen worden om het acellulaire vaccin aan te passen met toevoeging van een adjuvans dat een meer Th1 type immuunreactie induceert. Uiteraard zullen deze voorstellen eerst onderzocht moeten worden op de lange-termijn immuniteit tegen kinkhoest.

Op dit moment is, voor de korte termijn bescherming van zuigelingen met het hoogste risico op een ernstige of fatale kinkhoest infectie, maternale vaccinatie de beste optie om baby's te beschermen. Op de langere termijn zullen echter nieuwe vaccins ontwikkeld moeten worden die een betere en langdurigere bescherming bieden tegen kinkhoest. De resultaten vermeld in dit proefschrift laten zien dat het bepalen van de immuniteit die wordt opgewekt door kinkhoestvaccinaties belangrijk is om de kinkhoest immuniteit in de populatie te evalueren en daarmee het Rijksvaccinatieprogramma.

Appendix

Dankwoord

Yes! Daar is hij dan, mijn "boekje"! Wat een werk! Zonder de hulp van velen was dit niet mogelijk geweest. Ik wil daarom graag iedereen bedanken die op zijn of haar manier heeft bijgedragen aan de totstandkoming van dit proefschrift.

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A

Appendix

About the author

Saskia van der Lee was born on August 8, 1986 in Soest, the Netherlands. She attended secondary school at the Amersfoortse Berg in Amersfoort and completed her HAVO exam in 2003. In 2007, she graduated from the Hogeschool Utrecht at the faculty Life Sciences and Biology studying Biology and Medical Laboratory Research with a specialisation in Molecular Biology. During this study, she completed her first internship at the department of prenatal screening at the Academic Medical Centre (AMC), Amsterdam. Her final internship was performed at the National Institute for Public Health and the Environment (RIVM) in Bilthoven studying the Coxsackie virus A under the supervision of G.P. Smits.

After graduation, she worked for a year as a technician at the department of prenatal screening at the AMC. From 2008 till 2010 she worked at the RIVM where she studied the genomic profiles of circulating *Bordetella pertussis* strains. Thereafter, she worked for two years in the group of Prof. dr. J. Knol studying gut biology at Nutricia Research, Wageningen. In 2013, she started her PhD project on *Bordetella pertussis* vaccine induced memory immunity under the supervision of Prof. dr. E.A.M. Sanders at the Wilhelmina Children's Hospital, Utrecht and Dr. A.M. Buisman and Dr. G.A.M. Berbers at the RIVM. The results of this research are presented in this thesis. Saskia lives in Leusden together with Randolph Karels and their daughter Sarah.

Appendix

List of publications

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