Decoding mucosal immunity

Impact of SARS-CoV-2 and *Bordetella pertussis* vaccination and infection

Janeri Fröberg

RADBOUD UNIVERSITY PRESS

Radboud Dissertation Series

Decoding mucosal immunity

Impact of SARS-CoV-2 and *Bordetella pertussis* vaccination and infection

Janeri Fröberg

This Publication has been made possible by the PERISCOPE consortium, the Nierstichting, and ZonMW.

Decoding mucosal immunity - Impact of SARS-CoV-2 and *Bordetella pertussis* vaccination and infection
Janeri Fröberg

Radboud Dissertation Series
ISSN: 2950-2772 (Online); 2950-2780 (Print)

Published by RADBOUD UNIVERSITY PRESS
Postbus 9100, 6500 HA Nijmegen, The Netherlands
www.radbouduniversitypress.nl

Design: Proefschrift AIO | Guus Gijben
Cover: Proefschrift AIO | Guntra Laivacuma
Printing: DPN Rikken/Pumbo

ISBN: 9789465150659
DOI: 10.54195/9789465150659
Free download at: https://doi.org/10.54195/9789465150659

RADBOUD
UNIVERSITY
PRESS

Decoding mucosal immunity

Impact of SARS-CoV-2 and *Bordetella pertussis* vaccination and infection

Proefschrift ter verkrijging van de graad van doctor
aan de Radboud Universiteit Nijmegen
op gezag van de rector magnificus prof. dr. J.M. Sanders,
volgens besluit van het college voor promoties
in het openbaar te verdedigen op

donderdag 19 juni 2025
om 14.30 uur precies

door

Janeri Fröberg
geboren op 28 februari 1996
te Utrecht

Promotoren:
Prof. dr. M.I. de Jonge
Prof. dr. M.A. Huynen

Copromotor:
Dr. ir. D.A. Diavatopoulos

Manuscriptcommissie:
Prof. dr. A. Timen
Prof. dr. P.C.J. Bruijning-Verhagen (Universiteit Utrecht)
Dr. N. Mielcarek (Institut Pasteur de Lille, Frankrijk)

Table of contents

Chapter 1

Introduction

Throughout the day, we are always at risk to be exposed to pathogens, through the food we eat, the things we touch and the air we inhale. Pathogens that enter the body via the respiratory tract are called respiratory pathogens. Examples of respiratory pathogens are the viruses influenza and SARS-CoV-2, as well as the bacterium *Bordetella pertussis* (Bp). These microscopically small pathogens can cause respiratory diseases, which are the most common type of infectious diseases in the world and cause millions of deaths each year[1]. In 2022, among all infectious diseases, lower respiratory infections were the leading cause of disease burden[2], and pneumonia the leading cause of death in children under 5 years of age[1].

However, not all exposure events result in severe disease (**Figure 1**)[3,4]. One can be exposed to a pathogen that is eliminated from the airways before it causes infection. In case exposure does lead to infection of the respiratory tract, the outcome can be **asymptomatic** (also referred to as subclinical infections, or colonization when talking about bacterial infections), where the pathogen multiplies within the host without noticeable symptoms. Such cases will typically not be detected in the absence of routine, population-based screening. In case infection leads to **disease**, it can manifest mildly with general symptoms like a fever, runny nose, coughing, or sore throat. These cases are typically monitored through syndromic surveillance, such as "Influenza-like illness" and "acute respiratory infections" syndromes in the EU[5]. However, a large proportion of mild infections will likely stay undiagnosed[6,7]. Finally, when a pathogen becomes invasive and spreads to other sites within the host (i.e. the lower respiratory tract) it can lead to more severe disease with symptoms like shortness of breath and pneumonia, and specific manifestations such as whooping cough in the case of *Bordetella pertussis* infections.

On a population level, infections will mainly be detected when severe disease occurs; testing the presence of pathogens in healthy individuals is not routinely done. However, various studies have shown that asymptomatic respiratory infections can play a driving role in the maintenance and spread of such pathogens in the population, by creating a large undetected infectious reservoir[8-11]. To better understand the role and mechanisms of asymptomatic infections in disease dynamics, it is important to investigate host-pathogen interactions at all stages of infection, including the asymptomatic and mild disease stage[11].

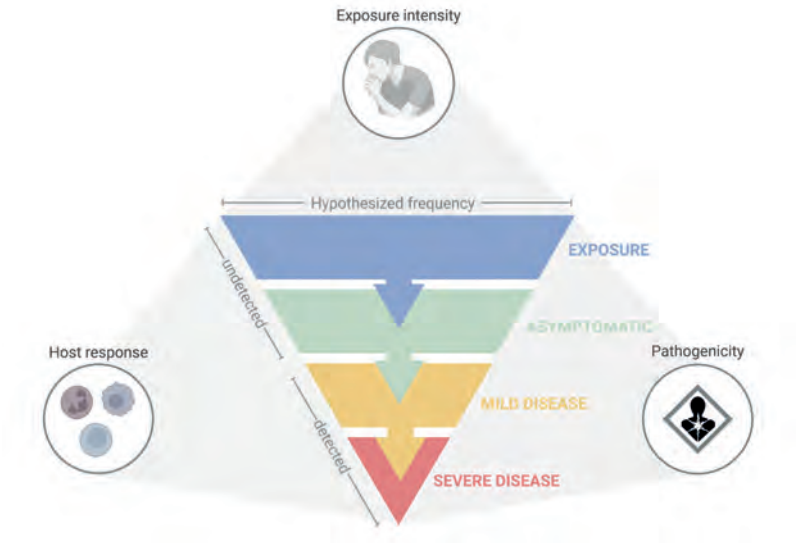


Figure 1: The possible outcomes of exposure to a pathogen. Exposure: the pathogen enters the respiratory tract, but is eliminated almost immediately through physical or immunological barriers. **Asymptomatic infection:** the pathogen is able to establish infection and multiply in the host, without causing notifiable symptoms. **Mild disease:** the pathogen multiplies in the host, causing damage and mild symptoms. **Severe disease:** the pathogen multiplies and causes extensive damage to the host, leading to severe symptoms (hospitalization). Whether a host-pathogen interaction leads to severe disease, depends on exposure intensity and duration, pathogenicity, and host response. Created in BioRender. Froberg, J. (2025) https://BioRender.com/v70c532

Whether a respiratory pathogen will cause (severe) symptoms, depends on several factors (**Figure 1**):

- **Exposure intensity and duration**; exposure to a high quantity of pathogen during a longer period of time will increase the chance of infection leading to damage. For instance, decreasing exposure intensity was one of the main goals of mouth mask wearing during the COVID-19 pandemic[12].
- **Pathogenicity**; the ability of the pathogen to invade and multiply within the host, as well as the ability of the pathogen to cause damage within the host will determine the chance of becoming symptomatic. For example, the capsule of Streptococcus pneumoniae protects the bacterium from phagocytosis, enhancing its virulence in the respiratory tract[13].
- **Host response**; the ability of the immune system to get rid of the pathogen before damage as a consequence of infection occurs. This can be influenced by previous infections or vaccinations[14], but also by other factors such as age and co-morbidity.

How vaccination influences the (local) host response during an infection, and *vice versa,* how prior infection influences the vaccine response remains an underappreciated field of research. In this thesis, I explore the interplay of respiratory infection vs. vaccination on the host's (mucosal) immune responses. First, I will give an overview of the mucosal immune system, the humoral immune response, and different methods of immunization, followed by an introduction of the pathogens focused on in this thesis.

Mucosal immunity as the first line of defence

After the gut and skin, the respiratory epithelium is the largest mucosal surface in the human body [15-17]. The respiratory epithelium varies in cellular composition, depending on the anatomical position; the upper respiratory tract (nose, trachea) mainly consist of ciliated epithelial cells and goblet cells that function as a barrier and contribute to pathogen removal[18,19], while the lower respiratory tract (bronchi) consists more of non-ciliated epithelial cells and club cells that under steady-state retain an anti-inflammatory environment to sustain lung function[18,20]. Underneath the epithelial cells lies the lamina propria, a layer of connective tissue that houses immune cells and organized structures such as the mucosa-associated lymphoid tissue[16,21], and is in close proximity to both the lymphatic system and the bloodstream. This enables a continuous interaction of the mucosal, lymphatic and systemic system.

In this thesis I will largely focus on immune responses in the upper respiratory tract, the first line of defence to airborne pathogens. To restrict pathogen replication and prevent damage and spread to other tissues, there are three main modes of defence in the upper respiratory (nasal) mucosa (Figure 2)[16,17,21]. These defence mechanisms have an overarching goal: preventing the pathogen from traveling towards the lower respiratory tract or the bloodstream, where damage or inflammation can result in severe disease[21].

The first line of defence is the mucosal barrier. The epithelial goblet cells produce mucus, also called mucosal lining fluid (MLF)[22], which contains antimicrobial compounds like mucins, antibodies, and many other soluble factors, providing a barrier function for the epithelial cells. The mucus can also trap pathogens, so they can be removed from the respiratory tract into the gastro-intestinal tract through ciliary beating of the epithelial cells; a rhythmic organized movement of the cilia[20,23].

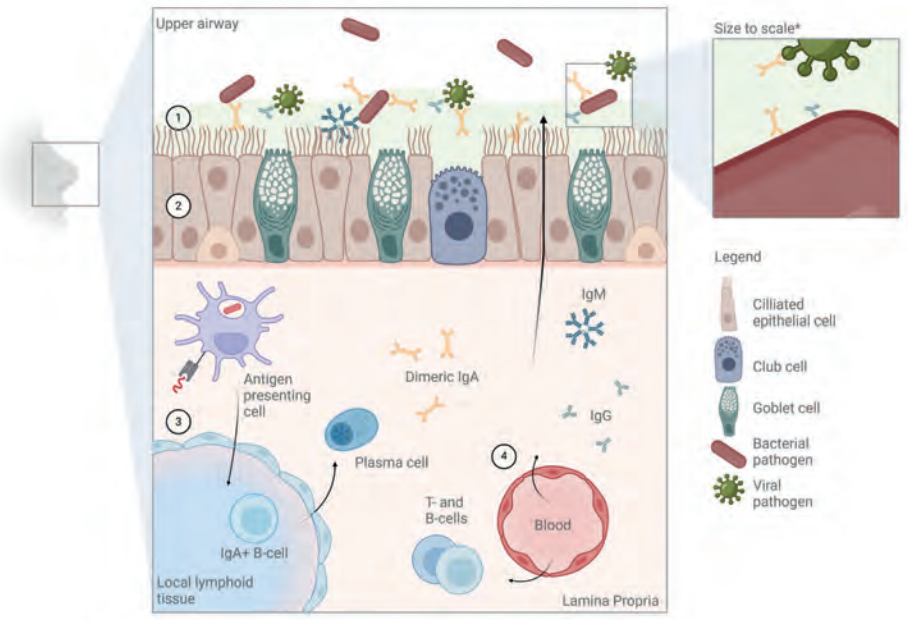


Figure 2: The different aspects of the mucosal immune system. 1) The physical barrier consists of goblet cells that produce a mucus layer that can trap pathogens, and ciliated epithelial cells that remove pathogens from the airways by ciliary beating. 2) **The innate immune response** is activated when pathogens bind to innate receptors expressed by the different types of epithelial cells or antigen presenting cells (APC), which will then initiate an inflammatory signalling cascade, resulting in immune cell recruitment and activation 3) **The adaptive immune response** consists of APCs that take up the pathogen, and present its antigens to T-cells in the local lymphoid tissue, which in turn leads to activation of B-cells, antibody production and memory cell activation. 4). **Mucosal infiltration** of immune cells occurs through chemokines that attract T- and B-cells, and antibodies move from the blood to the mucosal site via specific transporters. *size to scale is an approximation based on general viral, bacterial and antibody size. Created in BioRender. Fröberg, J. (2025) https://BioRender.com/a30r796

Secondly, innate immune responses are generated in the nasal mucosa. Although airway epithelial cells are not strictly considered immune cells, they do represent an important part of the innate immune system of the respiratory tract[23-26]. For instance, epithelial cells express a large variety of innate pattern-recognition receptors such as toll-like receptors, both on the epithelial surface as well as intracellularly [23,26]. When pathogen-associated molecular patterns are detected by these receptors, epithelial cells (mainly goblet and club cells) can rapidly excrete antimicrobial proteins, like CCL20 and mucin, that immobilize or kill pathogens before they can cause further damage[23,25]. Moreover, cytokines and chemokines are produced (IL-6, interferons, CXCL8), which recruit immune cells that are part of the adaptive immune response, like neutrophils, macrophages and T-cells[23,27].

The adaptive immune response is the third and last mode of protection, that gets activated when the innate response is not sufficient in stopping the infeciton[25]. Cytokines and chemokines produced by the innate immune system attract antigen-presenting cells like neutrophils and dendritic cells, leading to uptake of the pathogen at the epithelial surface or within the lamina propria. These cells then present the pathogen-derived antigens to locally residing or recruited T- and B-cells, in specialized lymphoid structures within the lamina propria as well as in local lymph nodes[28]. When the T-cells activate the B-cells to differentiate into plasma cells, these plasma cells move to the lamina propria to produce antigen-specific IgM, IgG or IgA[28]. In the mucosa, the presence of T-cells and IgA-associated cytokines, such as IL-6, TGF-b and IL-10, induce IgA class switching of B-cells, resulting in a large quantity of IgA in the mucosal surfaces[29,30]. Furthermore, cytokines produced in response to infection attract immune cells (macrophages, neutrophils, T- and B-cells) from the bloodstream through chemotaxis. These cytokines can additionally cause an influx of blood-derived antibodies, by making the blood vessels more permeable, a process called exudation, or by increasing the expression of immunoglobulin transporting receptors [31].

Together, these mechanisms form an integrated network that protect the host from infection and disease. Where the physical and innate mucosal defence mechanisms are non-specific, the adaptive immune system leads to a pathogen-specific immune response and specific immunological memory. This immune memory ensures a quicker response upon reencountering the same pathogen, as (tissue resident) memory T- and B-cells will get activated [21,32]. The ability to more rapidly contain the pathogenic threat is why one presents with milder disease upon reinfection, as the pathogen is cleared before it can replicate and cause damage (**Figure 4**)[21]. Of note, not all infections will generate a complete protective immunity. As mentioned above, the chance of disease is highly dependent on the exposure intensity and duration, the pathogenicity, and the host-response. Similarly, the strength of the immune response that is generated after a natural infection will differ per infection and pathogen. For example, infection with influenza virus is related to strong antibody responses and broad protection against re-infection, but this is not the case for Respiratory Syncytial Virus (RSV) infection, probably due to differential B- and T-cell activation[33].

IgA and IgG

This thesis will mainly focus on one aspect of the adaptive immune system, the antibody response. In the respiratory immune system, antibodies play an important role in pathogen eradication. They can bind to the pathogens on the basolateral side as well as the apical surface, being transported across the epithelial cells into the secretion[21]. Although there are more antibody subtypes, my focus here will be on IgG and IgA, two antibody isotypes that are produced following class switching of B-cells. Both play an essential role in the protection against pathogens, but there are some important differences.

IgG is the most common antibody of the blood stream (it accounts for 75% of serum antibodies[34]), is only present in monomeric form, and mainly plays a role in preventing infections and damage by promoting Fc-receptor mediated phagocytosis/killing, and activating the complement system[35]. Systemic IgG can cross the mucosal barrier through two different processes: active transport via the human neonatal Fc receptor (FcRn) that is expressed on the basolateral side of epithelial cells; and via exudation. Although FcRn expression by epithelial cells can be upregulated under inflammatory conditions, it also occurs in steady state[36]. Conversely, exudation is an inflammation-dependent process, primarily occurring during infection, where antibodies passively cross the mucosal barrier[15,31,37]. IgA is the most abundant antibody of the mucosal immune system, with a production of over 3 grams of IgA per day[38]. IgA can be monomeric but is mostly present as dimers and sometimes trimers in the mucosa[38]. It is produced in the local lymphoid tissue and can be actively transported across the epithelial barrier into the mucosal secretions via the polymeric immunoglobulin receptor (pIgR)[35]. During this process, a fraction of pIgR called the secretory component is cleaved off, creating secretory IgA (sIgA), which is more resistant to proteolysis[28,35]. Once IgA is transported into the secretions, it will stay there as sIgA which, especially in its dimer-form, is important in neutralization and agglutination of pathogens[38]. (s)IgA does not activate the complement system or Fc-receptors on immune cells, giving it a more anti-inflammatory profile compared to IgG [16].

Thus, during an infection, there is an anti-inflammatory IgA response, and a pro-inflammatory IgG response. It has been described that (mucosal) IgA is the first responder to an infection. It can play an important role in viral load reduction or bacterial agglutination, and in the prevention of tissue damage[38-40]. In a study that experimentally challenged healthy individuals with H1N1 influenza virus, individuals with pre-existing mucosal IgA but not systemic IgG were protected

from more severe disease[41], underlining the importance of mucosal IgA in disease prevention. On the other hand, IgG is essential for phagocyte function and complement activation, protecting the body from severe disease[34,42].

Mucosal lining fluid to measure mucosal antibodies

In this thesis, we investigate mucosal antibody responses using epithelial lining fluid. This fluid covers the epithelial cells of the nose and contains secreted cytokines, antibodies and many other soluble factors[22]. To collect this fluid, a nasosorption strip, consisting of a synthetic absorptive matrix, is inserted into the nose for 60 seconds. This strip absorbs the mucosal lining fluid without disturbing the epithelial surface (**Figure 3**). Because it is non-invasive and easy to use, it can be applied to collect samples even from very young infants. Additionally, it is suitable for self-collection by participants, enabling frequent sampling without the need to arrange home visits or visits to the clinical site.

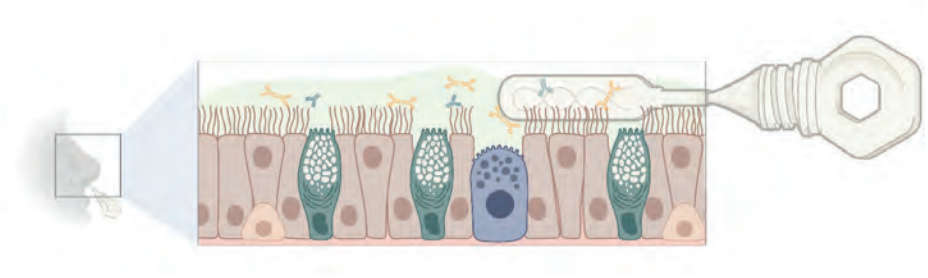


Figure 3: Mucosal lining fluid sampling. A nasosorption strip is inserted into the nose for 60 seconds. It will absorb the mucosal lining fluid, enabling the assessment of nasal antibodies. Created in BioRender. Fröberg, J. (2025) https://BioRender.com/n46o313

Vaccination induced immunity

Above, I introduced the mucosal immune system and how it responds to invading pathogens during a natural infection, and creates an immune memory when doing so. Next to natural infection, the immune system can be trained to detect and remove pathogens through vaccination. Vaccination is the most important public health measure to protect against infectious diseases[43,44]. Since the global commitment to vaccinate in 1974, vaccines have saved over 154 million lives from infectious diseases worldwide over the past 50 years; an equivalent of 6 lives saved every minute of every year[44]. Like natural infections, vaccines stimulate a strong

antigen-specific antibody response, activating T- and B-cells, and creating immune memory (Figure 4). However, they differ from infections several key ways.

First, vaccines are often targeting certain antigens or subunits of the pathogen, instead of the whole spectrum of antigens that is expressed during a natural infection[45]. Although this is essential to provide immunity without causing disease, a consequence of this selection could be that one builds an incomplete protection, that enables pathogen adaption. For example, the adaptation of the influenza virus to vaccine-derived immunity in the population is the reason why we have the seasonal flu-vaccine[46]. Influenza virus is known for its high mutation rate and antigenic flexibility. Where natural infection with a influenza virus strain elicits a relatively broad and long-lived protection, we have not yet been able to mimic this response with vaccines[47]. Another example of vaccine-driven pathogen adaption can be seen in countries that switched vaccines against *B. pertussis*. Many Western countries switched from a whole -cell (inactivated whole bacterium, wP) vaccine to a purified protein vaccine (the aP vaccine) against *B. pertussis*. Instead of the whole bacterium, this aP vaccine contains one to five Bp antigens; PT, FIM2/3, FHA, and PRN. Countries that have switched to the aP vaccine have been reporting an increase of Bp strains that lack the PRN antigen[48], indicating that these strains might have an advantage in the population compared to Bp strains that do have PRN expression, which is driven by the aP-vaccine[49,50].

Second, the different formulations of vaccines can also lead to differential activation of immune pathways. For instance, in the case of the Bp wP and aP vaccines, a difference in T-cell activation was observed. Where the wP vaccine elicits an immune response that is similar to the response after a natural infection, with a T-cell response that is important for bacterial clearance [51-53], the aP vaccines mainly generate a T-cell response that is less able to clear the infection[54]. In the case of influenza A vaccines, differences have been described between the live attenuated, whole inactivated, and subunit vaccines in their ability to elicit strong and broad antibody responses[47], illustrating that vaccine formulation can have a big effect on downstream immune-activation and protection.

Third, where natural infection happens at the mucosal site, most vaccinations are given intramuscularly (parenteral vaccination). Parenteral vaccination primarily triggers a systemic immune response[47], although mucosal responses have been described for some parenteral vaccines, like the whole-cell Bp vaccine[55]. The lack of mucosal immunity derived from parenteral vaccines has been linked to prolonged colonization and reduced nasal clearance after infection, in the case of

B. pertussis and *S. pneumoniae*[56,57]. Recently, more attention has been paid to the limitations of parenteral vaccines and the benefits of mucosal vaccines[16,58,59]. There are currently eight licensed mucosal vaccines, including the oral polio, cholera and rotavirus vaccines, and the intranasal influenza A/B vaccine[59]. The oral polio vaccine elicits strong mucosal sIgA responses that correlated with viral blocking[15], and the intranasal live attenuated influenza vaccine induced strong T cell and sIgA responses in children, which were not seen in the parenteral inactivated influenza vaccines[60]. However, many challenges in mucosal vaccine development remain, especially with regards to dosing and vaccine uptake[17]. Furthermore, for many pathogens, both mucosal and systemic immune responses are needed for optimal protection, especially when invasive disease is one of the concerns, like with *Neisseria meningitidis*[61].

In summary, although vaccination is essential to provide protection against disease, it does not always provide the same (mucosal) immune priming as natural infection. This can result in differences in the susceptibility of the host upon subsequent exposure. If vaccinated individuals remain vulnerable for asymptomatic or subclinical infection, these individuals could unknowingly spread the pathogen to vulnerable individuals upon infection, as illustrated in **Figure 4**.

Aim and scope of this thesis

To aid our understanding of the intricate mechanisms of immune development, this thesis aims to investigate differences and similarities between infection and vaccination-induced antibody responses. To achieve this, we used various cohorts, ranging from infant cohorts pre- and post-primary vaccination, to adult cohorts after clinical or subclinical infection. The immune responses are described in the context of two pathogens: the viral pathogen SARS-CoV-2 and the bacterial pathogen *Bordetella pertussis* (Bp).

SARS-CoV-2

At the end of 2019, cases of an emerging respiratory disease were first reported in China. By the beginning of 2020, the so-called severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) outbreak was declared a public health emergency of global concern[62]. SARS-CoV-2 is a single-stranded RNA virus belonging to the betacoronaviruses that causes the disease COVID-19, which has a large range of symptoms, varying from asymptomatic or flu-like symptoms to potentially fatal respiratory failure and long-COVID sequelae [63-66]. At the time of writing this

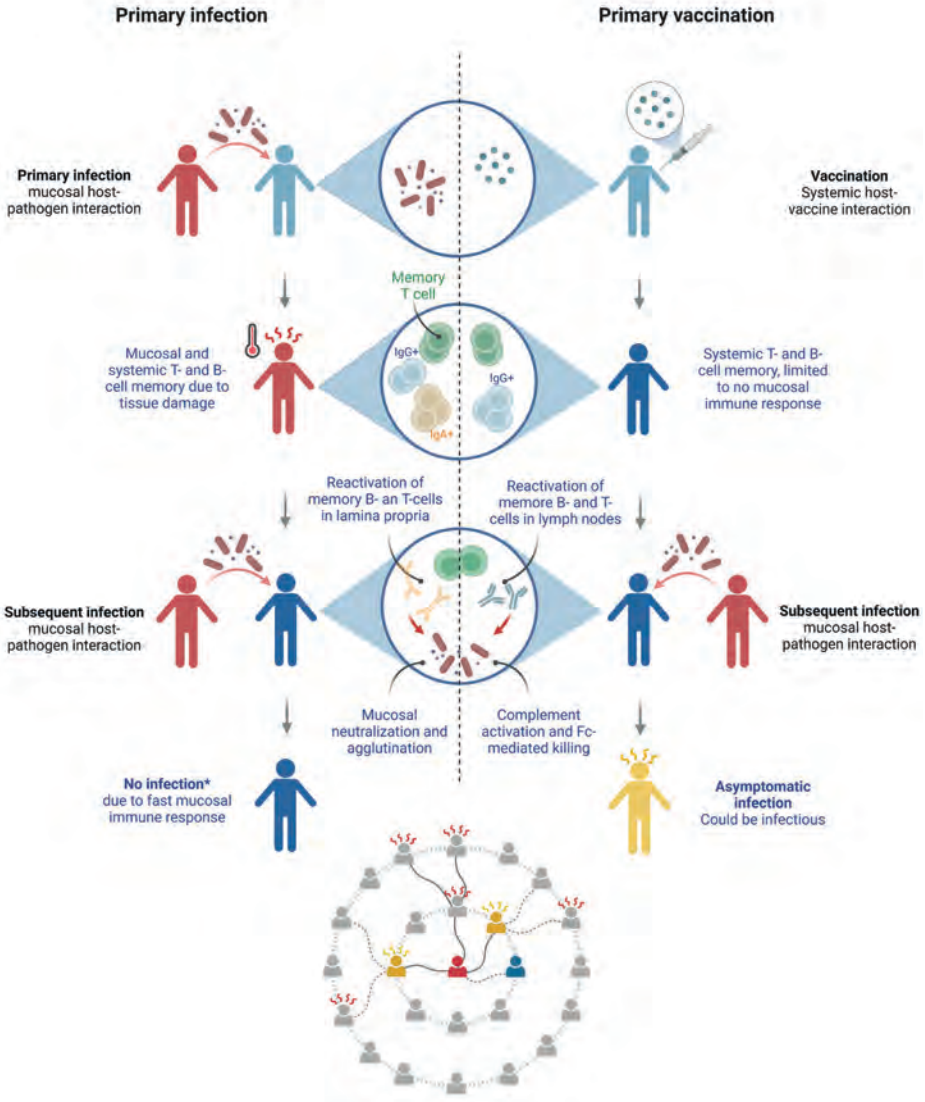


Figure 4. Primary infection vs. vaccination. Primary infection leads to a mucosal immune response against the pathogen, with mucosal and systemic T- and B-cell activation and production of (tissue resident) IgG+ and IgA+ memory cells. Upon re-infection, these memory cells get activated, resulting in quick mucosal neutralization and agglutination, no symptoms and no transmission. On the other hand, when a person gets primed through parenteral vaccination, they produce a systemic T- and B-cell memory against the vaccine-elements, which is more IgG focused. Upon natural infection, this systemic immune response will protect the individual from disease, but might not be sufficient to protect against mild symptoms and transmission into the community. *not all infections will lead to full immunity. Created in BioRender. Fröberg, J. (2025) https://BioRender.com/l48j622

thesis, there are more than 25 known (sub-) variants of SARS-CoV-2[67,68]. Although multiple types of vaccines are currently available[68], at the time this research was started, vaccines were still in development and the population consisted mostly of individuals who had no prior exposure to the virus. This gave us the unique opportunity to investigate the mucosal immune response after primary infection with SARS-CoV-2.

In **Chapter 2**, we conducted a literature review of mucosal immunity in the context of SARS-CoV-2. In **Chapter 3**, we performed a COVID-19 household contact study to investigate the mucosal antibody responses after primary infection and its relation with SARS-CoV-2 viral load and symptoms. In **Chapter 4**, we compared primary infection-induced mucosal immune responses, including the ability to neutralize SARS-CoV-2, to primary vaccination.

B. pertussis

Bordetella pertussis is a gram negative bacterium that primarily infects the upper respiratory tract, and causes the disease 'pertussis'. Pertussis is also called 'whooping cough', or the '100 day cough' because the main symptom of pertussis is a long lasting cough[69,70]. In the Netherlands, pertussis is one of the most prevalent vaccine preventable respiratory diseases, despite a national immunization programme against pertussis since 1957. Pre-COVID-19, pertussis annual disease incidence varied between 20-50 notifications/100.000, with one to two deaths each year[71]. Especially infants who are not (yet) or incompletely vaccinated are at risk of severe pertussis disease; of the infants below six months that were diagnosed with pertussis in the Netherlands in 2024, over 50% needed to be hospitalized[72]. At the time of writing this thesis, several countries are experiencing a large post-COVID-19 pertussis epidemic, with over 32 thousand clinical cases notified in Europe alone between January and March 2024[73,74], and 400-500/100.000 notifications in infants, six infant deaths and two elderly deaths in the Netherlands in 2024[72].

The reason for the high prevalence of pertussis cases despite vaccination is complex. Infant vaccination in the Netherlands has a vaccine effectiveness of 80-90% in the first three years post-vaccination, which decreases after approximately five years[71]. Since *B. pertussis* is highly contagious with an R0 of 12-17[75], vaccine coverage needed to provide herd protection should be >92%[76]. Unfortunately, infant vaccine coverage in the Netherlands has been steadily declining in recent years, with only 85-88% of infants born after 2019 completing their primary pertussis vaccination series[77]. Another potential contributing factor

is the switch in pertussis vaccine-type in many Western countries[8,9,75]. Because of reactogenicity and safety concerns, these countries switched from whole-cell pertussis vaccines (wP), containing whole, inactivated bacteria, to acellular pertussis vaccine (aP), consisting of a cocktail of 1-4 purified pertussis antigens[78,79]. Furthermore, to protect the young infants, some countries have implemented a pertussis vaccine during pregnancy[80]. Although immune responses to both the wP and the aP vaccine have been extensively investigated in animal models, there is a lack of data in humans, particularly regarding mucosal immunity. In **Chapter 5,** we investigate the influence of maternal vaccination and primary vaccination with different pertussis vaccine types on the mucosal immune response to Bp in Gambian infants. In **Chapter 6** we studied the effect of aP booster vaccination on the mucosal antibody responses of aP-primed individuals. We also examined the effect of prior exposure to Bp by measuring IgA responses. In **Chapter 7**, we followed up on this approach by investigating the prevalence of Bp infections in a large, cross-sectional cohort of children living in the Netherlands.

Together, these chapters describe the intricate interplay of vaccination and infection. We identify differences between vaccination-induced and infection-induced (mucosal) antibody responses, enabling the development and use of antibody biomarkers to monitor Bp infection in aP-primed populations. We describe how prior exposure may influence immune responses to subsequent vaccination or infection. Finally, In **Chapter 8**, we reflect on these findings and contemplate on further investigations on mucosal antibody responses that could potentially impact the control of respiratory infectious diseases.

References

1. Ferkol T, Schraufnagel D. The global burden of respiratory disease. *Annals of the American Thoracic Society* 2014; **11**(3): 404-6.
2. Murray CJ. Findings from the Global Burden of Disease Study 2021. *The Lancet* 2024; **403**(10440): 2259-62.
3. Casadevall A, Pirofski L-a. Host-pathogen interactions: basic concepts of microbial commensalism, colonization, infection, and disease. *Infection and immunity* 2000; **68**(12): 6511-8.
4. Pirofski L-a, Casadevall A. The meaning of microbial exposure, infection, colonisation, and disease in clinical practice. *The Lancet Infectious Diseases* 2002; **2**(10): 628-35.
5. Organization WH. Operational considerations for respiratory virus surveillance in Europe: World Health Organization. Regional Office for Europe, 2022.
6. Melbye H, Joensen L, Risør MB, Halvorsen PA. Symptoms of respiratory tract infection and associated care-seeking in subjects with and without obstructive lung disease; The Tromsø Study: Tromsø 6. *BMC Pulmonary Medicine* 2012; **12**: 1-9.
7. Zhang Q, Feng S, Wong IO, Ip DK, Cowling BJ, Lau EH. A population-based study on healthcare-seeking behaviour of persons with symptoms of respiratory and gastrointestinal-related infections in Hong Kong. *BMC Public Health* 2020; **20**: 1-10.
8. Althouse BM, Scarpino SV. Asymptomatic transmission and the resurgence of Bordetella pertussis. *BMC medicine* 2015; **13**: 1-12.
9. Gill C, Rohani P, Thea DM. The relationship between mucosal immunity, nasopharyngeal carriage, asymptomatic transmission and the resurgence of Bordetella pertussis. *F1000Research* 2017; **6**.
10. Chisholm RH, Campbell PT, Wu Y, Tong SY, McVernon J, Geard N. Implications of asymptomatic carriers for infectious disease transmission and control. *Royal Society open science* 2018; **5**(2): 172341.
11. Kim A-R, Sette A, da Silva Antunes R. Adaptive immune response to bordetella pertussis during vaccination and infection: emerging perspectives and unanswered questions. *Expert Review of Vaccines* 2024; **23**(1): 705-14.
12. Howard J, Huang A, Li Z, et al. An evidence review of face masks against COVID-19. *Proceedings of the National Academy of Sciences* 2021; **118**(4): e2014564118.
13. Hyams C, Camberlein E, Cohen JM, Bax K, Brown JS. The Streptococcus pneumoniae capsule inhibits complement activity and neutrophil phagocytosis by multiple mechanisms. *Infection and immunity* 2010; **78**(2): 704-15.
14. Hinojosa M, Shepard SS, Chung JR, et al. Impact of immune priming, vaccination, and infection on influenza A (H3N2) antibody landscapes in children. *The Journal of infectious diseases* 2021; **224**(3): 469-80.
15. Lamm ME. Interaction of antigens and antibodies at mucosal surfaces. *Annual review of microbiology* 1997; **51**(1): 311-40.
16. Kurono Y. The mucosal immune system of the upper respiratory tract and recent progress in mucosal vaccines. *Auris nasus larynx* 2022; **49**(1): 1-10.
17. Neutra MR, Kozlowski PA. Mucosal vaccines: the promise and the challenge. *Nature reviews immunology* 2006; **6**(2): 148-58.
18. Kageyama T, Ito T, Tanaka S, Nakajima H. Physiological and immunological barriers in the lung. Seminars in Immunopathology; 2024: Springer; 2024. p. 1-15.

19. Ali M. Histology of the human nasopharyngeal mucosa. *Journal of anatomy* 1965; **99**(Pt 3): 657.
20. Yaqub N, Wayne G, Birchall M, Song W. Recent advances in human respiratory epithelium models for drug discovery. *Biotechnology Advances* 2022; **54**: 107832.
21. Mettelman RC, Allen EK, Thomas PG. Mucosal immune responses to infection and vaccination in the respiratory tract. *Immunity* 2022; **55**(5): 749-80.
22. Wolsk HM, Chawes BL, Thorsen J, et al. Noninvasive sampling of mucosal lining fluid for the quantification of in vivo upper airway immune-mediator levels. *Journal of Visualized Experiments: JoVE* 2017; (126).
23. Davis JD, Wypych TP. Cellular and functional heterogeneity of the airway epithelium. *Mucosal immunology* 2021; **14**(5): 978-90.
24. Parker D, Prince A. Innate immunity in the respiratory epithelium. *American journal of respiratory cell and molecular biology* 2011; **45**(2): 189-201.
25. Schleimer RP, Kato A, Kern R, Kuperman D, Avila PC. Epithelium: at the interface of innate and adaptive immune responses. *Journal of Allergy and Clinical Immunology* 2007; **120**(6): 1279-84.
26. Bals R, Hiemstra P. Innate immunity in the lung: how epithelial cells fight against respiratory pathogens. *European Respiratory Journal* 2004; **23**(2): 327-33.
27. Vanderheiden A, Ralfs P, Chirkova T, et al. Type I and type III interferons restrict SARS-CoV-2 infection of human airway epithelial cultures. *Journal of virology* 2020; **94**(19): 10.1128/jvi. 00985-20.
28. Sato S, Kiyono H. The mucosal immune system of the respiratory tract. *Current opinion in virology* 2012; **2**(3): 225-32.
29. Cerutti A. The regulation of IgA class switching. *Nature reviews immunology* 2008; **8**(6): 421-34.
30. Isho B, Florescu A, Wang AA, Gommerman JL. Fantastic IgA plasma cells and where to find them. *Immunological reviews* 2021; **303**(1): 119-37.
31. Brandtzaeg P. Immunocompetent cells of the upper airway: functions in normal and diseased mucosa. *European archives of oto-rhino-laryngology* 1995; **252**: S8-S21.
32. Goulding J, Snelgrove R, Saldana J, et al. Respiratory infections: do we ever recover? *Proceedings of the American Thoracic Society* 2007; **4**(8): 618-25.
33. Ascough S, Paterson S, Chiu C. Induction and subversion of human protective immunity: contrasting influenza and respiratory syncytial virus. *Frontiers in Immunology* 2018; **9**: 323.
34. REYNOLDS HY. Immunoglobulin G and its function in the human respiratory tract. Mayo Clinic Proceedings; 1988: Elsevier; 1988. p. 161-74.
35. Turula H, Wobus CE. The role of the polymeric immunoglobulin receptor and secretory immunoglobulins during mucosal infection and immunity. *Viruses* 2018; **10**(5): 237.
36. Salik E, Tyorkin M, Mohan S, et al. Antigen trafficking and accessory cell function in respiratory epithelial cells. *American journal of respiratory cell and molecular biology* 1999; **21**(3): 365-79.
37. Butler WT, Waldmann TA, Rossen RD, Douglas Jr RG, Couch RB. Changes in IgA and IgG concentrations in nasal secretions prior to the appearance of antibody during viral respiratory infection in man. *The Journal of Immunology* 1970; **105**(3): 584-91.
38. Mestecky J, Lamm ME, Ogra PL, et al. Mucosal immunology: Elsevier; 2005.
39. Havervall S, Marking U, Svensson J, et al. Anti-spike mucosal IgA protection against SARS-CoV-2 omicron infection. *New England Journal of Medicine* 2022; **387**(14): 1333-6.

40. Pisanic N, Antar AA, Hetrich MK, et al. Early, robust mucosal secretory IgA but not IgG response to SARS-CoV-2 spike in oral fluid is associated with faster viral clearance and COVID-19 symptom resolution. *The Journal of Infectious Diseases* 2024: jiae447.
41. Gould VM, Francis JN, Anderson KJ, Georges B, Cope AV, Tregoning JS. Nasal IgA provides protection against human influenza challenge in volunteers with low serum influenza antibody titre. *Frontiers in microbiology* 2017; **8**: 900.
42. Bournazos S, Gupta A, Ravetch JV. The role of IgG Fc receptors in antibody-dependent enhancement. *Nature Reviews Immunology* 2020; **20**(10): 633-43.
43. Orenstein WA, Ahmed R. Simply put: Vaccination saves lives. National Acad Sciences; 2017. p. 4031-3.
44. Shattock AJ, Johnson HC, Sim SY, et al. Contribution of vaccination to improved survival and health: modelling 50 years of the Expanded Programme on Immunization. *The Lancet* 2024; **403**(10441): 2307-16.
45. Plotkin S. History of vaccination. *Proceedings of the National Academy of Sciences* 2014; **111**(34): 12283-7.
46. Gerdil C. The annual production cycle for influenza vaccine. *Vaccine* 2003; **21**(16): 1776-9.
47. Krammer F. The human antibody response to influenza A virus infection and vaccination. *Nature Reviews Immunology* 2019; **19**(6): 383-97.
48. Barkoff A-M, Mertsola J, Pierard D, et al. Pertactin-deficient Bordetella pertussis isolates: evidence of increased circulation in Europe, 1998 to 2015. *Eurosurveillance* 2019; **24**(7): 1700832.
49. Lesne E, Cavell BE, Freire-Martin I, et al. Acellular pertussis vaccines induce anti-pertactin bactericidal antibodies which drives the emergence of pertactin-negative strains. *Frontiers in Microbiology* 2020; **11**: 2108.
50. Ma L, Caulfield A, Dewan KK, Harvill ET. Pertactin-deficient Bordetella pertussis, vaccine-driven evolution, and reemergence of pertussis. *Emerging infectious diseases* 2021; **27**(6): 1561.
51. da Silva Antunes R, Babor M, Carpenter C, et al. Th1/Th17 polarization persists following whole-cell pertussis vaccination despite repeated acellular boosters. *The Journal of clinical investigation* 2018; **128**(9): 3853-65.
52. Ross PJ, Sutton CE, Higgins S, et al. Relative contribution of Th1 and Th17 cells in adaptive immunity to Bordetella pertussis: towards the rational design of an improved acellular pertussis vaccine. *PLoS pathogens* 2013; **9**(4): e1003264.
53. Higgs R, Higgins S, Ross P, Mills K. Immunity to the respiratory pathogen Bordetella pertussis. *Mucosal immunology* 2012; **5**(5): 485-500.
54. Warfel JM, Zimmerman LI, Merkel TJ. Acellular pertussis vaccines protect against disease but fail to prevent infection and transmission in a nonhuman primate model. *Proceedings of the National Academy of Sciences* 2014; **111**(2): 787-92.
55. McCarthy KN, Hone S, McLoughlin RM, Mills KH. IL-17 and IFN-γ–producing Respiratory Tissue-Resident Memory CD4 T Cells Persist for Decades in Adults Immunized as Children With Whole-Cell Pertussis Vaccines. *The Journal of Infectious Diseases* 2024: jiae034.
56. Kapil P, Merkel TJ. Pertussis vaccines and protective immunity. *Current opinion in immunology* 2019; **59**: 72-8.
57. Dagan R, Juergens C, Trammel J, et al. Efficacy of 13-valent pneumococcal conjugate vaccine (PCV13) versus that of 7-valent PCV (PCV7) against nasopharyngeal colonization of antibiotic-nonsusceptible Streptococcus pneumoniae. *The Journal of Infectious Diseases* 2015; **211**(7): 1144-53.
58. Russell MW, Moldoveanu Z, Ogra PL, Mestecky J. Mucosal immunity in COVID-19: a neglected but critical aspect of SARS-CoV-2 infection. *Frontiers in immunology* 2020; **11**: 611337.

59. Lavelle EC, Ward RW. Mucosal vaccines—fortifying the frontiers. *Nature Reviews Immunology* 2022; **22**(4): 236-50.
60. Hoft DF, Lottenbach KR, Blazevic A, et al. Comparisons of the humoral and cellular immune responses induced by live attenuated influenza vaccine and inactivated influenza vaccine in adults. *Clinical and Vaccine Immunology* 2017; **24**(1): e00414-16.
61. Pollard AJ, Frasch C. Development of natural immunity to Neisseria meningitidis. *Vaccine* 2001; **19**(11-12): 1327-46.
62. Wu F, Zhao S, Yu B, et al. A new coronavirus associated with human respiratory disease in China. *Nature* 2020; **579**(7798): 265-9.
63. Huang C, Wang Y, Li X, et al. Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. *The lancet* 2020; **395**(10223): 497-506.
64. Kim G-U, Kim M-J, Ra SH, et al. Clinical characteristics of asymptomatic and symptomatic patients with mild COVID-19. *Clinical microbiology and infection* 2020; **26**(7): 948. e1-. e3.
65. Mizrahi B, Shilo S, Rossman H, et al. Longitudinal symptom dynamics of COVID-19 infection. *Nature communications* 2020; **11**(1): 6208.
66. Raveendran A, Jayadevan R, Sashidharan S. Long COVID: an overview. *Diabetes & Metabolic Syndrome: Clinical Research & Reviews* 2021; **15**(3): 869-75.
67. Carabelli AM, Peacock TP, Thorne LG, et al. SARS-CoV-2 variant biology: immune escape, transmission and fitness. *Nature Reviews Microbiology* 2023; **21**(3): 162-77.
68. Hillary VE, Ceasar SA. An update on COVID-19: SARS-CoV-2 variants, antiviral drugs, and vaccines. *Heliyon* 2023; **9**(3).
69. Cherry JD, Tan T, Wirsing von König C-H, et al. Clinical definitions of pertussis: Summary of a Global Pertussis Initiative roundtable meeting, February 2011. *Clinical Infectious Diseases* 2012; **54**(12): 1756-64.
70. ECDC. Factsheet about pertussis. 28 Nov 2023 2023. https://www.ecdc.europa.eu/en/pertussis/facts (accessed 24 July 2024).
71. Pluijmakers A, de Melker H. The National Immunisation Programme in the Netherlands. Surveillance and developments in 2022-2023. 2023.
72. Current figures on whooping cough in the Netherlands. 20 Nov 2024 2024. https://www.rivm.nl/en/whooping-cough/current-figures-on-whooping-cough-in-netherlands (accessed 12 Dec 2024).
73. Khalil A, Samara A, Campbell H, Ladhani SN, Amirthalingam G. Recent increase in infant pertussis cases in Europe and the critical importance of antenatal immunizations: We must do better… now. *International Journal of Infectious Diseases* 2024; **146**: 107148.
74. ECDC. Increase of pertussis cases in the EU/EEA. 2024.
75. Torres RS, Santos TZ, Torres RA, et al. Resurgence of pertussis at the age of vaccination: clinical, epidemiological, and molecular aspects. *Jornal de pediatria* 2015; **91**(4): 333-8.
76. Fine P, Eames K, Heymann DL. "Herd immunity": a rough guide. *Clinical infectious diseases* 2011; **52**(7): 911-6.
77. EA van Lier JH, M Knijff, M Westra, et al. Vaccination coverage National Immunisation Programme in the Netherlands. Reporting year 2024: RIVM, 2024.
78. Guiso N, Meade BD, von König CHW. Pertussis vaccines: the first hundred years. *Vaccine* 2020; **38**(5): 1271-6.
79. Domenech de Cellès M, Rohani P. Pertussis vaccines, epidemiology and evolution. *Nature Reviews Microbiology* 2024: 1-14.
80. Saso A, Kampmann B. Maternal immunization: nature meets nurture. *Frontiers in Microbiology* 2020; **11**: 1499.

Section I: SARS-CoV-2

Chapter 2

Mucosal immunity to SARS-CoV-2 infection

Janeri Fröberg[1,2*] and Dimitri A. Diavatopoulos[1,2#]

[1.] Radboud university medical center, Radboud Institute for Molecular Life Sciences, Laboratory of Medical Immunology, Section Paediatric Infectious Diseases, Nijmegen, 6525 GA Nijmegen, The Netherlands

[2.] Radboud Center for Infectious Diseases, Radboudumc, Nijmegen

Curr Opin Infect Dis. 2021 Jun 1;34(3):181-186. doi: 10.1097/QCO.0000000000000724.

Abstract

Purpose of review

Despite its crucial role in protection against viral infections, mucosal immunity has been largely understudied in the context of COVID-19. This review outlines the current evidence about the role of mucosal immune responses in clearance of SARS-CoV-2 infection, as well as potential mucosal mechanisms of protection against (re-)infection.

Recent findings

The ACE-2 cellular entry receptor for SARS-CoV-2 is most highly expressed in the upper respiratory tract (URT) and most SARS-CoV-2 shedding occurs from the upper respiratory tract. Viral shedding peaks early during infection around the onset of symptoms, before dropping rapidly in most individuals within 7 days of symptom onset, suggesting mucosal inhibition of viral infection. Serum and mucosal IgG and IgM responses were found to be strongly correlated in infected patients, whereas correlations were much weaker for IgA. Mucosal IgA responses have been detected in infected cases in the absence of serum antibody responses, with mucosal antibody levels correlating strongly with virus neutralization. Bulk and single-cell RNA sequencing analysis of nasopharyngeal swabs and bronchoalveolar lavage samples of COVID-19 patients revealed the induction of mucosal chemokine and cytokine genes, complement pathways, JAK/STAT signaling and cytotoxic T cells.

Summary

Although most clinical studies focus on antibodies and cellular immunity in peripheral blood, mucosal immune responses in the respiratory tract play a key role in early restriction of viral replication and the clearance of SARS-CoV-2. Identification of mucosal biomarkers associated with viral clearance will allow monitoring of infection-induced immunity. Further studies are needed to understand how the systemic immunological endpoints measured in vaccination studies translate to mucosal protection against SARS-CoV-2 infection.

Introduction

Since the beginning of 2020, the world has been severely impacted by the COVID-19 pandemic, caused by severe acute respiratory syndrome virus 2 (SARS-CoV-2), a newly emerged coronavirus. Coronavirus disease is characterized by a large spectrum of clinical symptoms. Approximately 20% of infected cases develop severe or critical COVID-19[1,2]. Severe and critical COVID-19 is associated with respiratory failure, acute respiratory distress syndrome (ARDS), sepsis and septic shock, thromboembolism, multiorgan failures and other complications[2]. Several risk factors have been identified for severe morbidity of COVID-19 and/or mortality, including older age, obesity, high blood pressure and diabetes (reviewed elsewhere[3]). The majority of individuals infected with SARS-CoV-2 develop no or mild symptoms (40%) or moderate symptoms (40%)[2]. Young age in particular is an important determinant for mild and asymptomatic infection. Mild or moderate COVID-19 is characterized by flu-like symptoms, respiratory symptoms, gastro-intestinal symptoms and loss of smell[4-8]. Cognitive symptoms may also develop, including dizziness, confusion and memory loss[8]. Around 10% of mild cases develop what is now known as 'long COVID-19', characterized by longer-term sequelae[9].

Viral replication and shedding

Viral infectious dose and the duration and frequency of exposure are key determinants in the establishment of SARS-CoV-2 infection[10]. It is likely that many - if not most - transmission events of SARS-CoV-2, i.e. the transfer of virus particles between individuals, will not lead to establishment of infection. For instance, virus particles may get trapped in the mucous layer that lines the respiratory epithelium, a complex gel-like matrix of mediators containing e.g. mucins and glycans, and subsequently removed by mucociliary clearance[11]. Having overcome the mucous barrier, virus particles attach to host cells through binding of the viral spike protein to the cellular entry receptor angiotensin-converting enzyme 2 (ACE-2)[12]. Facilitated by host factors such as the transmembrane serine protease TMPRSS2[12], virus particles are then internalized and fusion occurs at the cellular or endosomal membrane. Viral genomic RNA is then released and viral replication initiates. The replication cycle of SARS-CoV-2 has been estimated at approximately 10 hrs[13], after which viral progeny is released by exocytosis[14]. Although the expression level and tissue distribution of ACE-2 and TMPRSS2 vary with genetic factors, sex, age and comorbidities like obesity[15,16], they are mostly highly expressed in the upper respiratory tract (URT)[17,18]. The salivary glands also act as a viral repository[19,20]. Thus, initial viral replication and shedding mostly takes place in the URT, and precedes viral replication in the lower respiratory tract (LRT)[1]. Once the virus reaches the

LRT, pulmonary cells are infected and patients may become viremic. The route of the virus and the timing of viral replication, first in the URT and later in the LRT, is reflected in the clinical presentation; URT symptoms including congestion, sore throat and loss of taste precede LRT symptoms such as dyspnea[1,21]. Investigations on transmission to close contacts indicate that shedding of SARS-CoV-2 viral particles is highest in the URT at the first stage of infection and can already occur during the incubation period, i.e. the time between exposure and symptom onset, known as the presymptomatic period[2]. This is underlined by research looking at the viral load dynamics, which found that viral load peaks approximately two days before symptom onset after which it declines[1,22,23]. Predictive modeling suggests that up to 80% of transmission occurs prior to symptom onset[24].

Role of mucosal immunity in viral clearance and protection against re-infection

Given that viral loads decrease rapidly after onset of (mostly) URT symptoms and that most infections with SARS-CoV-2 remain mild or asymptomatic, mucosal immune responses presumably play a central role in infection clearance. Despite this, surprisingly few studies have examined mucosal immunity in the context of SARS-CoV-2. This review outlines the current evidence about the role of human mucosal immune responses in clearance of SARS-CoV-2 infection, as well as the role of human infection- and vaccination-induced mucosal immunity in protection against re-infection.

Mucosal immune response to infection

To sustain essential respiratory function, the mucosal immune system in the respiratory tract must strike a careful balance between minimizing inflammation-induced tissue damage and acting adequately on threats such as SARS-CoV-2 infection. Although there have been many publications on profiling immune response dynamics in COVID-19 patients, including advanced single-cell analysis methods, virtually all of these studies focused on peripheral blood. Nonetheless, there have been some attempts to study local responses in the respiratory tract, albeit generally with low patient numbers. Single cell (sc) and bulk RNA sequencing of nasopharyngeal swabs and bronchoalveolar lavage samples - performed on a single timepoint - identified various genes and cell types associated with COVID-19 and severe disease, and show that cellular responses in the lungs differ from those in the URT. In nasopharyngeal swab samples, COVID-19 patients expressed gene sets involved in cytokine-cytokine interaction, complement pathways and JAK/

STAT signaling. These gene sets were found to be more highly expressed in severe/critical patients[25]. Cellular interactions between epithelial and immune cells were also linked to development of severe symptoms, reflected by increased activation of inflammatory macrophages and cytotoxic T-cells[26]. In the bronchoalveolar lavage fluid, a high expression of cytokines, chemokines, and antiviral interferon-stimulated genes[27] was found, as well as chemokine receptors, suggestive of macrophage and neutrophil recruitment[28]. scRNA-seq found that severe COVID-19 patients had CD8+ T-cell populations that were less expanded and phenotypically heterogeneous, while mild COVID-19 patients had higher CD8+ T-cell counts, with features of tissue residence[29]. These results suggest that during severe disease, inflammatory processes are dysregulated, leading to recruitment of inflammatory monocytes and neutrophils. In contrast, in mild cases, virus-specific T-cells are recruited and – presumably - able to more effectively control viral replication. It should be noted though that none of these studies examined temporal changes of the cellular responses in relation to viral infection kinetics.

Although mucosal B-cells were not identified in the scRNA-seq analysis, the production of mucosal antibodies through B-cell activation remains a key component of the antiviral response. In serum, virus-specific IgM can be detected approximately 7 days post symptom onset, after which class switching occurs and specific IgA and IgG are detected. IgG shows the strongest response and is present in high concentrations in convalescent plasma, while the IgA response is more transient[30,31]. The persistence of infection-induced serum antibodies ranges from only one month to at least 6 months[30-33], but it is not yet clear how this translates to mucosal persistence. There have been some studies focusing on mucosal antibodies. Mucosal IgM and IgG responses in saliva generally correlated well with those in serum[30,34]. In contrast, mucosal IgA showed a significantly weaker correlation with serum IgA[30], suggesting distinct regulatory mechanisms. A limited number of studies have examined nasal fluid, which can be obtained by nasal wash or through nasal absorption. A study examining nasal fluid and serum found that infected healthcare workers who failed to develop serum antibody responses against SARS-CoV-2 still developed mucosal IgA responses[35]. Although this study requires further validation, it is tempting to speculate that (mild) infection may not always induce systemic responses but can still activate local immunity. Another pre-print study found an inverse correlation between serum and nasal fluid antibody isotypes, with higher mucosal IgA levels correlated with lower IgG. Of note, viral neutralization of nasal fluid samples correlated most strongly with nasal IgA, while for serum strongest correlations with neutralization were observed for IgG[36].

Factors influencing mucosal immune responses

Both genetic and non-genetic factors may contribute to phenotypic variation in the mucosal immune response to SARS-CoV-2 infection. Non-genetic factors may include pre-existing cross-reactive memory to seasonal coronaviruses, SARS-CoV-1 and MERS has been described[37], in particular for the nucleocapsid (N)-protein. This applies to both cross-reactive T-cells[38] and antibodies[39]. Whether pre-existing memory may promote or hinder clearance is not yet known. It has also been hypothesized that previous vaccinations may contribute to protection against COVID-19 due to shared epitopes. For instance, diphtheria, tetanus, and pertussis (DTP) vaccines are predicted to share CD4 and CD8 T-cell epitopes and B-cell epitopes to SARS-CoV-2 spike protein[40]. Similarly, the BCG vaccine has been described to contain similar 9-amino acid sequences with SARS-CoV-2, which were related to HLA class I molecules[41]. However, it remains to be seen whether these small changes translate into partial protection or not. Another contributing factor may be the microbiome. Although the direct role of the microbiome on the host response is not yet investigated, several studies suggest that disruption of the gut and nasal microbiome results in more severe COVID-19 disease[42,43].

Vaccine-induced mucosal immunity

At the moment of writing, two COVID-19 vaccines have been authorized for emergency use, i.e. Pfizer-BioNTech BNT162b2 and Moderna mRNA-1273. Both vaccines contain mRNA molecules encoding the viral spike protein. The primary efficacy endpoint for all COVID-19 vaccines thus far has been protection against disease[44,45]. Although the phase 3 studies were not powered to detect differences in asymptomatic or subclinical infections, a key question is how well these vaccines provide herd immunity. Although larger studies are now ongoing that will hopefully soon be able to answer this question, protection against subclinical infection – even against homologous virus strains - will likely wane sooner than protection against disease, given that most non-replicating vaccines do not offer life-long protection[46]. It is therefore crucial to define the immunological correlates of protection for disease as well as for infection. Whereas all clinical studies to date have primarily focused on serum neutralizing antibodies as immunogenicity endpoint[47,48], it is not yet known if and how the new SARS-CoV-2 vaccines elicit durable mucosal immunity. Both the mRNA vaccines as well as the adenovirus vector vaccines currently in phase 3 trials induce strong CD4 T-cell responses in blood[49-51]. A similar induction has been observed for CD8 T-cells in blood[49,50]. An important question is whether parenteral COVID-19 vaccines will be able to induce tissue resident memory T cells (Trm) in the human respiratory tract. The ability to do so may vary between vaccine formulations. For instance, vaccination with acellular

subunit pertussis vaccines did not induce Trm cells in the lungs, whereas Trm cells were observed after vaccination with a whole cell pertussis vaccin[52].

Conclusion

Although severe SARS-CoV-2 infection can have far-reaching systemic effects, infection begins at the mucosal surface and - in most cases - remains restricted to the respiratory tract. It is therefore essential to not only focus on immunological endpoints in blood, but also to establish *mucosal* immunological correlates of protection. Two distinct immunological processes can be identified that both contribute to protection against infection. Firstly, complete prevention of infection is likely solely mediated by sufficiently high concentrations of neutralizing mucosal antibodies that block attachment of virus particles to host cells, inducing sterilizing immunity. Polymeric mucosal IgA and IgM may efficiently trap virus particles to facilitate their removal by cilial beating. Mucosal antibody responses are typified by high production of dimeric secretory IgA (sIgA). sIgA differs from monomeric serum IgA in its molecular form and effector functions[53,54]; with monomeric IgA not effectively being transported to the mucosal surface[54]. sIgA is highly effective against various viruses, can neutralize virus particles within epithelial cells[54], and has anti-inflammatory properties[53,55]. This is fundamentally different from the systemic antibody environment, which is dominated by IgG and is typically pro-inflammatory[56-58].

In case virus particles overcome this 'antibody barrier', either by waning of immunity, by exposure to high numbers of virus particles, or by suboptimal binding/neutralization due to e.g. mutations in the spike protein, antibodies may not fully block viral attachment to ACE-2, resulting in viral replication. In this case, additional processes will be required for clearance of viral infection. Antibodies may contribute to this process by binding to viral antigens on infected cells and induce antibody-dependent cellular cytotoxicity (ADCC) or other Fc-mediated effector functions[59]. Additionally, cytotoxic T-cells may kill infected cells through the release of granzymes, which induce apoptosis, and perforin that punches holes in the cell membrane.

There are both challenges and advantages to measuring mucosal immunity. Challenges are related to sampling standardization and validation and to availability of robust assays to measure mucosal immunity. Historically, there have been difficulties in standardization of mucosal sampling and dilution

effects are frequently observed with e.g. saliva. Furthermore, mucosal antibody concentrations are significantly lower than in serum, necessitating sensitive detection assays. However, many of these challenges can be or have already been overcome. For instance, precision sampling of undiluted nasal mucosal lining fluid by nasosorption allows robust measurements of antibodies [60]. Highly sensitive and specific detection methods have been established to measure antibodies to SARS-CoV-2[61]. Although mucosal antibodies are a more likely candidate for correlates of protection, mucosal cellular immunity may also be studied via sampling of mucosal tissue via swabs, curettage or brushing. Whilst cell numbers are typically low in these samples, advances in single-cell analysis methods allow unprecedented in-depth analysis. Importantly, all of these sampling techniques are non-invasive and can therefore be performed in people of all ages, including children. Expanding our knowledge on mucosal immunity to SARS-CoV-2 is essential to understand what causes protection against infection, and to properly monitor the immunological effects of vaccination. The ease of collecting mucosal samples provides the opportunity to analyse immune responses in a non-invasive manner at multiple time points. Mucosal findings will need to be bridged with existing data on systemic immunological endpoints, but recent advances in both sampling and analysis methods pave the way for mucosal correlates of protection.

References

1. Wölfel R, Corman VM, Guggemos W, et al. Virological assessment of hospitalized patients with COVID-2019. *Nature* 2020.
2. Clinical management of COVID-19. WHO.int: World Health Organization, 2020.
3. Chidambaram V, Tun NL, Haque WZ, et al. Factors associated with disease severity and mortality among patients with COVID-19: A systematic review and meta-analysis. *PloS one* 2020; **15**(11): e0241541.
4. Huang C, Wang Y, Li X, et al. Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. *Lancet* 2020; **395**(10223): 497-506.
5. Bello-Chavolla OY, Antonio-Villa NE, Vargas-Vázquez A, Fermín-Martínez CA, Márquez-Salinas A, Bahena-López JP. Profiling pre-symptomatic and asymptomatic cases with confirmed SARS-CoV-2 infection in Mexico City. *medRxiv* 2020.
6. Kim G-u, Kim M-J, Ra SH, et al. Clinical characteristics of asymptomatic and symptomatic patients with mild COVID-19. *Clinical Microbiology and Infection* 2020.
7. Hannum ME, Ramirez VA, Lipson SJ, et al. Objective sensory testing methods reveal a higher prevalence of olfactory loss in COVID-19 positive patients compared to subjective methods: A systematic review and meta-analysis. *medRxiv* 2020.
8. Mizrahi B, Shilo S, Rossman H, et al. Longitudinal symptom dynamics of COVID-19 infection. *Nature Communications* 2020; **11**(1): 6208.
9. Greenhalgh T, Knight M, Buxton M, Husain L. Management of post-acute covid-19 in primary care. *Bmj* 2020; **370**.
10. Calisti R. SARS-CoV-2: exposure to high external doses as determinants of higher viral loads and of increased risk for COVID-19. A systematic review of the literature. *Epidemiol Prev* 2020; **44**(5-6 Suppl 2): 152-9.
11. Chatterjee M, van Putten JPM, Strijbis K. Defensive Properties of Mucin Glycoproteins during Respiratory Infections-Relevance for SARS-CoV-2. *mBio* 2020; **11**(6).
12. Hoffmann M, Kleine-Weber H, Schroeder S, et al. SARS-CoV-2 Cell Entry Depends on ACE2 and TMPRSS2 and Is Blocked by a Clinically Proven Protease Inhibitor. *Cell* 2020; **181**(2): 271-80.e8.
13. Bar-On YM, Flamholz A, Phillips R, Milo R. SARS-CoV-2 (COVID-19) by the numbers. *Elife* 2020; **9**.
14. Ghosh S, Dellibovi-Ragheb TA, Kerviel A, et al. β-Coronaviruses Use Lysosomes for Egress Instead of the Biosynthetic Secretory Pathway. *Cell* 2020; **183**(6): 1520-35.e14.
15. Ortiz-Fernández L, Sawalha AH. Genetic variability in the expression of the SARS-CoV-2 host cell entry factors across populations. *Genes & Immunity* 2020; **21**(4): 269-72.
16. Bourgonje AR, Abdulle AE, Timens W, et al. Angiotensin-converting enzyme 2 (ACE2), SARS-CoV-2 and the pathophysiology of coronavirus disease 2019 (COVID-19). *The Journal of pathology* 2020; **251**(3): 228-48.
17. Sungnak W, Huang N, Bécavin C, et al. SARS-CoV-2 entry factors are highly expressed in nasal epithelial cells together with innate immune genes. *Nature medicine* 2020; **26**(5): 681-7.
18. Hou YJ, Okuda K, Edwards CE, et al. SARS-CoV-2 Reverse Genetics Reveals a Variable Infection Gradient in the Respiratory Tract. *Cell* 2020.
19. To KK-W, Tsang OT-Y, Yip CC-Y, et al. Consistent detection of 2019 novel coronavirus in saliva. *Clinical Infectious Diseases* 2020.

20. Xu J, Li Y, Gan F, Du Y, Yao Y. Salivary glands: potential reservoirs for COVID-19 asymptomatic infection. *Journal of Dental Research* 2020: 0022034520918518.
21. Kakodkar P, Kaka N, Baig MN. A Comprehensive Literature Review on the Clinical Presentation, and Management of the Pandemic Coronavirus Disease 2019 (COVID-19). *Cureus* 2020; **12**(4): e7560-e.
22. Benefield AE, Skrip LA, Clement A, Althouse RA, Chang S, Althouse BM. SARS-CoV-2 viral load peaks prior to symptom onset: a systematic review and individual-pooled analysis of coronavirus viral load from 66 studies. *medRxiv* 2020.
23. Lee S, Kim T, Lee E, et al. Clinical Course and Molecular Viral Shedding Among Asymptomatic and Symptomatic Patients With SARS-CoV-2 Infection in a Community Treatment Center in the Republic of Korea. *JAMA Intern Med* 2020.
24. Tindale LC, Stockdale JE, Coombe M, et al. Evidence for transmission of COVID-19 prior to symptom onset. *Elife* 2020; **9**: e57149.
25. Jain R, Ramaswamy S, Harilal D, et al. Host transcriptomic profiling of COVID-19 patients with mild, moderate, and severe clinical outcomes. *Computational and Structural Biotechnology Journal* 2020.
26. Chua RL, Lukassen S, Trump S, et al. COVID-19 severity correlates with airway epithelium–immune cell interactions identified by single-cell analysis. *Nature Biotechnology* 2020; **38**(8): 970-9.
27. Zhou Z, Ren L, Zhang L, et al. Heightened innate immune responses in the respiratory tract of COVID-19 patients. *Cell Host & Microbe* 2020.
28. Xiong Y, Liu Y, Cao L, et al. Transcriptomic characteristics of bronchoalveolar lavage fluid and peripheral blood mononuclear cells in COVID-19 patients. *Emerging microbes & infections* 2020; **9**(1): 761-70.
29. Liao M, Liu Y, Yuan J, et al. Single-cell landscape of bronchoalveolar immune cells in patients with COVID-19. *Nature medicine* 2020: 1-3.
30. Isho B, Abe KT, Zuo M, et al. Persistence of serum and saliva antibody responses to SARS-CoV-2 spike antigens in COVID-19 patients. *Science immunology* 2020; **5**(52).
31. Seow J, Graham C, Merrick B, et al. Longitudinal observation and decline of neutralizing antibody responses in the three months following SARS-CoV-2 infection in humans. *Nature Microbiology* 2020; **5**(12): 1598-607.
32. Wajnberg A, Amanat F, Firpo A, et al. Robust neutralizing antibodies to SARS-CoV-2 infection persist for months. *Science* 2020; **370**: 1227-30.
33. Bruni M, Cecatiello V, Diaz-Basabe A, et al. Persistence of anti-SARS-CoV-2 antibodies in non-hospitalized COVID-19 convalescent health care workers. *medRxiv* 2020.
34. Pisanic N, Randad PR, Kruczynski K, et al. COVID-19 serology at population scale: SARS-CoV-2-specific antibody responses in saliva. *Journal of Clinical Microbiology* 2020.
35. Cervia C, Nilsson J, Zurbuchen Y, et al. Systemic and mucosal antibody responses specific to SARS-CoV-2 during mild versus severe COVID-19. *Journal of Allergy and Clinical Immunology* 2020.
36. Butler SE, Crowley AR, Natarajan H, et al. Features and Functions of Systemic and Mucosal Humoral Immunity Among SARS-CoV-2 Convalescent Individuals. *medRxiv* 2020.
37. Altmann DM, Boyton RJ. SARS-CoV-2 T cell immunity: Specificity, function, durability, and role in protection. *Science Immunology* 2020; **5**.
38. Le Bert N, Tan AT, Kunasegaran K, et al. SARS-CoV-2-specific T cell immunity in cases of COVID-19 and SARS, and uninfected controls. *Nature* 2020; **584**(7821): 457-62.
39. Ng KW, Faulkner N, Cornish GH, et al. Preexisting and de novo humoral immunity to SARS-CoV-2 in humans. *Science* 2020; **370**: 1339-43.
40. Reche PA. Potential Cross-Reactive Immunity to SARS-CoV-2 From Common Human Pathogens and Vaccines. *Frontiers in Immunology* 2020; **11**(2694).

41. Tomita Y, Sato R, Ikeda T, Sakagami T. BCG vaccine may generate cross-reactive T cells against SARS-CoV-2: In silico analyses and a hypothesis. *Vaccine* 2020; **38**(41): 6352-6.
42. Yeoh YK, Zuo T, Lui GC, et al. Gut microbiota composition reflects disease severity and dysfunctional immune responses in patients with COVID-19. *Gut* 2021.
43. Di Stadio A, Costantini C, Renga G, Pariano M, Ricci G, Romani L. The Microbiota/Host Immune System Interaction in the Nose to Protect from COVID-19. *Life (Basel)* 2020; **10**(12).
44. Polack FP, Thomas SJ, Kitchin N, et al. Safety and efficacy of the BNT162b2 mRNA covid-19 vaccine. *New England Journal of Medicine* 2020.
45. Voysey M, Clemens SAC, Madhi SA, et al. Safety and efficacy of the ChAdOx1 nCoV-19 vaccine (AZD1222) against SARS-CoV-2: an interim analysis of four randomised controlled trials in Brazil, South Africa, and the UK. *The Lancet* 2020.
46. Klein NP, Bartlett J, Rowhani-Rahbar A, Fireman B, Baxter R. Waning protection after fifth dose of acellular pertussis vaccine in children. *New England Journal of Medicine* 2012; **367**(11): 1012-9.
47. Walsh EE, Frenck Jr RW, Falsey AR, et al. Safety and immunogenicity of two RNA-based Covid-19 vaccine candidates. *New England Journal of Medicine* 2020.
48. Mulligan MJ, Lyke KE, Kitchin N, et al. Phase I/II study of COVID-19 RNA vaccine BNT162b1 in adults. *Nature* 2020; **586**(7830): 589-93.
49. Ewer KJ, Barrett JR, Belij-Rammerstorfer S, et al. T cell and antibody responses induced by a single dose of ChAdOx1 nCoV-19 (AZD1222) vaccine in a phase 1/2 clinical trial. *Nature Medicine* 2020.
50. Sahin U, Muik A, Derhovanessian E, et al. COVID-19 vaccine BNT162b1 elicits human antibody and TH1 T cell responses. *Nature* 2020; **586**(7830): 594-9.
51. Anderson EJ, Rouphael NG, Widge AT, et al. Safety and Immunogenicity of SARS-CoV-2 mRNA-1273 Vaccine in Older Adults. *New England Journal of Medicine* 2020; **383**(25): 2427-38.
52. Wilk MM, Borkner L, Misiak A, Curham L, Allen AC, Mills KHG. Immunization with whole cell but not acellular pertussis vaccines primes CD4 T(RM) cells that sustain protective immunity against nasal colonization with Bordetella pertussis. *Emerg Microbes Infect* 2019; **8**(1): 169-85.
53. Russell MW, Mestecky J, Strober W, Lambrecht BN, Kelsall BL, Cheroutre H. Overview: the mucosal immune system. Mucosal immunology: Elsevier; 2015: 3-8.
54. Russell MW, Moldoveanu Z, Ogra PL, Mestecky J. Mucosal immunity in COVID-19: A neglected but critical aspect of SARS-CoV-2 infection. *Frontiers in Immunology* 2020; **11**: 3221.
55. Russell MW, Kilian M, Mantis NJ, Corthésy B. Biological activities of IgA. Mucosal immunology: Elsevier; 2015: 429-54.
56. Vabret N, Britton GJ, Gruber C, et al. Immunology of COVID-19: current state of the science. *Immunity* 2020.
57. Zhang B, Zhou X, Zhu C, et al. Immune phenotyping based on neutrophil-to-lymphocyte ratio and IgG predicts disease severity and outcome for patients with COVID-19. *medRxiv* 2020.
58. Zhao J, Yuan Q, Wang H, et al. Antibody responses to SARS-CoV-2 in patients of novel coronavirus disease 2019. *Clin Infect Dis* 2020.
59. Vanderven HA, Kent SJ. The protective potential of Fc-mediated antibody functions against influenza virus and other viral pathogens. *Immunol Cell Biol* 2020; **98**(4): 253-63.
60. Thwaites RS, Ito K, Chingono JMS, et al. Nasosorption as a Minimally Invasive Sampling Procedure: Mucosal Viral Load and Inflammation in Primary RSV Bronchiolitis. *J Infect Dis* 2017; **215**(8): 1240-4.
61. den Hartog G, Schepp RM, Kuijer M, et al. SARS-CoV-2–Specific Antibody Detection for Seroepidemiology: A Multiplex Analysis Approach Accounting for Accurate Seroprevalence. *The Journal of Infectious Diseases* 2020; **222**(9): 1452-61.

Chapter 3

SARS-CoV-2 mucosal antibody development and persistence and their relation to viral load and COVID-19 symptoms

Janeri Fröberg[1,2*], Joshua Gillard[1,2,3*], Ria Philipsen[1,2,4], Kjerstin Lanke[2], Joyce Rust[1,2,4], Diana van Tuijl[1,2,4], Karina Teelen[2], Teun Bousema[2], Elles Simonetti[1,2], Christa E. van der Gaast – de Jongh[1,2], Mariska Bos[1,2], Frank J. van Kuppeveld[5], Berend-Jan Bosch[5], Marrigje Nabuurs- Franssen[6], Nannet van der Geest-Blankert[7], Charlotte van Daal[7], Martijn A. Huynen[3], Marien I. de Jonge[1,2], Dimitri A. Diavatopoulos[1,2]

*contributed equally

1. Radboud University Medical Centre, Radboud Institute for Molecular Life Sciences, Laboratory of Medical Immunology, Section Paediatric Infectious Diseases, Nijmegen, 6525 GA Nijmegen, The Netherlands
2. Radboud Center for Infectious Diseases, Radboudumc, Nijmegen
3. Centre for molecular and Biomolecular Informatics, Radboud Institute for Molecular Life Sciences, Radboud University Medical Centre Nijmegen, 6525 GA Nijmegen, The Netherlands
4. RTC CS Radboud Technology Center Clinical Studies, Radboudumc, Nijmegen
5. Utrecht University, Faculty of Veterinary Medicine, Department of Biomolecular Health Sciences, Division Infectious Diseases and Immunology, Utrecht, The Netherlands
6. Department of Medical Microbiology and Infectious Diseases, Canisius Wilhelmina hospital, Nijmegen
7. Department of Occupational Health, Radboud University Medical Centre

Nat Commun. 12, 5621 (2021). doi:10.1038/s41467-021-25949-x

Abstract

Although serological studies have shown that antibodies against SARS-CoV-2 play an important role in protection against (re)infection, the dynamics of mucosal antibodies during primary infection and their potential impact on viral load and the resolution of disease symptoms remain unclear. During the first pandemic wave, we assessed the longitudinal nasal antibody response in index cases with mild COVID-19 and their household contacts. Nasal and serum antibody responses were analysed for up to nine months. Higher nasal receptor binding domain and Spike protein-specific antibody levels at study inclusion were associated with lower viral load. Older age was correlated with more frequent COVID-19 related symptoms. receptor binding domain and Spike protein-specific mucosal antibodies were associated with the resolution of systemic but not respiratory symptoms. Finally, receptor binding domain and Spike protein-specific mucosal antibodies remained elevated up to nine months after symptom onset.

Introduction

The rapid spread of SARS-CoV-2 in populations is attributed to several aspects, i.e. the route of transmission via respiratory droplets, rapid viral replication and shedding from the upper respiratory tract[1], early infectiousness with a peak viral load before onset of symptoms[2,3], and a high frequency of mild and asymptomatic infections[3-7]. These aspects have complicated effective control of SARS-CoV-2 spread, as containment strategies have primarily been dependent on symptomatic case detection[8,9]. Indeed, pre-symptomatic carriers are likely an important driver of community-based viral transmission [8,10]. Transmission within households contributes significantly to the spread of SARS-CoV-2 in communities, as close contact within households facilitates early-onset transmission of the virus[11-13].

Antibodies are considered to play a crucial role in protection against viral (re)infection. The SARS-CoV-2 virus enters human cells following binding to the ACE2 receptor with the receptor binding domain (RBD) of the viral spike (S) protein. Serological studies have shown that antibodies directed against the spike protein and RBD region are capable of neutralizing viral binding and entry, and vaccines inducing immunity against the S protein have been shown to be efficacious[1,3,14-17]. Infection with SARS-CoV-2 also induces humoral responses against the viral nucleocapsid (N) protein. The N protein of SARS-CoV-2 shares approximately 80% of its amino acid sequence with SARS-CoV-1 and other seasonal coronaviruses[18]. Therefore, pre-existing immunity against the N-protein may play a protective role during infection[18,19].

Studies investigating antibody response dynamics in mild cases have demonstrated the development of serum antibodies against SARS-CoV-2 approximately 10-15 days post symptom onset[1,20]. An understudied aspect of the immune response to SARS-CoV-2 infection is the magnitude, kinetics, and persistence of mucosal antibodies. Animal studies of other human coronaviruses have shown that mucosal antibodies play a key role in the reduction of viral load and may contribute to protection following re-exposure. Intranasal vaccination also induces strong and protective mucosal immune responses[21,22]. Viral entry and replication of SARS-CoV-2 first occurs in the upper respiratory tract, where ACE2 receptor expression is very high[23,24].

Together, these findings suggest that nasal antibodies may play a key role early in the infection. The composition of mucosal antibodies differs from serum, particularly with regards to secretory IgA (sIgA) and IgM (sIgM). sIgA is primarily dimeric, whereas serum IgA is predominately monomeric, which may affect both viral neutralization and the inflammatory response[25-27].

To obtain a comprehensive view on the development and persistence of mucosal antibodies following mild SARS-CoV-2 infection, we performed a prospective, observational household contact study in 50 households with at least one PCR confirmed case (index case) and two household members (contacts). We assessed the timing, magnitude and persistence of mucosal antibodies against SARS-CoV-2 antigens and examined their associations with viral load and COVID-19 related symptom development.

Based on PCR positivity and/or seropositivity, household contacts were classified into cases and non-cases. Baseline mucosal antibody levels were associated with variation in viral load. The development of COVID-19 symptoms during the 28-day follow-up period was analysed in relation to mucosal antibody dynamics. Our study provides new insights in mucosal antibody production during a primary mild SARS-CoV-2 infection and the longevity of antibodies in nasal fluid and serum.

Results

Cohort description and study design

The recruitment strategy for inclusion of households focused on healthcare workers with a PCR-confirmed infection who were in home isolation (index cases), with at least two participating household members. Between 26 March 2020 and 15 April 2020, i.e. during the first pandemic wave, we prospectively enrolled 50 index cases and 137 household members (**Figure 1a** and **1b**). Index cases were mostly female (76%), reflective of the gender distribution amongst healthcare workers, with a median age of 46 (IQR: 37-54). Consequently, household contacts were mostly male (61%) and younger, with a median age of 21 (IQR:13-46) (**Table S1**). An overview of the study design is shown in **Figure 1C**. Home visits were performed to collect naso- and oropharyngeal swab samples and nasal mucosal lining fluids (MLF) at study start (D0). Study participants self-sampled MLF on three subsequent timepoints as described in the Methods, and a serum sample was collected via fingerprick on day 28. Index cases were asked to report their first day of symptoms, and all participants completed a daily symptom survey during the 28-day follow-up to monitor symptom development (**Figure 1c**). Contacts were classified as cases or non-cases based on PCR and/or sero-positivity (see Methods). To analyse the persistence of nasal and serum antibodies, serum (N=100) and MLF (N=108) was collected from index and contact cases at nine months after study enrolment (**Figure 1a** and **1c**).

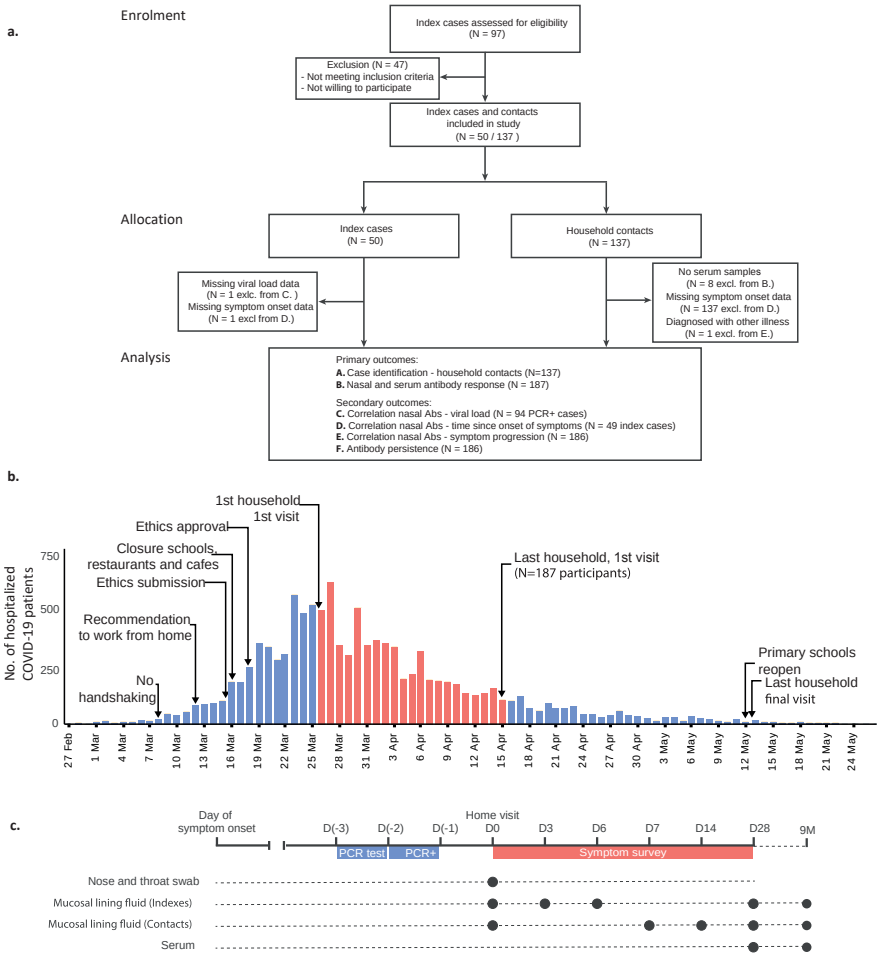


Figure 1. Flow diagram and study procedures. **a)** Flow diagram describing the recruitment of households, sample sizes, and study outcomes. We initially contacted 97 index cases that were tested positive for SARS-CoV-2. After exclusion of cases that did not meet the inclusion criteria or did not consent, 50 index cases and their household contacts (N=137) were recruited. Mucosal lining fluid (MLF) antibodies were analysed as a primary outcome in both indexes and household contacts. Secondary analyses (correlation of MLF antibodies with viral load and symptoms, serology, estimation of SARS-CoV-2 exposure) were performed. **b)** Study timeline, with respect to the number of hospitalizations due to COVID-19 over time and COVID-19 control measures in the Netherlands 60,61. The first home visit was conducted at the peak of hospitalizations at March 26, and the last visit was one day after the reopening of primary schools, at May 13. **c)** Overview of the study design and measurements. Home visits were initiated after the index was tested positive for SARS-CoV-2 by PCR, to collect naso- and oropharyngeal swabs for viral load determination as well as nasal MLF samples. Subsequent MLF samples were collected and stored by the participants, who also completed a daily symptom survey. At the end of the 28 day follow-up, blood samples were collected for serological analyses. A subset of cases (N= 108) were visited again nine months after enrolment. At this timepoint, a MLF and serum sample were taken.

High infection rate among household contacts

All participants were tested for SARS-CoV-2 infection at study day 0 by PCR on naso- and oropharyngeal swabs. Antibody levels in serum and MLF were measured using a fluorescent-bead-based multiplex immunoassay (MIA). IgG, IgA and IgM levels specific for S- N- and RBD- antigens were determined. To determine increases in antibody levels, we used 32 pre-pandemic serum control samples and 17 pre-pandemic MLF control samples. Antibody values were normalized by calculating the Log2-transformed antibody levels of study samples over the mean of the control samples. To evaluate the performance of the MIA, we performed a ROC analysis with the pre-pandemic controls as a negative control and the PCR positive samples as a positive control. For the RBD and S antigen, the MIA performed well for all antibodies in both MLF (AUC > 0.750) and serum (AUC > 0.915), with the S protein analysis in serum performing the best of the tested antigens (AUC> 0.970, **Figure S1a**). The MIA assay showed high reproducibility when two batches analysed nine months apart were compared, with Spearman correlations > 0.87 for all antibody/antigen combinations (**Figure S1b**). Based on the ROC results, serum anti-S IgA, IgM and IgG antibody responses on day 28 were selected as a measure to identify cases, in combination with the PCR analysis performed at study start.

Based on PCR positivity on day 0 and/or seropositivity against the S antigen on day 28, we identified 80 contact cases among the 137 household contacts (58.4%, **Table S1**).To examine potential age-related differences, we stratified household contacts into three age categories, i.e. <18y (n=46), 18-49y (n=54) and ≥50y (n=29). No age-related differences were observed with regards to the frequency of cases amongst household contacts, i.e. 57% for <18y, 58% for 18-49y and 61% for ≥50y. Of note, a large percentage of contacts was already PCR positive at study start, especially in the ≥50y age group (45.2%, see **Figure 2a**). Almost all PCR+ contact cases were seropositive (see **Table S2**). We also examined 'nasoconversion' against the S protein, which shows that 68% of the household contacts had become 'naso-positive' by day 28. Seropositivity at day 28 identified 32 additional cases among the PCR- household contacts (40%), of which 11 had also nasoconverted (34%, **Table S2**). Highest seroconversion among household contacts was observed for anti-S IgG and IgA, with lower seroconversion against N (**Figure 2a**). Of the index cases, 94% was still PCR positive at study start (**Figure 2b**). Of the index cases, 92% had 'nasoconverted' by day 28, most of whom were PCR+ and sero+ (**Table S2**). Similar to the contact cases, seropositivity rates were highest for S and lowest for N. IgG and IgA seropositivity rates were generally higher than IgM. (**Figure 2b**).

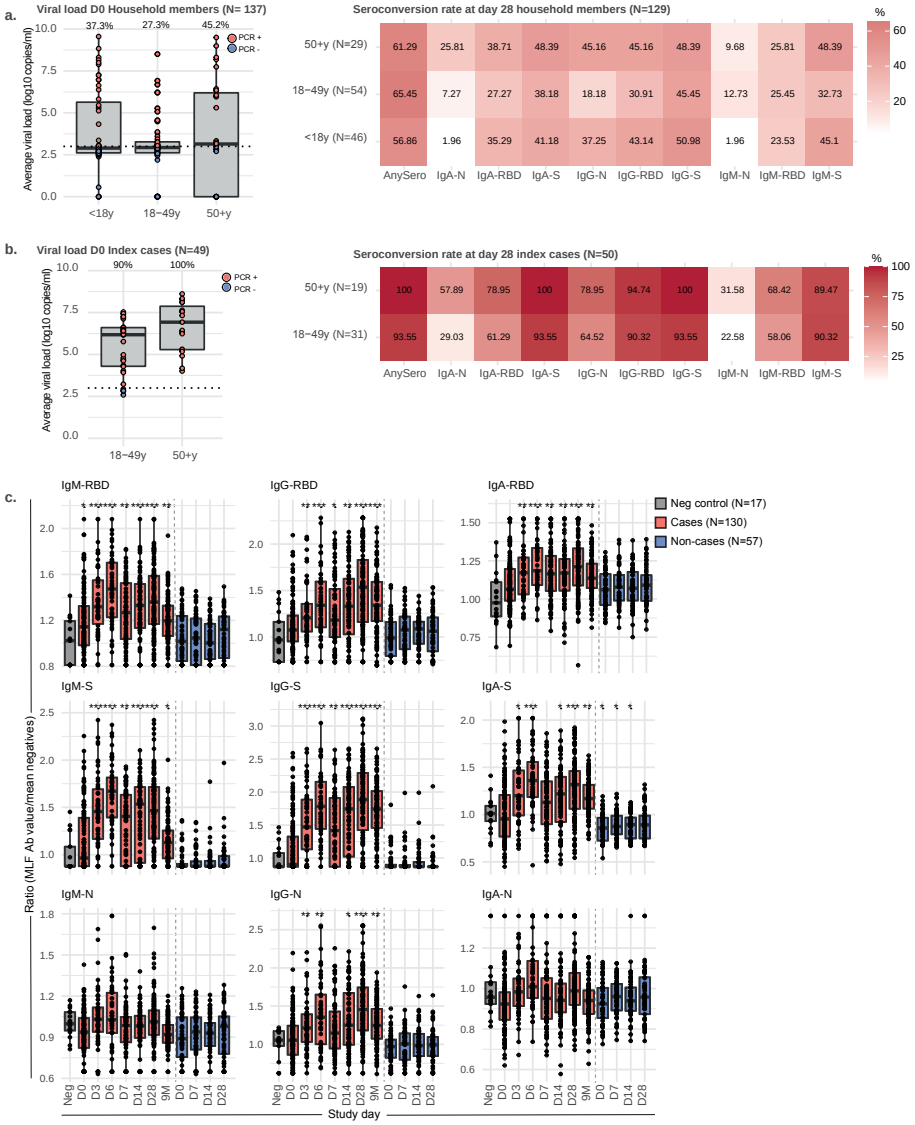


Figure 2. Mucosal antibodies against Spike increase after SARS-CoV-2 infection **a)** PCR and sero-positivity rate of household members (N=137), split into age groups. PCR positivity was defined as a Ct value<36, which corresponds to a viral load of at least 10^3 copies/ml extracted sample (dashed line).The boxplots display a median line, interquartile range (IQR) boxes, 1.5*IQR whiskers and individual data points (red=PCR positive, blue=PCR negative). The percentage of PCR positive individuals is depicted in the figure. Seroconversion was defined as an antibody titre that is higher than the mean + 2*standard deviation of the pre-pandemic control samples, and is given for each antigen and antibody isotype measurement, as well as for any single antibody measurement (anySero). **b)** PCR positivity and seroconversion rates of index cases (N=49), split into age groups. **c)** Mucosal antibody responses to RBD, S and N, plotted as a ratio to the pre-pandemic negative controls (n=17), per study

timepoint and split into cases (N= 130, red boxplots) and non-cases (N= 57, blue boxplots). Cases were defined with the PCR positivity on day 0 and seropositivity against the Spike protein on day 28. The boxplots display a median line, interquartile range (IQR) boxes, 1.5*IQR whiskers and individual data points. Pre-pandemic controls are presented in the grey boxplots for comparison (n=17). Values of each boxplot were compared with the negative controls with the two-sided Wilcoxon-signed rank test, * p <0.05, **p<0.01, *** p<0.001. There were no non-cases in the D3, D6 and 9M timepoint.

The magnitude of serum antibody levels in the index and contact cases did not differ between the three age-groups, except for anti-N IgA and IgG, which were significantly higher in the ≥50y olds (**Figure S2a**). To examine the correlation between nasal and serum antibody levels in cases, we focused on the day 28 and 9 month time points. For all antibody-antigen combinations, positive and significant correlations were observed between MLF and serum (**Figure S2b**).

Nasal antibodies against SARS-CoV-2 increase after infection and persist up to nine months

Next, we investigated nasal antibody response dynamics (**Figure 2c**). Day 0 and day 28 MLF samples were collected from both index cases as well as from all household contacts. MLF sampling time points for the index cases were chosen to capture the antibody response during the acute phase of infection, i.e. day 3 and 6. Because the infection status of the household contacts was not yet known at the moment of inclusion, MLF sampling time points from household contacts were selected to capture possible secondary infections, i.e. day 7 and 14. To examine antibody persistence, additional MLF and serum samples were collected from identified cases at 9 months.

By day 3 of the study, nasoconversion had already occurred for all antibody measurements, except for anti-N IgA and IgM. Although waning was observed between 28 days and nine months, all antibody isotypes against S and RBD as well as anti-N IgG remained significantly elevated **(Figure S3**). Thus, primary infection with SARS-CoV-2 induces a broad and persistent mucosal antibody response against Spike and RBD, whereas for Nucleocapsid protein the response was restricted to IgG.

Early nasal antibody production is correlated with lower viral load

Since participants were included at various stages of infection, there is significant variation in the viral load and mucosal antibody levels at the onset of the study, i.e. day 0. To examine potential relationships between antibody levels and viral load, we focused on day 0 for which we had paired viral load and mucosal antibody measurements. Correlation analysis indicated viral load was negatively correlated with anti-S and RBD mucosal antibodies, of which IgM-S showed the strongest

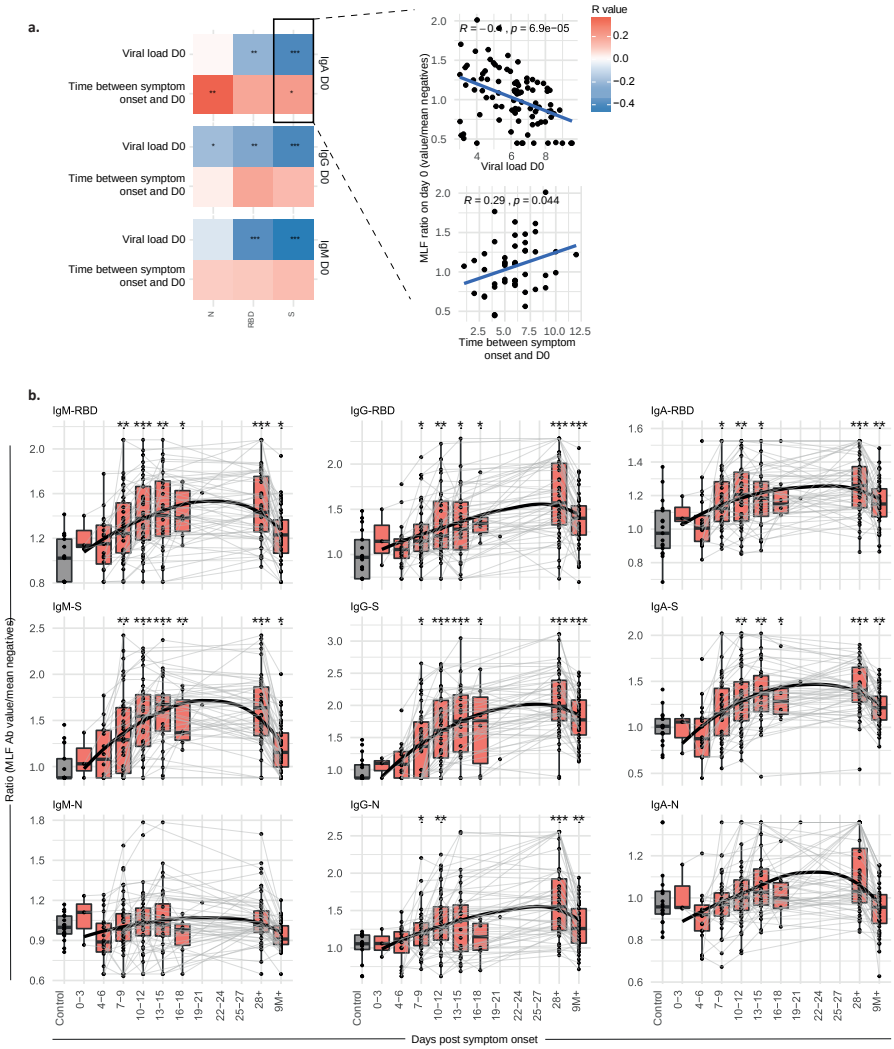


Figure 3. Mucosal antibody responses are correlated with viral load and persist up to nine months after symptom onset. **a)** IgM, IgG and IgA antibody responses against SARS-CoV-2 spike (S), receptor binding domain (RBD) or nucleocapsid (N) collected in mucosal lining fluid at study start were correlated with viral load at study start and time between symptom onset and study start. Two-sided Spearman correlations were calculated, * p <0.05, **p<0.01, *** p<0.001. The correlation plots of IgA-S are shown as an example, all correlation plots can be found in figure S4, together with the exact p-values of the correlations **b)** Longitudinal mucosal antibody responses to S, RBD and N, plotted as a ratio to mean of the pre-pandemic negative controls (n=17, grey boxplots), relative to the days post symptom onset. The boxplots display a median line, interquartile range (IQR) boxes, 1.5*IQR whiskers and individual data points. Values within each timeframe were compared with the controls with the two-sided Wilcoxon-signed rank test. A non-parametric Loess curve is shown to visualize the trend over time. Measurements from the same individual are connected with a grey line.

correlation (**Figure 3a** and **Figure S4a**). Of note, participants with a longer interval between onset of disease and inclusion into the study showed higher mucosal IgA levels (**Figure 3a** and **Figure S4b**).

Previous studies have shown that SARS-CoV-2-specific antibodies become detectable in serum at approximately two weeks PSO[28-30]. Here, we assessed the relationship between longitudinal mucosal antibody responses and symptom onset in more detail. This analysis was focused on index cases only, since information on the exact days post symptom onset (PSO) was available for this group only. Longitudinal nasal antibody responses were assessed by binning samples into three-day timeframes relative to the day of symptom onset and plotting the values alongside controls (Figure 3b). Mucosal IgM, IgA, and IgG antibody levels for S and RBD antigens were significantly elevated relative to controls between 7-9 days PSO, while for the N protein only IgG antibody responses were significantly higher than controls. Although IgA-N showed a similar response pattern, this did not reach statistical significance, presumably due to high variation in the pre-pandemic control samples. Nasal antibody responses that were increased after infection remained significantly elevated up to 9 months PSO.

Increases in nasal antibodies against S and RBD are associated with resolution of clinical symptoms

Next, we explored potential relationships between nasal antibodies and the progression of COVID-19 symptoms. Since none of the participants in our study required hospitalization or other medical intervention, our study population represents a cross-section of mild COVID-19 cases in a community setting. We examined the progression of 23 symptoms using a survey that all volunteers filled in daily throughout the 28 day follow-up. Symptoms were grouped into three categories: gastrointestinal symptoms (GS), systemic disease symptoms (SDS) and respiratory symptoms (RS). The most frequently reported symptoms were respiratory symptoms, which were also frequently reported by non-cases (**Figure S5a**). Anosmia/dysnosmia, i.e. change or loss of taste and smell, and systemic symptoms including a loss of appetite, muscle pain, joint pain, chills, fatigue and fever were reported significantly more often in cases than in non-cases. We examined whether the symptom duration varied between different symptom categories by generating a Kaplan-Meier curve for each symptom type. GS was excluded from this analysis as it only contained three symptoms. We found that systemic disease symptoms generally resolved faster than respiratory symptoms (p-value: 0.02, **Figure S5b**), with 50% of the cases being SDS free by day 14 after study inclusion. To examine longitudinal changes in the number of COVID-19 symptoms and identify potential

differences between different age groups, we binned symptom notifications into three-day timeframes relative to the study day, similar to the nasal antibody analysis described above. Subsequently, we analysed symptom progression per age group. Although faster resolution of SDS than RS was observed across all age groups, the number of reported symptoms per 3-day period was lowest in the <18y group and increased in both older age groups (**Figure 4a**). To assess the contribution of age to clinical symptom progression, we constructed a linear mixed-effects model per symptom group with the number of reported symptoms as the response and study day, age, viral load at study start and sex as covariates. Such a linear model was a good fit for our longitudinal symptom data (**Figure S6**). While time was – as expected– significantly associated with decreases in symptoms, increased age was significantly associated with increased SDS and RS when correcting for the effect of time (p-value: 0.0013 and 0.0001 respectively, **Figure 4b**). Female sex was associated with marginally more SDS (p-value: 0.02, **Figure 4b**). Viral load at day 0 was not related to SDS or RS progression (p-values: 0.78 and 0.92, respectively). To ascertain whether the induction of nasal antibodies was associated with COVID-19 symptoms, changes in nasal antibody levels over time were univariately added to the mixed effects model. This way, the effect of an increase of antibody signal on the changes in number of reported symptoms could be assessed while correcting for the effect of time, viral load, age and sex. Overall, increases in all nasal antibodies that were increased after infection were associated with decreased SDS and RS. After correction for multiple testing, a significant association was only found for SDS, where high levels of all isotypes against S, IgA and IgM against RBD and IgG against N were related to a decrease in symptoms. The largest effect was seen in the relation between IgA-RBD and SDS (**Figure 4c**). The age effect remained significant for all combinations.

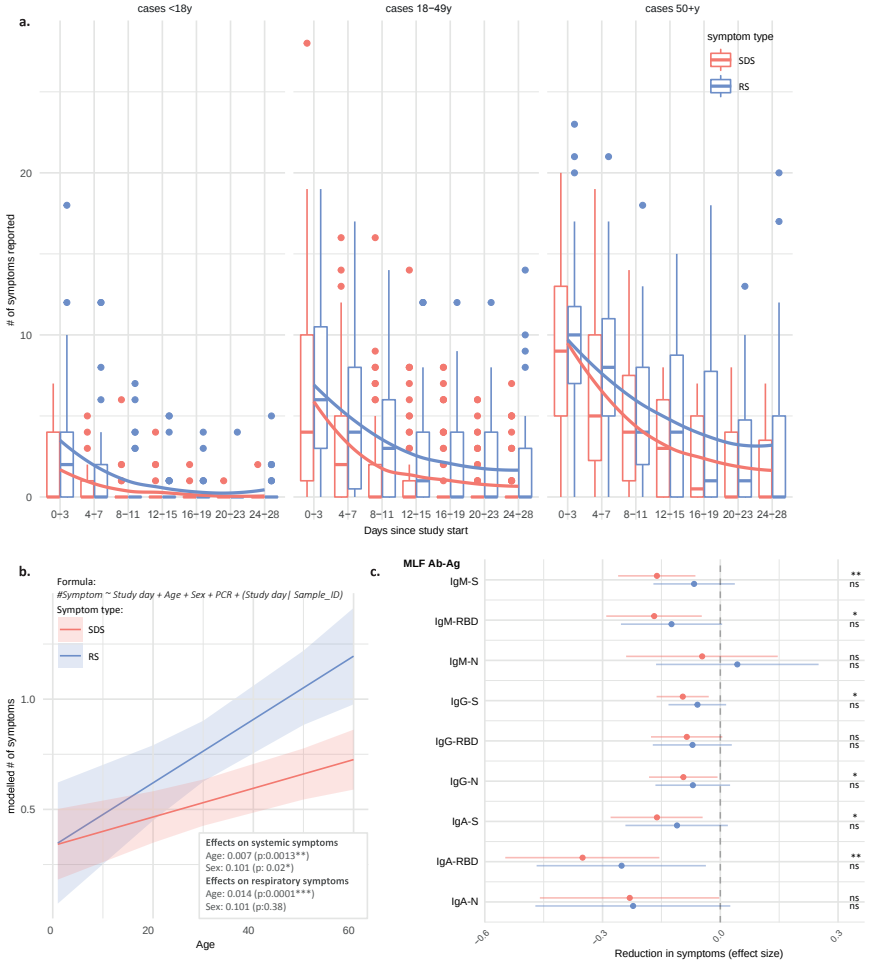


Figure 4. Age and mucosal antibody levels influence the presence and reduction of COVID-19 related symptoms. **a)** The number of respiratory (RS, blue) and systemic disease (SDS, red) symptoms were recorded for all cases (N=130) for each day during the 28-day study period. Data are plotted relative to the study day and values were binned into 3-day time frames. The boxplots display a median line, interquartile range (IQR) boxes, 1.5*IQR whiskers and outlier data points. A non-parametric Loess curve is shown as a red (SDS) or blue (RS) line in order to visualize the trend over time. Cases were grouped into three age-groups: <18y, 18-49y, and 50+y. **b)** A linear mixed-effect model (MEM) was fit to the data per symptom group. The response was specified as the number of symptoms on a given day, and explanatory fixed effects variables were: Study day, age, sex, and viral load at study start (PCR). Study day was also specified as a random slope, and Sample ID as a random intercept. The model fit per individual symptom trajectory can be found in supplementary figure S5. A significant effect of age was demonstrated for RS and SDS, as well as an effect of female sex on SDS. The mean predicted symptom values are plotted against age with 95% confidence interval bands (based on the standard error of the mean), and model estimates and p-values are depicted in the figure, together with the used model formula. **c)** Scaled longitudinal mucosal antibody measures were univariately added to the already

existing MEM formula depicted in figure 4b. The predicted change in symptoms per unit increase of the scaled relative antibody level is presented with 95% confidence intervals based on the standard error of the mean, and two-sided p-values for the association are plotted on the right, corrected for multiple testing with the Benjamini & Hochberg method (ns p>0.05, * p=0.01 for IgG-S, IgA-S and IgM-RBD, and p=0.046 for IgG-N , ** p=0.004 for IgM-S and IgA-RBD on SDS. None of the antibody effects on RS were significant).

Discussion

In this study, we examined the development and persistence of nasal antibodies following infection with SARS-CoV-2 in a household-contact study. Out of the 137 household contacts, we identified 80 cases. We found that mucosal antibodies against S and RBD increase 7-9 days after symptom onset and remain elevated for at least nine months. Anti-S and RBD mucosal antibodies were found to be correlated with a lower viral load, and were related to a faster decline in systemic symptoms.

We identified a very high frequency of cases among the household contacts (54.7%), which is higher than previously reported (11-37%)[31-33]. Most household transmission studies conducted during the first wave identified cases based on a single PCR test. This likely underestimates the true number of cases within a cluster, as PCR positivity is dependent on the time of sampling in relation to the infection. By combining PCR with seropositivity at 28 days after study inclusion, we were able to identify an additional 32 cases compared to PCR testing alone, increasing the total number of contact cases by 40%. This underlines the importance of antibody testing to assess disease exposure and transmission, especially in settings where PCR testing is limited and/or rapid antigen tests are not available.

We assessed the antibody production in the nose at multiple timepoints and observed a significant increase in nasal antibody levels in response to infection, with similar kinetics as previously described for serum and saliva[3,29,30]. The majority of sero- and PCR-positive contacts were also naso-positive by day 28 (Table S2). Nasal antibodies correlated strongly with serum antibodies, although correlations were noticeably weaker for IgA, which has previously also been observed in saliva[29,30,34]. As expected, both serum and nasal antibody levels decreased over time, but remained above detection threshold for at least nine months. These results substantiate the possibility of using nasal fluid as an epidemiological tool to monitor disease exposure until long after infection.

An effective early antibody response can modulate the clinical course of infection, as observed with e.g. influenza virus and Chikungunya infections[35,36]. We therefore

examined the relationship between the early nasal antibody response following SARS-CoV-2 infection and the progression of symptoms over time. Our study found that most anti-SARS-CoV-2 nasal antibodies, but in particular anti-RBD IgA correlated with the resolution of systemic disease symptoms. Surprisingly, no relation was identified with resolution with respiratory symptoms. It should be noted that the trajectory of respiratory symptoms was different compared to systemic symptoms. Furthermore, respiratory symptoms were reported much more frequently, including in non-cases, and were less specific to COVID-19 (**Figure S5a**). Although the exact nature of the relationship between nasal antibodies and clinical symptoms requires further investigation, higher anti-spike/RBD nasal antibodies at study baseline did correlate with lower viral load, particularly for anti-RBD/S IgM. Possibly, early control of viral infection in the upper respiratory tract reduces shedding of virus and viral replication in the lower respiratory tract and the periphery, resulting in less systemic symptoms. These findings are in line with a study by Butler *et al*., who also noted a prominent role for salivary anti-RBD IgA[37] neutralizing antibodies in reducing clinical severity.

The nucleocapsid is highly immunogenic and abundant in coronaviruses and is conserved across both SARS-CoV-2 and pre-COVID-19 seasonal coronaviruses. Although IgG and IgA antibodies against the nucleocapsid increased following infection, anti-N IgM levels did not increase in cases in our study. These results suggest the response to N is a memory recall response rather than a primary response, although variation between patients existed and some cases did show an anti-N IgM response. We used pre-COVID-19 MLF samples to determine 'naso-conversion'. Although this exploratory approach worked well for S/RBD for which no pre-existing antibodies are present, the presence of pre-existing anti-N nasal antibodies makes it more difficult to establish a valid 'cut-off' level.

Our study has several limitations. First, the starting point was the inclusion of healthcare workers, most of whom were female, and thus not entirely representative for the larger population. It should be noted that this study was performed during the first wave in March-April of 2020, when all schools in the Netherlands were closed (**Figure 1b**) and therefore no conclusions can be drawn in relation to child-child contacts in the transmission of SARS-CoV-2. When we performed the study, we were unable to collect additional samples for viral PCR due to national shortages in swabs and transport medium, limiting our ability to further examine the dynamic interactions between viral infection and antibody responses. To minimize the study burden, we made an initial decision to limit the number of serum samples to the day 28 timepoint, to which we later added the nine months timepoint.

Consequently, no comparison of serum and nasal antibody levels is possible at the early timepoints before day 28 of the study. Nonetheless, the possibility for participants to self-sample nasal MLF repetitively in a non-invasive manner removes an important obstacle for use in age groups that are normally difficult to sample, such as children, or hard-to-reach locations.

Taken together, our study shows that an early and higher nasal antibody response may play a key role in limiting disease by initiating early viral clearance and facilitating the resolution of systemic symptoms. Further research is needed to validate the role of nasal antibodies in clinical protection. Nasal IgA and IgG antibodies remain detectable for at least nine months after infection and likely confer at least partial protection against re-infection. Since mucosal antibodies are the first line of defence against viral infection, monitoring post-infection and post-vaccination nasal antibody levels may allow us to identify early signs of waning of immunity against infection. Finally, the study design and analysis strategy presented here can be used as a blueprint for follow-up investigations not only for COVID-19, but also for other infectious diseases.

Methods

Recruitment

This observational prospective cohort study was conducted among COVID-19 cases with a laboratory confirmed infection, as well as their household members that remained in home quarantine at the same address. The study was conducted in accordance with the provisions of the Declaration of Helsinki (1996) and the International Conference on Harmonization Guidelines for Good Clinical Practice. The study was approved by the local medical research ethics committee (CMO Regio Arnhem-Nijmegen) and is registered with ClinicalTrials.gov (NCT04590352; ethical committee reference NL73418.091.20). All index cases in this study were healthcare workers (HCW) from three hospitals (Radboudumc, CWZ and Rijnstate) in the provinces of Gelderland in the Netherlands, with a confirmed SARS-CoV-2 infection. Study participants were included from the 26th of March 2020 until the 15th of April, when the inclusion number of 50 households was reached. Participants were introduced to the study through the occupational health and safety services (OHS) of the participating hospitals. HCWs were included if they had a positive Polymerase Chain Reaction test (PCR-test) for the SARS-CoV-2 virus, tested and judged by the OHS of their hospital, with a positive indication for home isolation, and had at least two household members willing to participate.

Study design

Before the first home visit, all index cases of the family had a telephone interview, where they were asked about their first day of symptom onset, whether they were in isolation from the rest of the household, whether physical contact was restricted with other household members, whether they were still symptomatic, and whether they thought they were the primary case in the household. Households were visited within 1-2 days of a positive PCR in the index case. Following informed consent, nasopharyngeal and oropharyngeal swabs were taken for viral PCR, as per diagnostic guidelines[38]. A nasal mucosal lining fluid (MLF) sample was obtained from all participants by the use of the Nasosorption™ FX·i nasal sampling device (Hunt Developments, UK). A synthetic absorptive matrix (SAM) strip was gently inserted into the nostril of the participant and placed along the surface of the inferior turbinate. The index finger was lightly pressed against the side of the nostril to keep the SAM strip in place and to allow MLF absorption for 60 seconds, after which the SAM strip was placed back in the protective plastic tube. Participants were instructed on how to self-sample MLF at home. Finally, participants were asked about their symptoms of that day.

Participants were followed up for approximately 28 days, starting on the day of the first home visit (day 0) and ending on the last home visit (day 28-33). This range in the last visit was due to logistical difficulties during the summer holidays; 14 index cases were visited on their day 29, three on day 30, five on day 31, four on day 32 and one on day 33. All participants registered their symptoms for 28 days. During follow-up, clinical symptoms were registered three times daily and MLF was collected at three different study days via self-sampling. For the index case, MLF was collected on day 0, 3, 6 and one of the days 28-33 and for the household contacts this was on day 0, 7, 14 and one of the days 28-33 (**Figure 1c**). Self-sampled MLF samples were stored in biosafety bags in the participants' own freezer (temperature around -20 °C).

At the final home visit, MLF samples were picked up and transported to the Radboudumc on dry ice, where they were stored at -80°C until further testing. For antibody analysis, Nasosorption™ FX·i nasal sampling devices were thawed on ice, after which the synthetic adsorptive matrix (SAM) was removed using sterile forceps. The SAM was placed in a spin-X filter Eppendorf tube with 300 µl of elution buffer (PBS/1% BSA) for a minimum of 10 minutes, followed by centrifugation at 16000x*g* for 10 minutes at 4°C. To prevent unspecific binding, the spin-X filter columns were pre-incubated with the elution buffer for 30 minutes. The filter cups were then removed from the Eppendorf tubes using sterile forceps. To inactivate

live SARS-CoV-2, the eluate was incubated for a minimum of 45 minutes at 56 °C, spun down, aliquoted and stored at -20°C for further testing.

Finger-prick blood (~0.3 ml) was collected from all participants consenting to the finger-prick at day 28 by the use of a sterile disposable lancet device (BD Microtainer Lancet) and a sterile capillary tube. Blood samples were kept at room temperature until processing at the Radboudumc laboratory site, after which serum was stored at -20°C until further testing.

All collected symptom diaries were digitalized into Castor EDC, clinical trial software for electronic data capture and clinical data management.

Nine months after the first visit, cases (N=108) were visited again and a serum and MLF sample were taken and processed in the manner described above (**Figure 1c**). Nobody had been vaccinated yet at that time.

Detection of SARS-CoV-2

Presence or absence of SARS-CoV-2 and viral copy number per ml was determined on the combined nasopharyngeal and oropharyngeal swab, using a PCR protocol that was developed at the National Institute of Health and the Environment (RIVM), and has been widely used for the diagnosis of SARS-CoV-2 in the Netherlands[39]. The protocol was slightly adjusted for the use of a different reaction mix by the Medical Microbiology Laboratory of the Radboudumc. In short, nasopharyngeal and oropharyngeal swabs were collected in GLY medium and stored at -80°C until processing. Samples were thawed, vortexed and 500 µl of sample was lysed in 450 µl MagNAPure Lysis/binding buffer (Roche). An ivRNA internal extraction control was added and samples were extracted on the automated MagNAPure LC 2.0 system using the MagNAPure LC Total Nucleic Acid isolation kit - High Performance (Roche). Samples were eluted in 50 ul of which 5 µl was used in the RT-qPCR using the Luna Universal Probe One-Step RT-qPCR kit (NEB) with 400 nM E-gene primers (FW: 5'- acaggtacgttaatagttaatagcgt-3' RV: 5'- atattgcagcagtacgcacaca-3') and 200 nM E-gene probe (5'-FAM- ACACTAGCCATCCTTACTGCGCTTCG-BHQ1-3' (Biolegio)) on a CFX96 Real-Time PCR Detection System (BioRad) See table S3 for a summary of the primers used. Transcript quantities were calculated using a 10-fold dilution series of E-gene ivRNA. The extraction efficiency was checked in a separate RT-qPCR using the Luna Universal Probe One-Step RT-qPCR kit (NEB) with primers targeting the ivRNA that was added prior to extraction.

Antibody measurements

For antibody analysis, a fluorescent-bead-based multiplex immunoassay (MIA) was developed. The stabilized pre-fusion conformation of the ectodomain of the Spike protein (amino acids 1 – 1,213) fused with the trimerization motif GCN4 (S-protein) and the receptor binding domain of the S-protein (RBD) as previously described by Wang C. *et al.*[40], and the Nucleocapsid-His recombinant Protein (N) (40588-V08B, Sino Biologicals), were each coupled to beads or microspheres with distinct fluorescence excitation and emission spectra, essentially as described in the paper by den Hartog *et al*.[41].

A total of six reference serum samples were selected from PCR confirmed COVID-19 patients with varying immunoglobulin G (IgG) concentrations, and pooled to create standard curves for IgG, IgA and IgM. Next to this, four different samples from the same cohort were used as quality control samples. As negative control samples, we used historical serum (n=32) and MLF (n=17) samples collected prior to the SARS-CoV-2 pandemic.

MLF samples were diluted 1:5 in assay buffer (PBS/1% BSA/0.05% tween-20) and serum samples were diluted 1:500 in assay buffer, incubated with antigen-coated microspheres for 30 minutes at room temperature while shaking at 450 rpm. Following incubation, the microspheres were washed two times with PBS, incubated with phycoerythrin-conjugated goat anti-human, IgG (Jackson Immunoresearch, 109-116-170), IgM (Southern Biotech, 2022-09) and IgA (Southern Biotech, 2052-09) for 20 minutes and washed twice. Data were acquired on the Luminex FlexMap3D System. Validation of the detection antibodies was obtained from a recent publication using the same antibodies and the same assay[41], and specificity was checked using rabbit anti-SARS SIA-ST serum.

S- and N-coupled microspheres were combined to measure antibodies directed against multiple antigens (or epitopes) in one single sample. Since antibodies against the S-protein and RBD may compete for the same epitopes, antibody binding to RBD was measured separately. Using different conjugates, IgG, IgA, and IgM-specific antibodies concentrations were measured in MLF and serum.

MFI was converted to arbitrary units (AU/ml) by interpolation from a log-5PL-parameter logistic standard curve and log-log axis transformation, using Bioplex Manager 6.2 (Bio-Rad Laboratories) software and exported to R-studio. Negative control samples (MLF and serum) were used to filter out background signal in the antibody measures. The MLF samples originated from the KIRA-study performed

at the Radboudumc, in which healthy healthcare workers are vaccinated against pertussis as per routine care, and gave consent to the use of the MLF samples for other research. The serum samples originated from the Radboudumc Biobank, that allows the use of serum samples for research as long as privacy of the donors is guaranteed. The standard dilution range plus four quality control samples were added to each plate. A ROC analysis was performed to analyse the performance of the MIA (**Figure S1a**). During the analysis of the samples taken nine months after study start, an aliquot of the day 28 samples was thawed and re-analysed, to ensure reproducibility of the assay and enable batch correction if needed (**Figure S1b**).

Symptom categorization

To analyse the relation between symptom clearance in index cases and the mucosal antibody response, we categorized our set of symptoms into three categories, based on their clinical presentation. This resulted in a set of 23 symptoms, which were categorized into three categories, i.e. respiratory symptoms (RS) systemic disease symptoms (SDS), and gastrointestinal symptoms (GS). RS includes chest pain, sneezing, nose bleeding, pain when breathing, coughing with mucus, dyspnoea, sore throat, loss or change of taste/smell (dysnosmia), coughing, and rhinorrhoea. SDS includes dizziness, headache, fever, temperature, chills, joint pain, muscle pain, swollen lymph nodes, low appetite and fatigue. GS includes vomiting, diarrhoea, and nausea.

Case definition

For analysis of SARS-CoV-2 exposure within households, we categorized the household contacts into cases and non-cases. Cases were defined as being either PCR positive at study start and/or seropositive for IgA, IgG or IgM against S at study day 28. PCR positivity was set on a Ct value<36, which corresponds to a viral load of at least 10^3 copies/ml extracted sample. The seroconversion threshold was based on the mean + 2*SD of the historic negative control samples, which were collected before SARS-CoV-2 was introduced in the Netherlands. For explorative analyses, a nasoconversion threshold was based on the mean + 2*SD of the historic negative control samples of the MLF samples, and a participants was called "nasoconverted" at day 28 when they had at least one antibody isotype targeted against S above this threshold.

Statistical analyses and reproducibility

Analysis of Luminex data was performed with Bio-Plex 200 in combination with Bio-Plex manager software (Bio-Rad Laboratories, Hercules, CA). All MIA experiments were performed once, but standard reference curves and quality control samples

were identical throughout the experiment, to control for possible batch control. Demographical data was exported from Castor EDC, and double checked with the paper records by two members of the research team. All statistical analyses were performed using the Rstudio environment, with libraries 'stats' (hypothesis tests and correlations), "lme4" [42], "lmerTest" [43] for mixed-effects modelling and associated p-values, "ROCit" for the ROC analysis of the MIA, and "survival" [44] for Kaplan-Meier survival analysis. The libraries "survminer", "patchwork", and "ggplot2" were used for visualization. Changes in serum or mucosal antibodies compared to negative controls were tested using a two-tailed paired Wilcoxon signed rank test, and then corrected for multiple testing with the Benjamini-Hochberg method[45]. Statistical parameters including the sample sizes, measures of distribution, and p-value thresholds for significance are reported directly in the figures and figure legends. In order to determine if a sample was seropositive for a given combination of antigen and antibody isotype, a cut-off value (mean + 2 standard deviations) was calculated from the negative control samples. Samples above this threshold were classified as seropositive for that antigen and isotype combination. Samples that were seropositive for any of the antibodies tested were classified as such ("anySero", **Figure 2A**). Where correlations are presented, the spearman correlation coefficient and associated p-value were calculated. The differences in symptom reporting between cases and non-cases was calculated using the Fisher's exact test, the probability of becoming symptom-free was estimated using Kaplan-Meier's method, and the hypothesis testing was performed using log-rank test. In order to estimate the effect of patient characteristics and antibodies on symptoms over time, we constructed a mixed-effects model. For each subject and for each timepoint, we added together the number of complaints per symptom category. We specified a mixed-effects model per symptom category with symptoms as the response and study day, age, and sex, as explanatory variables. We also added study day and Sample_ID as random effects. The formula for the model (in R notation):

*Symptom_count ~ Study day + Age + Sex + Viral load + (Study day | Sample_ID)*In order to determine the effect of antibodies on the symptom response, the model above was updated in a univariate fashion with each antibody measurement as a covariate. The formula of the updated model:

*Symptom_count ~ Study day + Age + Sex + Viral load + Antibody + (Study day | Sample_ID).*Estimates for the covariates, as well as 95% confidence intervals and p-values (Satterthwaite's approximation to degrees of freedom) were extracted and plotted.

Acknowledgements

We would like to thank all MuCo study participants for their willingness to participate and their support for the study. Additionally, we thank M. Boonstra, M. Blok, D. van der Giessen, E. Lenssen, E. Reuvers, M. Roek and E. Wijnhoven for assisting with performing the first home visit, H. Lyoo from Utrecht university for experimental assistance, J. Heijnen, M. Dautzenberg, and A. Voss for recruiting participants from the occupational health and safety officers at Rijnstate and CWZ hospitals, and the respective medical microbiology departments involved in PCR testing of hospital employees. This study was funded by the Laboratory of Medical Immunology, Radboud university medical center.

References

1. Wölfel R, Corman VM, Guggemos W, et al. Virological assessment of hospitalized patients with COVID-2019. *Nature* 2020.
2. Lee S, Kim T, Lee E, et al. Clinical Course and Molecular Viral Shedding Among Asymptomatic and Symptomatic Patients With SARS-CoV-2 Infection in a Community Treatment Center in the Republic of Korea. *JAMA Intern Med* 2020.
3. To KK, Tsang OT, Leung WS, et al. Temporal profiles of viral load in posterior oropharyngeal saliva samples and serum antibody responses during infection by SARS-CoV-2: an observational cohort study. *Lancet Infect Dis* 2020.
4. Wu Z, McGoogan JM. Characteristics of and Important Lessons From the Coronavirus Disease 2019 (COVID-19) Outbreak in China: Summary of a Report of 72 314 Cases From the Chinese Center for Disease Control and Prevention. *Jama* 2020.
5. Kim G-u, Kim M-J, Ra SH, et al. Clinical characteristics of asymptomatic and symptomatic patients with mild COVID-19. *Clinical Microbiology and Infection* 2020.
6. Oran DP, Topol EJ. Prevalence of Asymptomatic SARS-CoV-2 Infection: A Narrative Review. *Ann Intern Med* 2020.
7. RIVM. Epidemiologische situatie COVID-19 in Nederland: Rijksinstituut voor Volksgezondheid en Milieu, 2020.
8. Gandhi M, Yokoe DS, Havlir DV. Asymptomatic Transmission, the Achilles' Heel of Current Strategies to Control Covid-19. *New England Journal of Medicine* 2020; **382**(22): 2158-60.
9. WHO. Public health surveillance for COVID-19 WHO, 2020.
10. Arons MM, Hatfield KM, Reddy SC, et al. Presymptomatic SARS-CoV-2 infections and transmission in a skilled nursing facility. *New England journal of medicine* 2020.
11. Lewis NM, Duca LM, Marcenac P, et al. Early Release-Characteristics and Timing of Initial Virus Shedding in Severe Acute Respiratory Syndrome Coronavirus 2, Utah, USA.
12. Grijalva CG, Rolfes MA, Zhu Y, et al. Transmission of SARS-COV-2 infections in households—Tennessee and Wisconsin, April–September 2020. *Morbidity and Mortality Weekly Report* 2020; **69**(44): 1631.
13. Tosif S, Neeland MR, Sutton P, et al. Immune responses to SARS-CoV-2 in three children of parents with symptomatic COVID-19. *Nature Communications* 2020; **11**(1): 5703.
14. Voysey M, Clemens SAC, Madhi SA, et al. Safety and efficacy of the ChAdOx1 nCoV-19 vaccine (AZD1222) against SARS-CoV-2: an interim analysis of four randomised controlled trials in Brazil, South Africa, and the UK. *The Lancet* 2020.
15. Polack FP, Thomas SJ, Kitchin N, et al. Safety and efficacy of the BNT162b2 mRNA covid-19 vaccine. *New England Journal of Medicine* 2020.
16. Ewer KJ, Barrett JR, Belij-Rammerstorfer S, et al. T cell and antibody responses induced by a single dose of ChAdOx1 nCoV-19 (AZD1222) vaccine in a phase 1/2 clinical trial. *Nature Medicine* 2020.
17. Mulligan MJ, Lyke KE, Kitchin N, et al. Phase I/II study of COVID-19 RNA vaccine BNT162b1 in adults. *Nature* 2020; **586**(7830): 589-93.
18. Dutta NK, Mazumdar K, Gordy JT. The nucleocapsid protein of SARS–CoV-2: a target for vaccine development. *Journal of virology* 2020; **94**(13).
19. McAndrews KM, Dowlatshahi DP, Dai J, et al. Heterogeneous antibodies against SARS-CoV-2 spike receptor binding domain and nucleocapsid with implications for COVID-19 immunity. *JCI insight* 2020; **5**(18).

20. Zhao J, Yuan Q, Wang H, et al. Antibody responses to SARS-CoV-2 in patients of novel coronavirus disease 2019. *Clin Infect Dis* 2020.
21. Du L, Zhao G, Lin Y, et al. Intranasal vaccination of recombinant adeno-associated virus encoding receptor-binding domain of severe acute respiratory syndrome coronavirus (SARS-CoV) spike protein induces strong mucosal immune responses and provides long-term protection against SARS-CoV infection. *J Immunol* 2008; **180**(2): 948-56.
22. Lee J-S, Poo H, Han DP, et al. Mucosal Immunization with Surface-Displayed Severe Acute Respiratory Syndrome Coronavirus Spike Protein on Lactobacillus casei Induces Neutralizing Antibodies in Mice. *Journal of Virology* 2006; **80**: 4079-87.
23. Sungnak W, Huang N, Bécavin C, et al. SARS-CoV-2 entry factors are highly expressed in nasal epithelial cells together with innate immune genes. *Nature medicine* 2020; **26**(5): 681-7.
24. Hou YJ, Okuda K, Edwards CE, et al. SARS-CoV-2 Reverse Genetics Reveals a Variable Infection Gradient in the Respiratory Tract. *Cell* 2020.
25. Russell MW, Kilian M, Mantis NJ, Corthésy B. Biological activities of IgA. Mucosal immunology: Elsevier; 2015: 429-54.
26. Russell MW, Moldoveanu Z, Ogra PL, Mestecky J. Mucosal immunity in COVID-19: A neglected but critical aspect of SARS-CoV-2 infection. *Frontiers in Immunology* 2020; **11**: 3221.
27. Russell MW, Mestecky J, Strober W, Lambrecht BN, Kelsall BL, Cheroutre H. Overview: the mucosal immune system. Mucosal immunology: Elsevier; 2015: 3-8.
28. Seow J, Graham C, Merrick B, et al. Longitudinal evaluation and decline of antibody responses in SARS-CoV-2 infection. *medRxiv* 2020.
29. Isho B, Abe KT, Zuo M, et al. Persistence of serum and saliva antibody responses to SARS-CoV-2 spike antigens in COVID-19 patients. *Science immunology* 2020; **5**(52).
30. Pisanic N, Randad PR, Kruczynski K, et al. COVID-19 serology at population scale: SARS-CoV-2-specific antibody responses in saliva.. *J Clin Microbiol*; (JCM.02204-20).
31. Bi Q, Wu Y, Mei S, et al. Epidemiology and transmission of COVID-19 in 391 cases and 1286 of their close contacts in Shenzhen, China: a retrospective cohort study. *The Lancet Infectious Diseases* 2020.
32. Koh WC, Naing L, Rosledzana MA, et al. What do we know about SARS-CoV-2 transmission? A systematic review and meta-analysis of the secondary attack rate, serial interval, and asymptomatic infection. *medRxiv* 2020.
33. Lopez Bernal J, Panagiotopoulos N, Byers C, et al. Transmission dynamics of COVID-19 in household and community settings in the United Kingdom. *medRxiv* 2020.
34. Faustini SE, Jossi SE, Perez-Toledo M, et al. Detection of antibodies to the SARS-CoV-2 spike glycoprotein in both serum and saliva enhances detection of infection. *medRxiv* 2020.
35. Kam Y-W, Simarmata D, Chow A, et al. Early appearance of neutralizing immunoglobulin G3 antibodies is associated with chikungunya virus clearance and long-term clinical protection. *Journal of Infectious Diseases* 2012; **205**(7): 1147-54.
36. Haran JP, Hoaglin DC, Chen H, Boyer EW, Lu S. Antigen-specific H1N1 influenza antibody responses in acute respiratory tract infections and their relation to influenza infection and disease course. *Journal of Clinical Virology* 2014; **60**(4): 367-73.
37. Butler SE, Crowley AR, Natarajan H, et al. Features and Functions of Systemic and Mucosal Humoral Immunity Among SARS-CoV-2 Convalescent Individuals. *medRxiv* 2020.

38. Richtlijnen COVID-19. 2020. https://lci.rivm.nl/richtlijnen/covid-19 (accessed January 14 2021).
39. Corman VM, Landt O, Kaiser M, et al. Detection of 2019 novel coronavirus (2019-nCoV) by real-time RT-PCR. *Eurosurveillance* 2020; **25**(3): 2000045.
40. Wang C, Li W, Drabek D, et al. A human monoclonal antibody blocking SARS-CoV-2 infection. *Nature Communications* 2020; **11**(1): 2251.
41. den Hartog G, Schepp RM, Kuijer M, et al. SARS-CoV-2–Specific Antibody Detection for Seroepidemiology: A Multiplex Analysis Approach Accounting for Accurate Seroprevalence. *The Journal of Infectious Diseases* 2020; **222**(9): 1452-61.
42. Bates D, Mächler M, Bolker B, Walker S. Fitting linear mixed-effects models using lme4. *arXiv preprint arXiv:14065823* 2014.
43. Kuznetsova A, Brockhoff PB, Christensen RH. lmerTest package: tests in linear mixed effects models. *Journal of statistical software* 2017; **82**(13): 1-26.
44. Therneau TM, Grambsch PM. The Cox model. Modeling survival data: extending the Cox model: Springer; 2000: 39-77.
45. Benjamini Y, Hochberg Y. Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. *Journal of the Royal Statistical Society Series B (Methodological)* 1995; **57**(1): 289-300.

Supplementary materials

Scan the QR code for the supplementary materials:

Chapter 4

Primary exposure to SARS-CoV-2 via infection or vaccination determines mucosal antibody-dependent ACE2 binding inhibition

Janeri Fröberg[1,2*], Vera J.C.H. Koomen[1,2,3*], Christa E. van der Gaast-de Jongh[1], Ria Philipsen[4], Corine H. GeurtsvanKessel[5], Rory D. de Vries[5], Marije C. Baas[3], Renate G. van der Molen[1], Marien I. de Jonge[1,2], Luuk B. Hilbrands[3#], Martijn A. Huynen[6#], Dimitri A. Diavatopoulos[1,2], on behalf of RECOVAC

[*] and [#] contributed equally

[1.] Department of Laboratory Medicine, Laboratory of Medical Immunology, Radboud University Medical Center, Nijmegen, The Netherlands

[2.] Center of Infectious Diseases, Radboud University Medical Center, Nijmegen, The Netherlands

[3.] Department of Nephrology, Radboud University Medical Center, Radboud University, Nijmegen, The Netherlands

[4.] RTC CS Radboud Technology Center Clinical Studies, Radboudumc, Nijmegen, The Netherlands

[5.] Department of Viroscience, Erasmus Medical Center, Rotterdam, the Netherlands

[6.] Department of Medical BioSciences, Radboud University Medical Centre Nijmegen, Nijmegen, The Netherlands

J Infect Dis. 2024 Jan 12;229(1):137-146. doi: 10.1093/infdis/jiad385.

Short summary

Comparison of longitudinal changes in nasal antibody levels and their ACE2-inhibiting activity shows that, although ACE2-inhibiting activity was similar, the inhibition after primary SARS-CoV-2 infection depended mostly on mucosal IgA while post-vaccination inhibition was mainly associated with mucosal IgG.

Abstract

Background

Mucosal antibodies play a critical role in preventing SARS-CoV-2 (re)infections by blocking the interaction of receptor binding domain (RBD) with angiotensin converting enzyme 2 receptor (ACE2) on the cell surface. In this study, we investigated the difference between the mucosal antibody response after primary infection or vaccination.

Methods

We assessed longitudinal changes in the quantity and capacity of nasal antibodies to neutralize the interaction of RBD with the ACE2 receptor using Spike protein and RBD from ancestral SARS-CoV-2 (Wuhan-Hu-1), as well as RBD from the Delta and Omicron variant.

Results

Significantly higher mucosal IgA concentrations were detected post-infection compared to post-vaccination, while vaccination induced higher IgG concentrations. However, ACE2 inhibiting activity did not differ between the cohorts. When investigating if IgA or IgG drove the ACE2 inhibition, infection-induced binding inhibition was driven by both isotypes, while post-vaccination binding inhibition was mainly driven by IgG.

Conclusions

Our study provides new insights into the relationship between antibody isotypes and neutralization by using a sensitive and high-throughput ACE2 binding inhibition assay. Key differences are highlighted between vaccination and infection at the mucosal level, showing that despite differences in the quantity of the response, post-infection and post-vaccination ACE2 binding inhibition capacity did not differ.

Introduction

The COVID-19 pandemic, caused by the introduction of SARS-CoV-2 in an immunologically naive population, has provided a unique opportunity to study *de novo* immune responses induced by infection or vaccination. Most immunological studies on SARS-CoV-2 to date have focused on serum antibodies and not mucosal antibodies. Mucosal antibodies play a critical role in preventing SARS-CoV-2 (re) infections, by blocking the interaction of the receptor binding domain (RBD) on the viral spike (S) protein with the angiotensin converting enzyme 2 (ACE2) receptor that is expressed on the surface of host cells[1-4].

Given the key role of mucosal antibodies in providing a first line of defense against infection, improved knowledge about local antibody concentration and function could provide important insights into interrupting SARS-CoV-2 transmission[1,5,6]. We and others have previously shown that SARS-CoV-2 infection generates a strong mucosal antibody response against the S protein, which is not always correlated to the serum response[5,7-9], and that early induction of such antibodies is associated with faster symptom resolution and lower viral loads compared to when mucosal antibodies develop later[5,10]. Several studies have shown that SARS-CoV-2 infection induces mucosal antigen-specific B cells[7,11,12], suggesting that mucosal antibodies are produced locally. Although studies have shown that COVID-19 vaccination also induces mucosal IgG to the S protein[11,13], nasal IgG after parenteral vaccination is likely not due to local production, but primarily the result of active transport of serum IgG via the neonatal Fc receptor[11].

The composition of the immune response and its capacity to neutralize SARS-CoV-2 may differ after infection or vaccination. This has not been extensively investigated, largely because mucosal specimens are difficult to analyze in the plaque reduction neutralization test (PRNT) because of lower antibody concentrations compared to serum. In this study, we compared the mucosal antibody response after primary infection or after primary vaccination with the Spikevax vaccine. We used a multiplex bead-based approach to assess and compare longitudinal changes in the quantity and neutralizing capacity of mucosal antibodies against ancestral SARS-CoV-2 (Wuhan-Hu-1), as well as Delta and Omicron BA.1 RBD. Moreover, we analyzed and compared how the relationships between antibody concentration and ACE2 inhibition capacity vary between infection and vaccination.

Methods

Cohort description

To investigate the differences between infection- and vaccination-induced mucosal antibody responses, we used clinical data and samples from two cohorts, henceforth called the 'infection' and 'vaccination' cohort. All participants signed an informed consent form before participating.

The infection cohort consisted of individuals who participated in the MuCo-study [NCT04590352] [5]. In this study, conducted during the first COVID-19 wave between March-April 2020, 50 hospital workers with a PCR confirmed SARS-CoV-2 infection and their household members were included. In the current analysis, we only included 'cases', i.e., participants who tested positive by PCR at study start (n=84, 82%) or had a positive serology test at 28 days after study start (n=19, 18%). Nasal mucosal lining fluid (MLF) was obtained by nasosorption at study start, at 7 and 28 days, and at 9 months after study start. COVID-19 vaccines were not yet available during this study period.

The vaccination cohort included participants from the RECOVAC-IR study, a prospective, controlled multicenter study designed to investigate the immunogenicity and safety of COVID-19 vaccination in specific kidney-patient groups, who received two vaccinations between February 24 and April 8 2021[14] [NCT05030974]. For the current analysis, we included samples from the 'control' group, representing individuals without known kidney disease (n=46). All included participants had no measurable serum antibodies against the nucleocapsid protein (N-serology) at study start and at 28 days. One individual had a positive N-serology at 6 months and was excluded from analysis at this timepoint. All participants were vaccinated with one dose of Spikevax (mRNA-1273; Moderna) at study start and again at 28 days after study start. MLF was obtained at study start, 28 days after study start, at 28 days and 6 months after the second vaccination.

To compare mucosal antibodies between the two cohorts, this study focused on the samples collected at study start, at 28 days post infection *or* second vaccination (+28D), and at the 6 or 9-months follow-up timepoints.

Mucosal lining fluid preparation

The method of MLF sampling and elution has been described previously[5]. In short, a nasosorption™ FX·i nasal sampling device (Hunt Developments, UK) was inserted gently into the nose, after which a finger was pressed against the nostril for

60 seconds. MLF strips were frozen immediately after collection and stored at -80°C until elution for the infection cohort and at -20°C for the vaccination cohort.

Antibody quantification

Antibodies in MLF were quantified using a bead-based multiplex immunoassay (MIA), as described previously[5]. Wuhan-Hu-1 trimeric spike (D614G mutant, ExcellGene), Wuhan-Hu-1 Nucleocapsid-His recombinant (Sino biologicals), Wuhan-Hu-1 RBD (ExcellGene), Delta RBD (L452R/T478K, Sino Biologicals) and Omicron BA.1 RBD (B.1.1.529/Y508H, Sino Biologicals) antigens were conjugated to Luminex MagPlex beads 02, 24, 60, 28, and 45 respectively. MLF samples were thawed, heat-inactivated at 56°C for 1 hour and incubated in a dilution of 1:5 with the antigen-coated beads at room temperature. Following a 45-minute incubation, the beads were washed and incubated in a 1:200 dilution with phycoerythrin-conjugated goat anti-human IgG or IgA (Southern Biotech) for 20 min and washed again twice. Samples were measured on the Luminex machine using the Flexmap 3D and xPONENT software. MFI values were converted to binding antibody units (BAU/mL) by arbitrarily assigning the reference serum (calibrated to the WHO international standard[15]) a starting concentration of 1000 BAU/ml for both IgG and IgA. A dilution factor of 3 and 2 for the reference serum was used for IgG and IgA, respectively. Samples were interpolated to the IgA or IgG standard curve with a log-5PL-parameter logistic regression and log–log axis transformation, using Bioplex Manager 6.2 (Bio-Rad Laboratories) software, and exported to R-studio.

ACE2 binding competition assay

To assess the neutralizing capacity of the MLF samples, we established an in-house ACE2 binding competition assay based on the ACE2-RBD assay by Junker *et al.*[16]. The same antigen-conjugated magnetic beads that were used in the MIA assay were vortexed and sonicated before preparing a bead solution of 2000 beads/25 µL (8x10E4 beads/ml) in assay buffer, with 500 beads/25 µL used per analyte. In a 96-well plate set-up, 25 µL beads were mixed with 25 µL of MLF sample and incubated for 30 minutes at room temperature (RT). Subsequently, 25 µL of human recombinant (His-Tag) biotinylated ACE2 protein dilution (Sino Biological; corresponding to 0.25 mg/mL) was added and incubated for 20 minutes at RT. For quality control, two wells containing only buffer and three wells containing the same QC sample of pooled serum from individuals with confirmed recent infection in the Omicron era were added to each plate. A 10-point dilution of an in-house 'standard' was added to each plate, based on the assay by den Hartog *et al.*[17], consisting of pooled serum from infected and vaccinated individuals, calibrated to the WHO international standard[15]. Samples were measured using the MIA-assay

method described above. A unit of inhibiting arbitrary units per ml (IAU/mL) was calculated using the same method as the MIA-assay and values were exported to R-studio.

To compare the ACE2 binding inhibition assay to the plaque reduction neutralization test (PRNT[18]), serum samples from participants in the RECOVAC study that were previously measured by PRNT were selected (n=74[14]). These serum samples were also measured in the ACE2 binding inhibition assay, showing high correlation with the PRNT, with an $R > 0.9$ for all variant combinations except for RBD Omicron ($R = 0.82$, **Figure S1**).

Statistical analysis

All analyses were performed in R-studio 2022.02.1, using R software version 4.1.3[19]. Data wrangling and statistical analyses were performed using the "dplyr", "tidyr", "lmer" and "lme4" package, and the "ggplot" and "patchwork" packages were used for data visualization. Samples with a value below the lower limit of detection for a particular analyte were manually assigned a value of 0.5 times the lowest measurable value for that analyte. Samples with a value above the limit of detection for a particular analyte were remeasured in a higher dilution (1:80 & 1:1200). When a sample was measured more than once, the mean of the value was used for data analysis.

Differences between paired timepoints were calculated using the non-parametric two-tailed Friedman's test with Dunn's post-hoc testing and Bonferroni adjustment for multiple testing. Differences between cohorts were calculated with the two-tailed Wilcoxon rank sum test. Correlations were calculated with a Spearman rank correlation. Statistical parameters including the sample sizes, measures of distribution and P value thresholds for significance are reported directly in the figures and figure legends. The significance threshold was set at a (corrected) p-value <0.05.

In order to estimate the effect of virus antigen-specific IgA and IgG antibodies on ACE2 binding inhibition over time, we constructed a random intercept mixed-effects model. We specified a separate model per cohort and per variant-antigen combination. IgA and IgG BAU/mL values were separately scaled from 0 to 1 to enable comparison of the isotype-specific effects. Besides antibody concentrations, the model included study day and age as explanatory variables, which were included after univariate analysis showed a significant effect on ACE2 binding inhibition, and participant ID as a random effect.

The formula for the model (in R notation):

ACE2 binding inhibition ~ IgA_scaled + IgG_scaled + Study day + Age + (1|ID)

To evaluate the goodness of fit of the model, the predicted ACE2 inhibition activity was plotted and the Akaike information criterion (AIC) of the final model and the univariate models were compared. Furthermore, the normality and variation of the residuals and homoscedasticity of the data were examined, using the R "performance" package. Estimates for the covariates, as well as 95% confidence intervals and P values (Satterthwaite's approximation to degrees of freedom), were extracted and plotted, and the AIC and residual ranges of the final models were reported.

Results

Cohort description

This study includes participants from two cohorts. The infection cohort consisted of 103 confirmed cases with mild SARS-CoV-2 infection. The median age of the infection cohort was 41 (IQR 20-52) and 58% was female. A more complete description of the infection cohort was published previously[5]. The vaccination cohort included 46 individuals (59% female). Participants were defined as being non-infected before and during the study by having a serum antibody response against the Nucleocapsid protein <19.7AU/mL[17]. The median age of the vaccination cohort was significantly higher than of the infection cohort (56 years, IQR:49-65, p=0.009e-6, **Figure S2**).

Comparable ACE2 binding inhibition between vaccinated and infected individuals

To assess the capacity of mucosal antibodies to inhibit binding of viral antigens to ACE2, we measured ACE2 binding inhibition in MLF samples for Wuhan-Hu-1 Spike, and for Wuhan-Hu-1, Delta and Omicron RBD. Wuhan-Hu-1 was the circulating virus at the time of the infection study and is incorporated into the Spikevax vaccine. Delta and Omicron were included to analyze the immune response against variants of concern (VOCs). At baseline, the ACE2 binding inhibition was higher in the infection cohort against all but the Omicron variant, as these samples were collected at 2-12 days after the onset of symptoms. Consequently, the infection cohort showed more variable increases compared to the vaccination cohort (**Figure S3**,[5]). Both cohorts showed a rapid and significant increase in ACE2 binding

inhibition after baseline at the early sampling time points, i.e., at 7 days post infection and at 28 days after the first vaccination dose (**Figure 1**). ACE2 binding inhibiting activity peaked at 28 days post infection/second vaccination (+28D) and had significantly decreased for all viral antigens at the follow-up timepoint, i.e., 6 or 9 months. In the infection cohort, at nine months post infection, ACE2 binding inhibition was not significantly different anymore from baseline activity for all the viral antigens ($p=0.059$ for Wuhan-Hu-1_S). In the vaccination cohort, at 6 months post second vaccination, ACE2 binding inhibition of Wuhan Spike (Wuhan-Hu-1_S) and Delta RBD were still significantly elevated as compared to baseline ($p=0.003$ and $p=0.022$, respectively, **Figure 1**). The peak ACE2 binding inhibition activity at +28D was not significantly different between the two cohorts, except for Wuhan-Hu-1_S, which was higher in the vaccination cohort ($p=0.0025$, **Figure S4a**). At the follow-up timepoints, no statistically significant differences were found between the cohorts (**Figure S4b**).

Infection and vaccination induce distinct mucosal antibody responses

To determine whether mucosal IgA or IgG are induced by SARS-CoV-2 infection or vaccination, we quantified the concentrations of SARS-CoV-2 antigen-specific IgG and IgA in MLF samples of all participants. Again, we observed higher concentrations of IgG and IgA at study start for the infection cohort (**Figure S5**). IgG generally revealed a similar pattern to the ACE2 binding inhibition capacity. Both cohorts showed a significant increase over baseline against all antigen-variant combinations (**Figure 2a**). Although antibody concentrations in both cohorts significantly waned from 28 days to 6 and 9 months, mucosal IgG antibody concentrations in both cohorts were significantly higher compared to baseline for all antigen-variant combinations ($p<0.001$). Infection significantly induced IgA antibodies against all antigen-variant combinations, which remained elevated up to nine months. In contrast, vaccination did not result in significant increases, except for Wuhan-Hu-1 S and RBD-Delta (**Figure 2a**). Likewise, although peak IgG concentrations in the vaccination cohort were significantly higher for all variants compared to the infection cohort, an opposite pattern was observed for IgA with higher peak concentrations in the infection cohort (**Figure 2b**). This pattern was maintained at the later timepoints (**Figure 2c**).

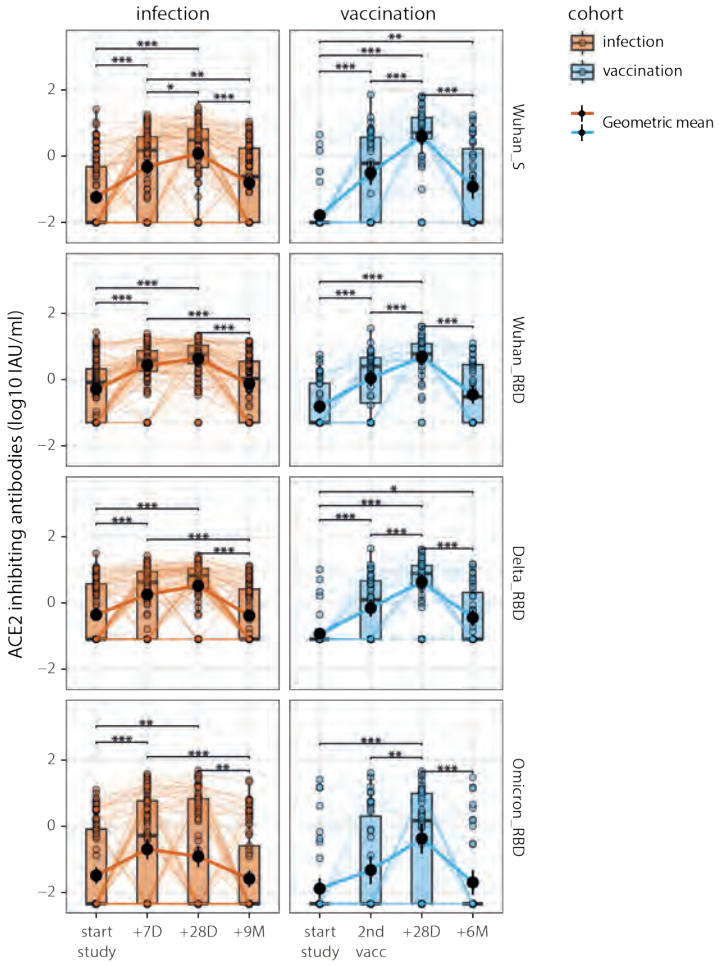
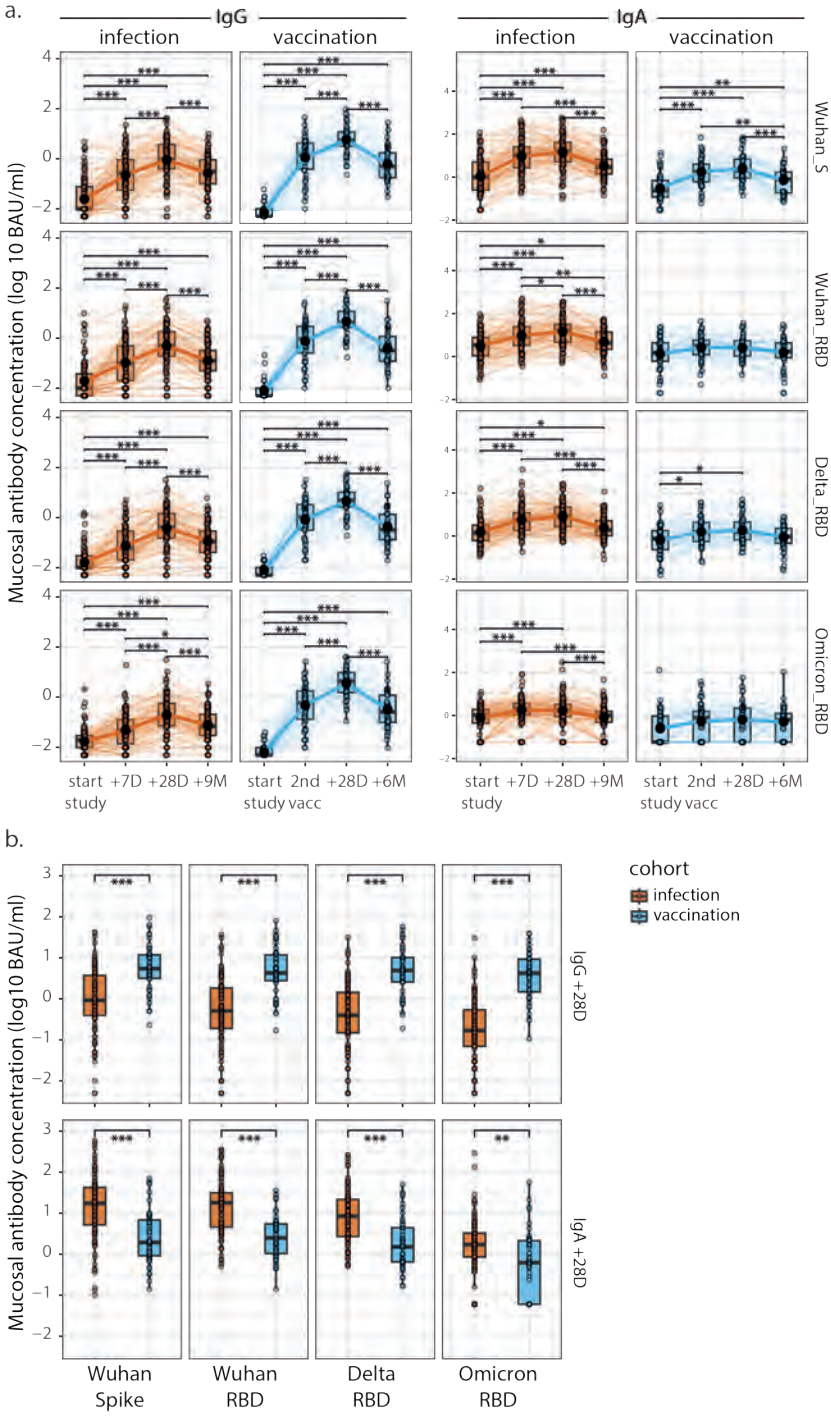


Figure 1. ACE2 inhibiting activity of mucosal antibodies increases shortly after infection and vaccination, but wanes over time. ACE2 binding inhibition levels (log10 BAU/mL) against the ancestral Wuhan-Hu-1 S and RBD, and Delta and Omicron RBD, at the different timepoints (infection cohort: start study, +7 days, +28 days and +9 months post study start; vaccination cohort: start study, moment of second vaccination (28 days post first), +28 days, and +6 months post second vaccination. The geometric mean of each cohort is depicted as a solid line and black solid dots, together with the confidence interval. Differences over time are measured using the Friedman's with post hoc Dunn's test. Orange: infection cohort, blue: vaccination cohort. P-values *p <0.05, **p<0.01, *** p<0.001.


a.
IgG
IgA
infection
vaccination
infection
vaccination
Wuhan_S
Wuhan_RBD
Delta_RBD
Omicron_RBD
Mucosal antibody concentration (log 10 BAU/ml)
start +7D +28D +9M
study
start 2nd +28D +6M
study vacc
b.
cohort
infection
vaccination
IgG +28D
IgA +28D
Mucosal antibody concentration (log10 BAU/ml)
Wuhan Spike
Wuhan RBD
Delta RBD
Omicron RBD

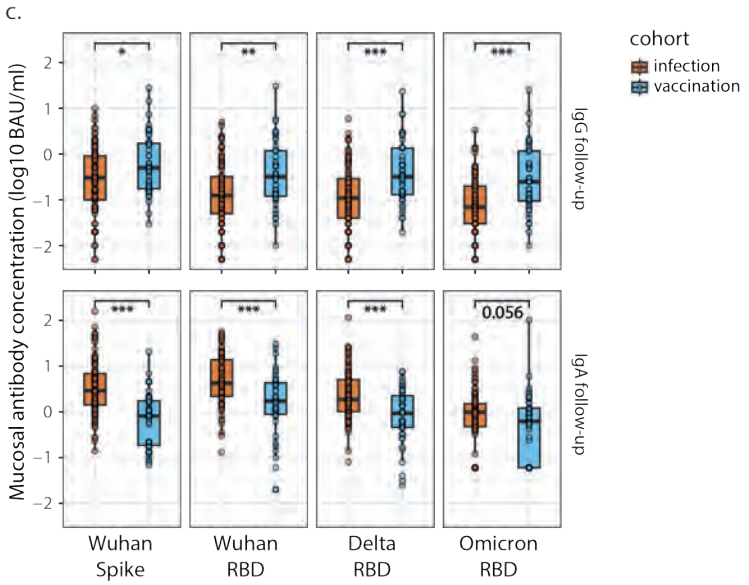


Figure 2. Concentrations of mucosal IgA and IgG depend on exposure type. **a)** Mucosal IgG and IgA concentrations (log10 IU/mL) against the ancestral Wuhan-Hu-1 S and RBD, and Delta and Omicron RBD, at the various timepoints (infection cohort: start study, +7 days, +28 days and +9 months post study start; vaccination cohort: start study, moment of second vaccination (28 days post first), +28 days, and +6 months post second vaccination). The geometric mean of each cohort is depicted as a solid line and black solid dots, together with the confidence interval. **b)** Peak (+28D) IgG and IgA concentrations against the ancestral Wuhan-Hu-1 S and RBD and Delta and Omicron RBD. **c)** Follow-up (+9M for the infection and +6M for the vaccination cohort) IgG and IgA concentrations against the ancestral Wuhan-Hu-1 S and RBD and Delta and Omicron RBD. Differences over time are measured using the Friedman's with post hoc Dunn's test. Differences between the groups are calculated using the Wilcoxon rank sum test. Orange: infection cohort, blue: vaccination cohort. P-values *p <0.05, **p<0.01, *** p<0.001.

Type of primary exposure determines underlying mechanisms of ACE2 inhibition

To investigate the relationship between mucosal antibody concentrations and ACE2 binding inhibition, we correlated the antibody responses with the ACE2 binding inhibition values. For both cohorts, IgG and IgA concentrations showed significant positive correlations with ACE2 binding inhibition. Interestingly, many participants in the infection cohort showed high ACE2 inhibition capacity at low IgG concentrations, suggesting that other antibody classes contribute to neutralization. Indeed, in the infection cohort ACE2 inhibition correlated stronger with IgA than with IgG (R ranges from 0.09-0.65 for IgG and 0.40-0.81 for IgA, **Figure 3a**). Vaccination-induced ACE2 binding inhibition was most strongly correlated with IgG (R ranges from 0.82-0.95 for IgG and 0.51-0.87 for IgA, **Figure 3a**).

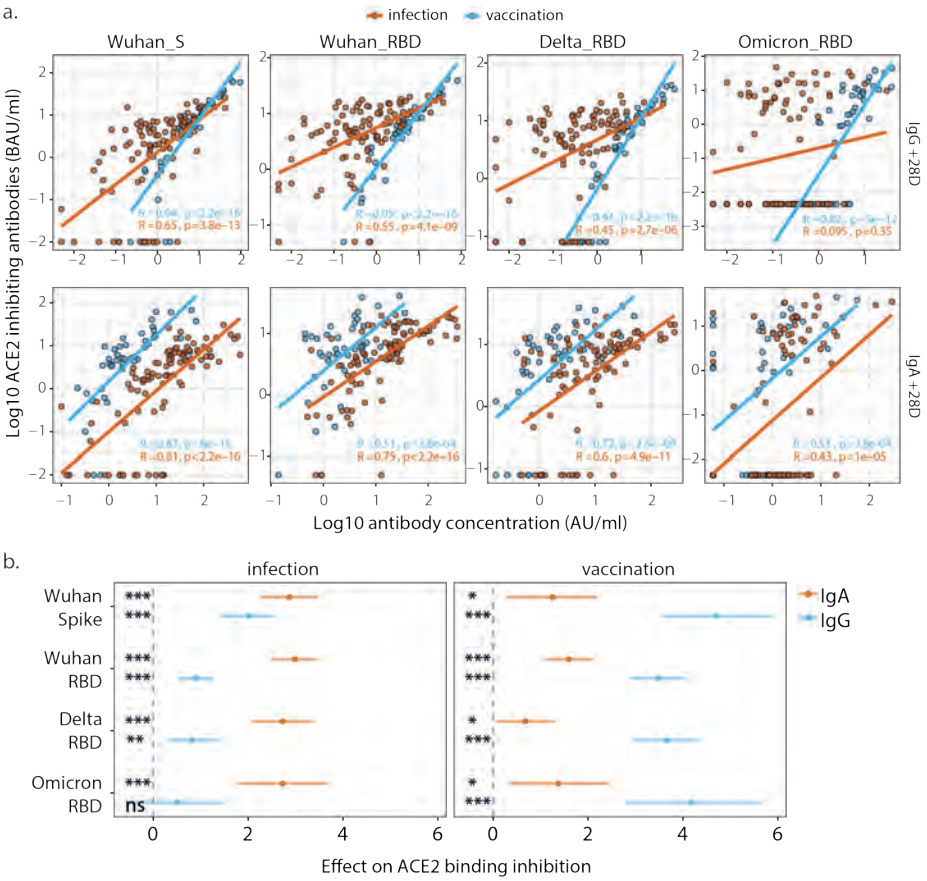


Figure 3. ACE2 inhibiting activity after infection is driven more by IgA, while post-vaccination inhibition is mainly IgG driven. **a.)** Correlations of IgA and IgG concentrations (log10 BAU/mL) and ACE2 inhibiting activity of mucosal antibodies (log10 IAU/mL). Spearman correlations were performed, and the R and p-value are depicted for each cohort. Orange: infection cohort, blue: vaccination cohort. P-values *p <0.05, **p<0.01, *** p<0.001. **b.)** The predicted change in ACE2 binding inhibition per unit increase of the scaled antibody level is presented with 95% confidence intervals based on the standard error of the mean, and two-sided P values for the association are plotted on the right, corrected for multiple testing with the Bonferroni method.

We know that other factors contribute to the variation in ACE2 binding inhibition, like time since exposure and age of the participant. To assess the contribution of antibody isotype to ACE2 inhibition while correcting for the influence of age and time, we performed a linear mixed effects model per cohort and variant. In this model, ACE2 inhibition was the outcome and study day, age, and the scaled IgA and IgG concentrations were covariates. The predictions from this linear model fitted well with the observed data, although the out-of-range values were not predicted as well

and increased the AIC (**Figure S6a** and **S6b**, and **Table S1**). We observed that for both cohorts the quantity of IgG and IgA had a significant and positive effect on the ACE2 inhibiting capacity. After infection, mainly IgA contributes to ACE2 inhibition. This IgA dominance becomes more prominent against the VOCs, with IgA having a larger effect on ACE2 inhibition than IgG in Delta and Omicron (**Figure 3b**). Conversely, ACE2 inhibition was mostly driven by IgG in the vaccination cohort, against both the ancestral variant and the VOCs.

Discussion

Blocking the interaction of SARS-CoV-2 with the ACE2 receptor is an essential function of mucosal antibodies to prevent or reduce SARS-CoV-2 infection and replication[1,20,21]. Differences in functionality between infection and vaccination-induced mucosal antibodies have not been extensively investigated. We compared the mucosal antibody response after primary infection and primary vaccination. Although ACE2 inhibiting activity did not differ between the infection and the vaccination cohort, we found that infection induced higher mucosal IgA, both at 28 days, and at 6 or 9 months. Importantly, we show that the way mucosal antibodies neutralize SARS-CoV-2 differs based on the type of primary exposure. After correcting for age and timepoints, infection-induced ACE2 binding inhibition was mainly driven by mucosal IgA, while post-vaccination this was mostly mucosal IgG.

Our cohorts consisted of individuals exposed to the Wuhan-Hu-1 strain, by vaccination or by infection. Consequently, and similar to previously published data, we observed lower responses towards the Omicron variant[22-26]. The vaccination cohort showed higher inhibition against the Wuhan Spike protein at day 28 than the infection cohort, in line with previous publications[27,28]. No differences between the infection and the vaccination cohort were found with regards to ACE2 binding inhibition against the other VOCs. An explanation for the lower ACE2 inhibiting activity post-infection for Wuhan Spike may be that, as an effect of viral load and duration of infection, there is a more variable level of antigen exposure post-infection. Contrastingly, vaccination is performed with a standard human dose of mRNA.

Although we show that both infection and vaccination induce mucosal IgA, the IgA concentrations after infection were significantly higher than after vaccination, especially at the follow-up timepoint, which was striking considering that the post-infection samples were collected three months later than the post-vaccination

samples. Furthermore, increased IgA concentrations post-vaccination were only found against the Wuhan-Hu-1 Spike protein. This is not unexpected, as it is known that intramuscular vaccination mainly elicits an IgG response[29,30] and IgA is generally not effectively transported to the mucosal surface[6,11,31].

For IgG, we found that the infection cohort had less significant and more variable responses over baseline than the vaccination cohort. This could partly be due to the cohort selection, as some participants of the infection cohort were not yet infected at study start, whilst others had already been infected for ~6 days at study start (**Figure S3**). The antibody response will therefore not be completely synchronized for the infection cohort, as opposed to the vaccination cohort.

The observation that infection induces a broader antibody profile than vaccination has been described previously[32-35]. In the case of parenteral influenza vaccines, vaccination induces an IgG response, but fails to induce mucosal IgA responses, while infection gives rise to both IgG and IgA formation[33]. A study examining memory B-cell responses after SARS-CoV-2 infection and vaccination showed that the memory B-cells after infection evolved for a longer period, resulting in a greater potency and breadth than vaccine-induced memory B-cells[36].

In this study, we have used an ACE2 binding inhibition assay to analyze MLF, following a similar approach as has been published before[16]. By including a dilution series of an external standard on each plate and fitting the results to the standard curve, we were able to account for batch- and dilution effects (**Figure S7a** and **S7b**). However, we recognize that the sensitivity of the ACE2 binding inhibition assay on MLF as compared to the MIA assay is lower, resulting in some out-of-range samples that were given an arbitrary low value (**Figure 3a**). We chose to include these samples in the correlation assay and modeling, to account for the limitations of the measurement. Overall trends in the correlation and modeling analyses stayed the same when we performed a sensitivity analysis with only the in-range values, although confidence intervals increased due to a significantly lower sample size. Our assay showed high correlation with the PRNT, the gold standard for measuring neutralizing capacity in serum (**Figure S1**). The ACE2 binding inhibition assay is not dependent on cell-culture and because of the bead-based approach, additional variants can be easily added. Moreover, we demonstrate that the assay, albeit being a bit less sensitive, is able to detect changes in functional mucosal antibodies, making it an attractive method for clinical or epidemiological studies.

This study has several limitations. First, because of the use of two cohorts from different studies, age and sampling timepoints did not completely match. The vaccination cohort was significantly older than the infection cohort, mostly due to the fact that the infection cohort also included children. However, in multivariable analysis, the effect of age did not remain significant in any of the models, so we do not consider this to be an issue in the final model. Second, the late follow-up timepoints for the two cohorts differed, precluding direct comparison between the cohorts. We thus cannot exclude that the observations at this time point are the result of differences in timing. However, given the fact that the IgA concentrations in the infection cohort are higher at nine months than the IgA concentrations in the vaccination cohort at six months, the higher IgA concentration in the infection cohort as compared to the vaccination cohort most likely represents a true, biological effect. Finally, we recognize that antibodies do not tell the complete story of protection. We did not analyze (memory) T and B cell responses, or other antibody functionalities, which could play an important role in protection too[37,38].

In conclusion, we have analyzed the mucosal antibody response to primary exposure to SARS-CoV-2 as a result of infection or of Spikevax vaccination. Our study found that although the ACE2 binding inhibition capacities in both cohorts were similar, it was dependent on different antibody isotypes.

Acknowledgements

We would like to thank all MuCo and RECOVAC study participants for their willingness to participate and their support for the study. Moreover, we thank J.F. Sanders, D. van Baarle, A.L. Messchendorp, C.C. Baan, F.J. Bemelman, M.E.J. Reinders, G. den Hartog, R. van Binnendijk, R.T. Gansevoort, M.M.L. Kho and E.B.M. Remmerswaal of the RECOVAC consortium for their help and support. Special thanks to Fred van Opzeeland, Liana Barenbrug, Joyce Rust and Wouter Mattheussens for their contribution to the experimental work and study logistics. The MuCo study was funded by the Laboratory of Medical Immunology, Radboud University Medical Center, and the RECOVAC consortium was funded by ZonMW and the Nierstichting.

References

1. Froberg J, Diavatopoulos DA. Mucosal immunity to severe acute respiratory syndrome coronavirus 2 infection. *Curr Opin Infect Dis* 2021; **34**(3): 181-6.
2. Hoffmann M, Kleine-Weber H, Schroeder S, et al. SARS-CoV-2 cell entry depends on ACE2 and TMPRSS2 and is blocked by a clinically proven protease inhibitor. *Cell* 2020; **181**(2): 271-80. e8.
3. Shang J, Wan Y, Luo C, et al. Cell entry mechanisms of SARS-CoV-2. *PNAS* 2020; **117**(21): 11727-34.
4. Khatri I, Staal FJ, Van Dongen JJ. Blocking of the high-affinity interaction-synapse between SARS-CoV-2 spike and human ACE2 proteins likely requires multiple high-affinity antibodies: an immune perspective. *Front Immunol* 2020; **11**: 570018.
5. Fröberg J, Gillard J, Philipsen R, et al. SARS-CoV-2 mucosal antibody development and persistence and their relation to viral load and COVID-19 symptoms. *Nat Commun* 2021; **12**(1): 1-11.
6. Russell MW, Moldoveanu Z, Ogra PL, Mestecky J. Mucosal Immunity in COVID-19: A Neglected but Critical Aspect of SARS-CoV-2 Infection. *Front Immunol* 2020; **11**: 611337.
7. Roltgen K, Boyd SD. Antibody and B cell responses to SARS-CoV-2 infection and vaccination. *Cell Host Microbe* 2021; **29**(7): 1063-75.
8. Ravichandran S, Grubbs G, Tang J, et al. Systemic and mucosal immune profiling in asymptomatic and symptomatic SARS-CoV-2–infected individuals reveal unlinked immune signatures. *Sci Adv* 2021; **7**(42): eabi6533.
9. Wright PF, Prevost-Reilly AC, Natarajan H, et al. Longitudinal systemic and mucosal immune responses to SARS-CoV-2 infection. *J Infect Dis* 2022; **226**(7): 1204-14.
10. Callow KA. Effect of specific humoral immunity and some non-specific factors on resistance of volunteers to respiratory coronavirus infection. *J Hyg (Lond)* 1985; **95**(1): 173-89.
11. Tang J, Zeng C, Cox TM, et al. Respiratory mucosal immunity against SARS-CoV-2 after mRNA vaccination. *Sci Immunol* 2022; **7**(76): eadd4853.
12. Laidlaw BJ, Ellebedy AH. The germinal centre B cell response to SARS-CoV-2. *Nat Rev Immunol* 2022; **22**(1): 7-18.
13. Guerrieri M, Francavilla B, Fiorelli D, et al. Nasal and Salivary Mucosal Humoral Immune Response Elicited by mRNA BNT162b2 COVID-19 Vaccine Compared to SARS-CoV-2 Natural Infection. *Vaccines (Basel)* 2021; **9**(12).
14. Sanders JF, Bemelman FJ, Messchendorp AL, et al. The RECOVAC Immune-response Study: The Immunogenicity, Tolerability, and Safety of COVID-19 Vaccination in Patients With Chronic Kidney Disease, on Dialysis, or Living With a Kidney Transplant. *Transplantation* 2022; **106**(4): 821-34.
15. WHO. First WHO International Standard for anti-SARS-CoV-2 immunoglobulin (human). 2020.
16. Junker D, Dulovic A, Becker M, et al. COVID-19 patient serum less potently inhibits ACE2-RBD binding for various SARS-CoV-2 RBD mutants. *Sci Rep* 2022; **12**(1): 7168.
17. den Hartog G, Schepp RM, Kuijer M, et al. SARS-CoV-2-Specific Antibody Detection for Seroepidemiology: A Multiplex Analysis Approach Accounting for Accurate Seroprevalence. *J Infect Dis* 2020; **222**(9): 1452-61.
18. Okba NM, Müller MA, Li W, et al. Severe acute respiratory syndrome coronavirus 2– specific antibody responses in coronavirus disease patients. *Emerg Infect Dis* 2020; **26**(7): 1478.
19. R Core Team R. R: A language and environment for statistical computing. 2013.
20. Jiang S, Zhang X, Yang Y, Hotez PJ, Du L. Neutralizing antibodies for the treatment of COVID-19. *Nat Biomed Eng* 2020; **4**(12): 1134-9.

21. Shi R, Shan C, Duan X, et al. A human neutralizing antibody targets the receptor-binding site of SARS-CoV-2. *Nature* 2020; **584**(7819): 120-4.

22. Toh ZQ, Mazarakis N, Nguyen J, et al. Comparison of antibody responses to SARS-CoV-2 variants in Australian children. *Nat Commun* 2022; **13**(1): 7185.

23. Cheng SMS, Mok CKP, Leung YWY, et al. Neutralizing antibodies against the SARS-CoV-2 Omicron variant BA.1 following homologous and heterologous CoronaVac or BNT162b2 vaccination. *Nat Med* 2022; **28**(3): 486-9.

24. Qu P, Faraone JN, Evans JP, et al. Enhanced evasion of neutralizing antibody response by Omicron XBB.1.5, CH.1.1, and CA.3.1 variants. *Cell Rep* 2023; **42**(5): 112443.

25. Dejnirattisai W, Shaw RH, Supasa P, et al. Reduced neutralisation of SARS-CoV-2 omicron B.1.1.529 variant by post-immunisation serum. *Lancet* 2022; **399**(10321): 234-6.

26. Pajon R, Doria-Rose NA, Shen X, et al. SARS-CoV-2 Omicron Variant Neutralization after mRNA-1273 Booster Vaccination. *N Engl J Med* 2022; **386**(11): 1088-91.

27. Bekliz M, Adea K, Vetter P, et al. Neutralization capacity of antibodies elicited through homologous or heterologous infection or vaccination against SARS-CoV-2 VOCs. *Nat Commun* 2022; **13**(1): 3840.

28. Hojjat Jodaylami M, Djaileb A, Ricard P, et al. Cross-reactivity of antibodies from non-hospitalized COVID-19 positive individuals against the native, B.1.351, B.1.617.2, and P.1 SARS-CoV-2 spike proteins. *Sci Rep* 2021; **11**(1): 21601.

29. Durrer P, Gluck U, Spyr C, et al. Mucosal antibody response induced with a nasal virosome-based influenza vaccine. *Vaccine* 2003; **21**(27-30): 4328-34.

30. Brokstad KA, Eriksson JC, Cox RJ, et al. Parenteral vaccination against influenza does not induce a local antigen-specific immune response in the nasal mucosa. *J Infect Dis* 2002; **185**(7): 878-84.

31. Horton RE, Vidarsson G. Antibodies and their receptors: different potential roles in mucosal defense. *Front Immunol* 2013; **4**: 200.

32. Nachbagauer R, Choi A, Hirsh A, et al. Defining the antibody cross-reactome directed against the influenza virus surface glycoproteins. *Nat Immunol* 2017; **18**(4): 464-73.

33. Krammer F. The human antibody response to influenza A virus infection and vaccination. *Nat Rev Immunol* 2019; **19**(6): 383-97.

34. Meyers J, Windau A, Schmotzer C, et al. SARS-CoV-2 antibody profile of naturally infected and vaccinated individuals detected using qualitative, semi-quantitative and multiplex immunoassays. *Diagn Microbiol Infect Dis* 2022; **104**(4): 115803.

35. Assis R, Jain A, Nakajima R, et al. Distinct SARS-CoV-2 antibody reactivity patterns elicited by natural infection and mRNA vaccination. *NPJ Vaccines* 2021; **6**(1): 132.

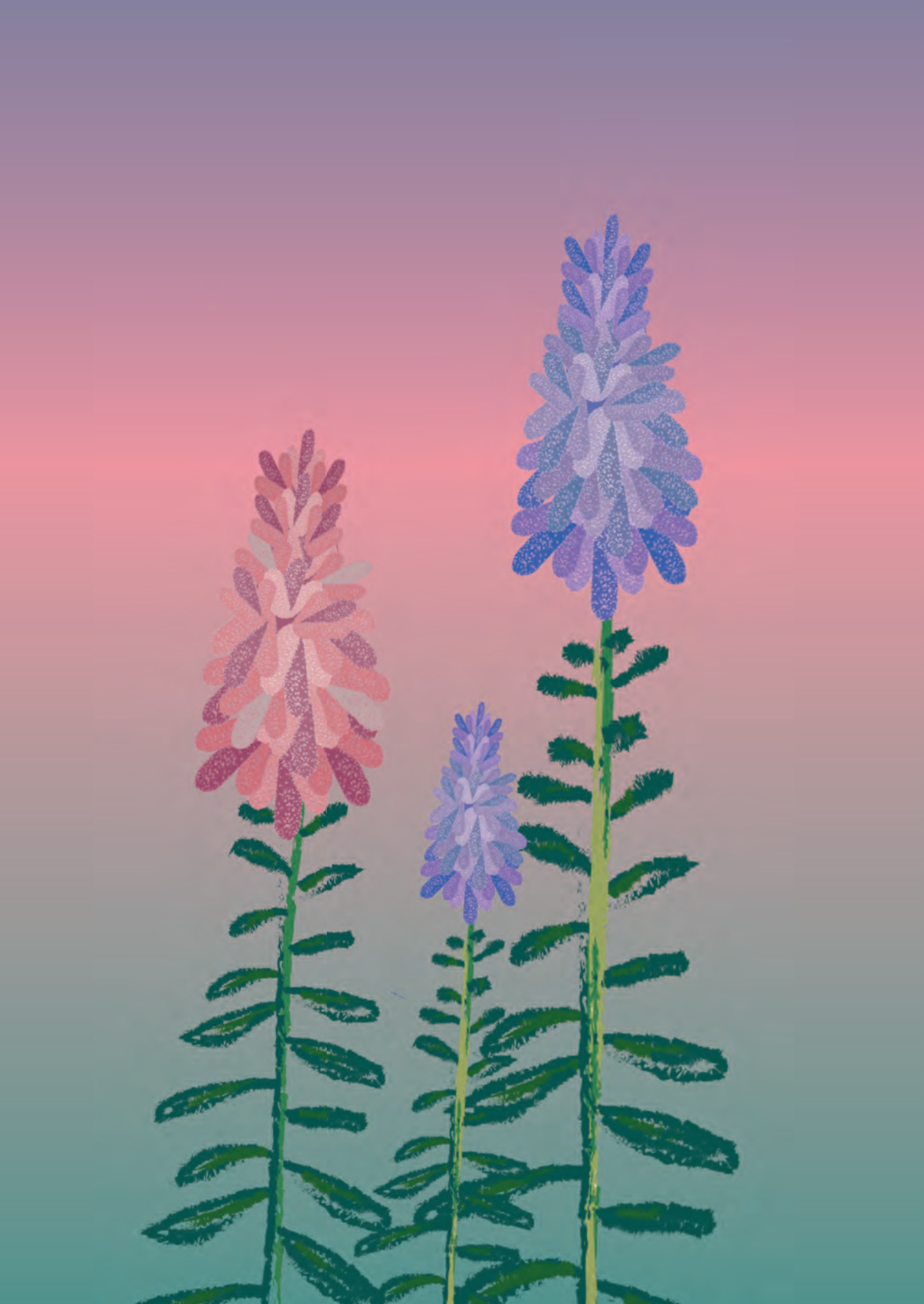
36. Cho A, Muecksch F, Schaefer-Babajew D, et al. Anti-SARS-CoV-2 receptor-binding domain antibody evolution after mRNA vaccination. *Nature* 2021; **600**(7889): 517-22.

37. Cox RJ, Brokstad KA. Not just antibodies: B cells and T cells mediate immunity to COVID-19. *Nat Rev Immunol* 2020; **20**(10): 581-2.

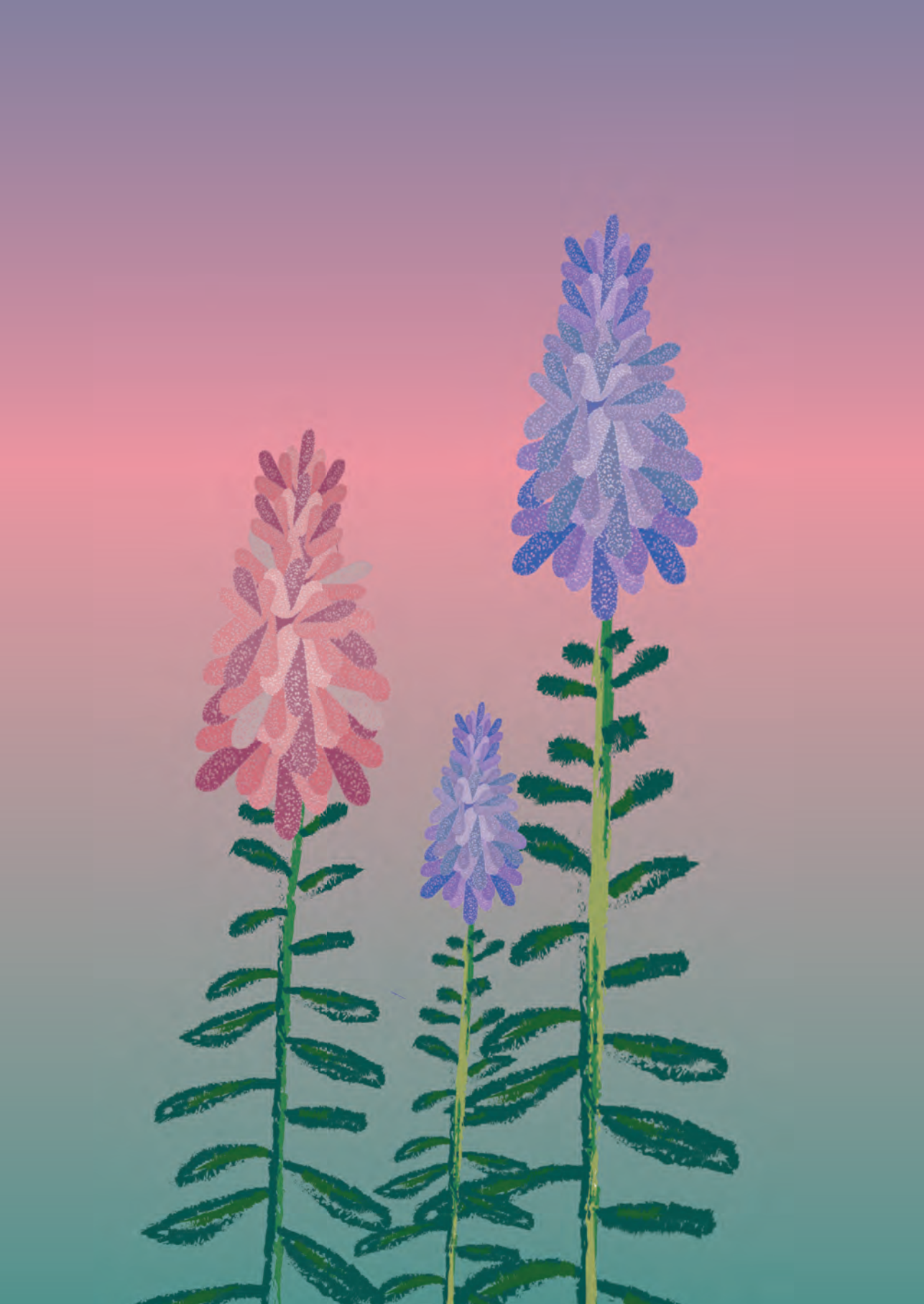
38. Moss P. The T cell immune response against SARS-CoV-2. *Nat Immunol* 2022; **23**(2): 186-93.

Supplementary materials

Scan the QR code for the supplementary materials:



Section II: *Bordetella pertussis*



Chapter 5

Mucosal immune responses to *Bordetella pertussis* in Gambian infants following maternal and primary vaccination: an immunological sub-study of a double-blinded, randomized, controlled, phase 4 trial

Fröberg J,[1 2*] Saso A,[3 4*] Jobe H,[3] Eleveld M,[1,2] Okoye M,[3] Kanteh E,[3] van Opzeeland F,[1,2] Kumado M,[3] Faal A,[3] Roberts E,[3] Fofana M,[3] Baldeh Aru-Kumba,[3] Conteh Karamo,[3] van Cranenbroek B,[1,2], Roetynck S,[3] de Silva TI,[3 4 5 6] Huynen M,[7] Kampmann B,[3 4 8**] Diavatopoulos D A,[1 2**] on behalf of the GaPs Study Team.[1]

** and ** contributed equally*

[1.] Laboratory Medical Immunology, RadboudUMC, Nijmegen, the Netherlands.

[2.] Research Center of Infectious Diseases, RadboudUMC, Nijmegen, the Netherlands

[3.] Vaccines & Immunity Theme, MRC Unit The Gambia at the London School of Hygiene and Tropical Medicine, Fajara, The Gambia

[4.] Faculty of Infectious and Tropical Diseases, London School of Hygiene & Tropical Medicine, London, United Kingdom

[5.] Division of Clinical Medicine, School of Medicine and Population Health, University of Sheffield, Sheffield, United Kingdom

[6.] Florey Institute of Infection and NIHR Sheffield Biomedical Research Centre, University of Sheffield, Sheffield, United Kingdom

[7.] Center for Molecular and Biomolecular Informatics, Radboudumc, 6525 GA Nijmegen, the Netherlands.

[8.] Charité Centre for Global Health, Charité Universitätsmedizin Berlin, Germany.

Under review

Abstract

Background

Pertussis, or whooping cough, is a highly contagious respiratory disease caused by the bacterium *Bordetella pertussis* (*Bp*). Mucosal protection relies on the induction of antibodies and the activation of mucosal tissue-resident memory cells which, together, recognise and neutralise *Bp* and/or its secreted products and mediate *Bp* clearance. Evidence from epidemiological and animal studies suggest differences in the ability of available pertussis vaccines, acellular (aP) versus whole-cell (wP), to induce effective mucosal immunity. We aimed to investigate the impact of primary aP versus wP vaccination on mucosal immunity to *Bp* in infants, in context of their mothers having received aP in pregnancy or not.

Methods

We conducted an immunological sub-study embedded within the Gambian Pertussis Study (GaPs), a randomized, controlled phase 4 trial which compared the immunogenicity of aP versus wP infant vaccines in Gambian infants, born to women who had received aP during pregnancy and a control group who had received Tetanus-Toxoid only. Using nasosorption devices, nasal mucosal lining fluid (MLF) was collected from 160 infants at multiple time points before and after completion of their primary immunization series. We measured antibody binding to whole *Bp* bacteria, as well as levels of T-cell associated cytokines in MLF.

Findings

We demonstrated that maternally-derived pertussis-specific IgG but not IgA antibodies are detectable in infant MLF up to 2 months of age. Post-vaccination, wP-vaccinated infants showed significantly higher nasal *Bp*-binding IgG levels, as well as activation of a broad nasal T-cell associated cytokine response. There was significant blunting of *Bp*-binding IgG levels in aP-vaccinated infants born to mothers who had received aP during pregnancy, even in infants with low *Bp*-binding IgG pre-primary immunization.

Interpretation

IgG antibodies migrate from the mother to their infant's upper airway, where they recognise and bind to *Bp,* thereby playing a potential role in *Bp* clearance. Furthermore, our findings indicate local cellular activation following wP-vaccination in infants, supporting previous observations in animal models.

Research in context

Evidence before this study

On July 2, 2024, we searched PubMed for articles that investigated the effects of maternal and primary pertussis vaccination on the mucosal antibody levels of the infant. We used the search (pertussis [ti] OR "whooping cough" [ti]) AND (vaccination[tiab] OR immunization [tiab] OR Tdap [tiab] OR dtap [tiab] OR DTP [tiab] OR aP [tiab] OR wP [tiab] OR whole-cell [tiab] OR acellular [tiab]) AND (antibody OR antibodies OR IgG OR IgA) AND (mucosal[tiab] OR nasal [tiab] OR local [tiab] OR secretory [tiab] OR nose [tiab] OR airway [tiab] OR lungs[tiab]). Based on the results generated, we could not identify any human studies that systematically compared immune responses following aP versus wP primary vaccination in the context of either the presence or absence of maternal pertussis immunization during pregnancy, nor any that determined mucosal immune responses in infants, particularly in low-and-middle-income countries (LMICs). Animal studies, however, have shown that aP vaccination is effective at preventing severe disease but may not prevent colonization and subsequent transmission. This may be attributable to a lack of induction of local cellular responses in the upper airways, although whether the same applies to infants has not yet been investigated.

Added value of this study

Our study aimed to assess the impact of maternal and primary pertussis vaccination on the immune response in the upper airway of Gambian infants. The GaPs trial had a unique study design and methodology that allowed us to investigate the effects of four different vaccination strategies at the mucosal site in infants. We were able to measure mucosal antibody binding to *Bordetella pertussis (Bp)*, as well as the presence of cytokines in nasal fluid following vaccination. Prior to primary vaccination, we found high levels of mucosal *Bp*-binding IgG in infants born to TdaP-IPV vaccinated mothers. Following vaccination, we observed that - unlike wP vaccination - aP following a maternal TdaP-IPV vaccine blunted mucosal IgG antibody binding to *Bp* in the infants, even when maternal antibody levels were low.

Implication of all available evidence

Our study shows the positive impact of pertussis vaccination in pregnancy on mucosal antibody levels in the early postnatal period. However, primary vaccination with aP following maternal pertussis immunization resulted in lower *Bp*-binding IgG levels in infants post-vaccination (blunting). Importantly, aP vaccination did not generate strong mucosal cytokine responses when compared to wP vaccination. Although the clinical consequences of these results remain unknown, our study

supports the hypothesis that aP vaccination is less protective against colonization, and an improved vaccine is therefore needed. Furthermore, minimally-invasive mucosal sampling in combination with standardized immune assays may be useful and relevant in future trials involving vaccinating pregnant women and/or their infants. Mucosal responses, being the first line of defence, should be considered in the decision-making on immunization schedules, especially if introducing vaccination during pregnancy or switching from wP to aP primary vaccination schedules in LMICs.

Introduction

Pertussis, or whooping cough, is an acute respiratory disease caused by the bacterium *Bordetella pertussis* (*Bp*).[1] It remains a significant, vaccine-preventable respiratory disease worldwide, with notable outbreaks recently, even in areas of high vaccine coverage.[2] Currently, two types of childhood vaccines are licensed: whole-cell pertussis vaccines (wP), which are mainly used in low- and middle-income countries (LMICs), and acellular pertussis vaccines (aP), which are mainly used in high-income countries (HICs).[3-5] wP vaccines are considered more effective at reducing disease and transmission, at the cost of higher reactogenicity.[3] Conversely, although aP vaccines provide robust protection against severe pertussis and have fewer side effects, they are also less effective at preventing *Bp* infection and therefore onward transmission.[3,6-8] Mucosal immunity in the upper airway is crucial for providing protection at the population level, by limiting *Bp* transmission, and the individual level, by preventing *Bp* spread to the lungs.[9,10] This protection is thought to rely on the induction of antibodies and the activation of mucosal tissue-resident memory cells which, together, recognise and neutralise *Bp* and/or its secreted products and mediate *Bp* clearance.[9,11,12]

Infants who are not yet fully vaccinated are at the highest risk of severe pertussis complications, hospitalisation, and death.[1] To mitigate the risk of severe pertussis in young infants, many HICs have introduced aP vaccinations during pregnancy. This allows maternally derived antibodies to be transferred across the placenta or through breast milk postnatally, thereby providing passive, disease-specific protection for infants[13]. A recent systematic review showed that maternal vaccination is 78-96% clinically effective against pertussis in infants from 0-3 months old.[14] However, studies have also shown that maternal antibodies may reduce or 'blunt' antibody responses to subsequent routine childhood vaccines.[15-17] At present, to the best of our knowledge, there are no studies that

have comprehensively investigated how maternal and primary infant pertussis vaccination affect mucosal immunity to *Bp*. Understanding this interplay may guide future vaccination strategies, including decision-making on the schedule and timing of routine childhood immunization programmes.

Here we address this knowledge-gap through an immunological sub-study embedded in the randomized, controlled phase 4 mother-infant vaccine trial, the Gambian Pertussis Study (GaPs), which investigated the effect of pertussis immunization during pregnancy and primary pertussis vaccination with aP or wP series on mucosal immunity to *Bp* in infants within four vaccination groups [protocol accepted publication]. Using a minimally-invasive nasosorption device, we collected nasal mucosal lining fluid (MLF) from infants at multiple time points before and after completion of their primary immunization series. We measured antibody binding to whole *Bp* bacteria, as well as levels of T-cell associated cytokines in MLF.

Methods

Study design and participants

The Gambian Pertussis Study (GaPs) was a phase 4 double-blinded, open-label, randomized, controlled, single-centre trial completed in The Gambia, a LMIC in West Africa. GaPs was conducted as part of the PERISCOPE (PERtussis Correlates Of Protection Europe) programme and full details of the trial, including primary endpoints, have been described elsewhere[18]. In brief, pregnant women (n=343) were randomized (1:1) to receive a dose of either Tetanus-Toxoid (TT, SIPPL) (n=172), or a Tetanus-diphtheria-aP-inactivated-polio-virus combination vaccine (Tdap-IPV, GSK) (n=171) at 28-34 weeks' gestation. Their (future) infants were randomized (1:1) at the same step and went on to receive the wP-containing (DTwP-HBV-HiB, 'DTwP' from herein) (n=161) or aP-containing (DTaP-HBV-HiB-IPV, 'DTaP' from herein) (n=166) primary immunization series, as part of the Expanded Programme of Immunization (EPI) schedule at 8 (8W), 12 (12W), and 16 weeks (16W) [protocol paper]. This paper describes the results of one of the trial's exploratory outcomes, antibody and cytokine profiles in the infant upper airway pre- and post-immunization. These endpoints were measured in a subset of infants from the GaPs trial (n=160, **Figure 1**), randomly selected across the four vaccine groups, for whom MLF and paired cord blood and serum samples were available. All participants in this substudy had completed the study per protocol [protocol paper].

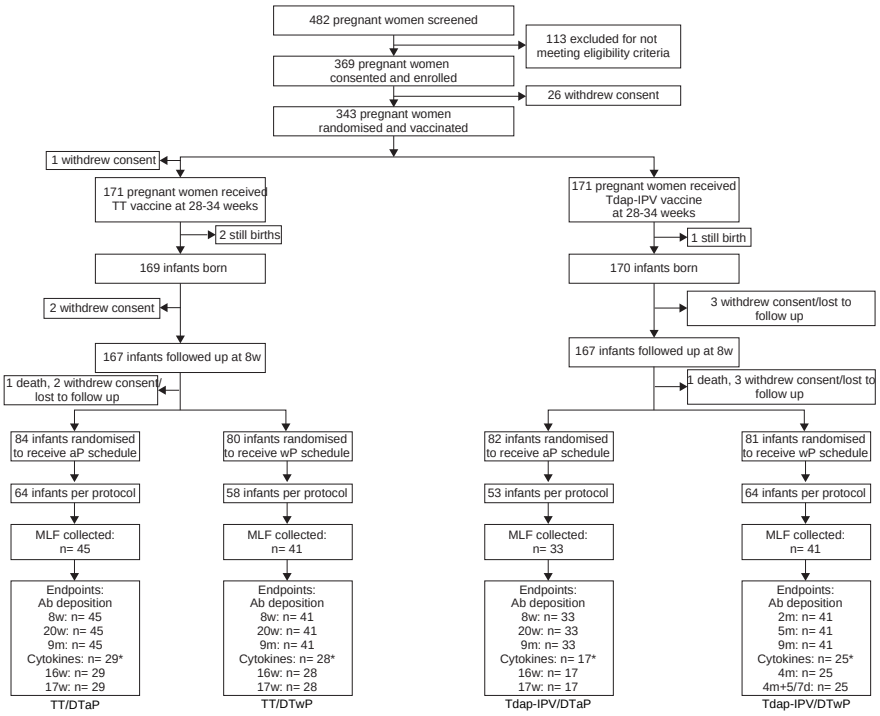


Figure 1. Flow of study participants through the GaPs trial and our embedded immunological substudy. *cytokine measurements were only done if sufficient nasal mucosal lining fluid sample was available.

The trial was registered under NCT03606096 and conducted in accordance with the Declaration of Helsinki (2013) and Good Clinical Practice guidelines. Approval was given by the Gambian Government and MRCG Joint Ethics Committee, the LSHTM Research Ethics Committee, and the Gambian Medicines Control Agency.

Collection mucosal lining fluid (MLF) and blood samples

The sampling timepoints and vaccine schedule are summarised in **Figures 1** and **S1**. We implemented a standardized, minimally-invasive sampling method to collect nasal MLF from infants at 8, 16, 17, and 20-weeks (8W, 16W, 17W, 20W), and at 9-months (9M) of age. A nasosorption device, consisting of a synthetic absorptive matrix (SAM) strip, was gently inserted into infant's nostril and placed along the surface of the inferior turbinate ('mucosal lining'). The index finger was lightly pressed against the side of the nostril for 60 seconds, after which the SAM strip was removed and kept at 4°C until elution.

MLF was eluted within 4 hours of collection, by adding 300uL buffer (AB-33K, Millipore) containing 0.05% Tween-20 and 1% BSA at 4°C, and vortexing for 30 seconds[19]. Using sterile forceps, the SAM strip was then transferred into a spin column (without 0.22 µm cellulose acetate filter) and centrifuged in the original microcentrifuge tube (16000xg, 20mins, 4°C)[19]. Cord blood was collected at birth and peripheral blood samples were collected from infants at 8W, 20W and 9M of age, and serum was subsequently separated. All sample types were aliquoted and stored at -80°C prior to analysis.

Quantification of Bp-specific IgG/IgA antibody binding in MLF and blood

Bp strain B1917 was cultured in complete THIJS medium according to standard conditions[20], labelled with Oregon-Green 488 (Thermo Fisher, catnr: O6149) and cryopreserved at -80°C in DMSO in single-use aliquots. Heat-inactivated (56°C), undiluted MLF or 1:200 diluted serum/cord blood was incubated with $2x10^6$ CFUs of fluorescently-labelled bacteria in 96-wells plates for 30 minutes, at 37°C with 5% CO2, while shaking. Following incubation, bacteria were washed and fixed in 2% paraformaldehyde for 20 minutes at room temperature. Next, the bacteria were washed and stained with a secondary antibody mix, containing PBS + 2% BSA with 1:500 Alexa Fluor® 647 AffiniPure Goat Anti-Human Serum IgA, α-chain-specific (Jackson immunoResearch, Lot. 162504) and 1:500 DyLight™ 405 AffiniPure Goat Anti-Human IgG, Fcγ-fragment-specific (Jackson immunoResearch, Lot. 145455). After incubation for 15 minutes at room temperature, samples were measured by flow cytometry (Beckman Coulter CytoFLEX®). Bacteria were gated based on Oregon Green 488 staining and FSC-SSC parameters, resulting in mean fluorescent intensities (MFI) of IgG and IgA. MFI was subsequently fitted to a standard curve consisting of pooled plasma from vaccinated and convalescent pertussis patients with a 5-parameter logistic regression, resulting in arbitrary units (AU).

For each analysis, longitudinal paired MLF or serum/cord blood samples from the same individual were run on the same plate; with the four vaccine groups randomized across each plate. *Bp* incubated with PBS + 2% BSA were used to correct for background signal. Plasma samples of three convalescent pertussis patients were used as quality control on every plate. Data were analysed in FlowJo™ 10.8 and GraphPad Prism 9.

Quantification of cytokine concentrations in MLF

The preconfigured Human Th17 magnetic bead-based multiplex immunoassay kit (MILLIPLEX MAP Kit, #HTH17MAG-14K, EMD Millipore / Merck) was used to

measure concentrations of T-cell associated cytokines in MLF, using Luminex xMAP technology. In total, 17 analytes (GM-CSF, IFNy, IL10, IL12, IL13, IL17A, IL2, IL21, IL22, IL4, IL5, IL9, IL15, IL17F, IL1b, TNFa, IL23) were quantified according to the manufacturer's instructions with samples incubated overnight to increase sensitivity. Data were acquired on a validated and calibrated Flexmap3D (Luminex) and analysed with Bio-Plex Manager 6.2 software (Bio-Rad). The measurement quality was assured in the same manner as the antibody deposition data described above, with internal controls and participant randomisation.

Statistical analysis

IgG and IgA antibody deposition on *Bp* was measured in paired mucosal and serum/cord blood samples taken at 8W, 20W and 9M of age. Antibody deposition levels were compared both within (longitudinal) and between (cross-sectional) each intervention group. Longitudinal analyses were performed using Wilcox signed-rank (2 timepoints) or Friedman (3+ timepoints) testing. Cross-sectional analyses were performed using Mann-Whitney U (2 groups) or Kruskal-Wallis (3+ groups) testing.

For antibody deposition analysis at 8W, infants were stratified into maternal vaccine given, i.e. TT versus TdaP-IPV. Post-vaccination responses were analysed across the four vaccination groups. *p*-values were calculated using Dunn's test for multiple comparisons with Benjamin-Hochberg multiple-testing correction. Geometric mean concentrations (GMCs) were calculated for each vaccination group and time point. A 'nasoconversion' threshold was defined as a 2-fold increase from the GMC of the TT group at 8 weeks, and the percentage of infants above this threshold was calculated for each vaccine group and timepoint.

Univariate regression analysis of mucosal antibody-deposition levels with cord or serum levels as well as with *a-priori* selected clinical and demographic variables (maternal vaccination, gestational age at birth, maternal age and infant sex) were done. Co-variates that had a significant or near-significant influence ($p<0.1$) were included in the final multivariate linear regression models.

Mucosal cytokine levels were measured pre (16W) and post 3rd primary vaccine dose (17W). Overall, levels were low, and analysis was only performed on the cytokines that had in-range values for both timepoints in 25% of the infants (**Figure S5**). Values of all included cytokines with unadjusted significance levels can be found in **Figure S6**. Statistical analysis used the same methods as described for the antibody deposition data, but without adjustment of *p*-values due to the explorative nature

of the analysis. The GMC of each cytokine was calculated per vaccination group, and normalized per cytokine, to get a relative number between 0 and 1 for each cytokine (1 being the highest value and 0 being the lowest).

All statistical analyses were conducted in R-studio 2022.02.1, using R-version 4.1.3.

Results

Demographic and clinical characteristics were not significantly different between the four infant groups (**Table 1**). Additional information on the GaPs cohort is described elsewhere [main GaPs paper under submission].

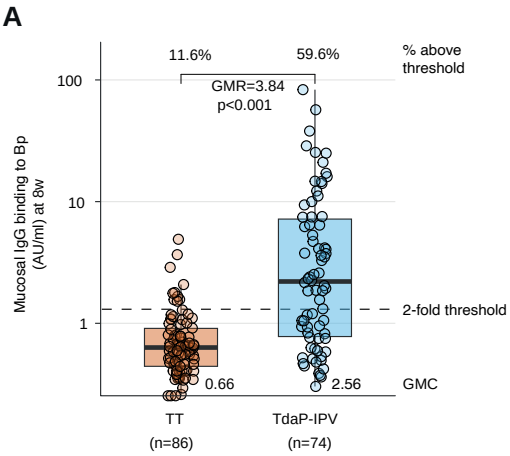
Before vaccination, mucosal *Bp*-binding IgG was almost four times higher in infants born to mothers who had received TdaP-IPV during pregnancy, compared to TT (GMR 3.84 [3.22-4.59], p<0.001) (**Figure 2a**). Of the TdaP-IPV group, approximately 60% had *Bp*-binding IgG levels above the 'nasoconversion' threshold, defined as the 2-fold level of the TT-group at 8W. The mucosal *Bp*-binding IgG levels of the TdaP-IPV group, but not the TT group, significantly correlated with *Bp*-binding IgG levels measured in serum at 8W (r=0.60, p<0.001) and cord blood at delivery (r=0.67, p<0.001) (**Figure 2b**). These observations were confirmed by multivariate linear regression analysis. Of note, male infants tended to have significantly higher baseline mucosal *Bp*-binding IgG (p=0.037, **Figures 2c**, **Table S1**), with no significant impact of other clinical or demographic factors such as gestational age at vaccination and/or interval between maternal vaccination and baseline infant sampling (**Figure 2c**, **Table S1**). In contrast to IgG, no differences were observed in mucosal *Bp*-binding IgA between the TT and Tdap-IPV groups (p=0.75, **Figure S2a**). Furthermore, there was no correlation between *Bp*-binding IgA in MLF and serum or cord blood (**Figure S2b**), consistent with previous studies showing that IgA cannot cross the placenta.

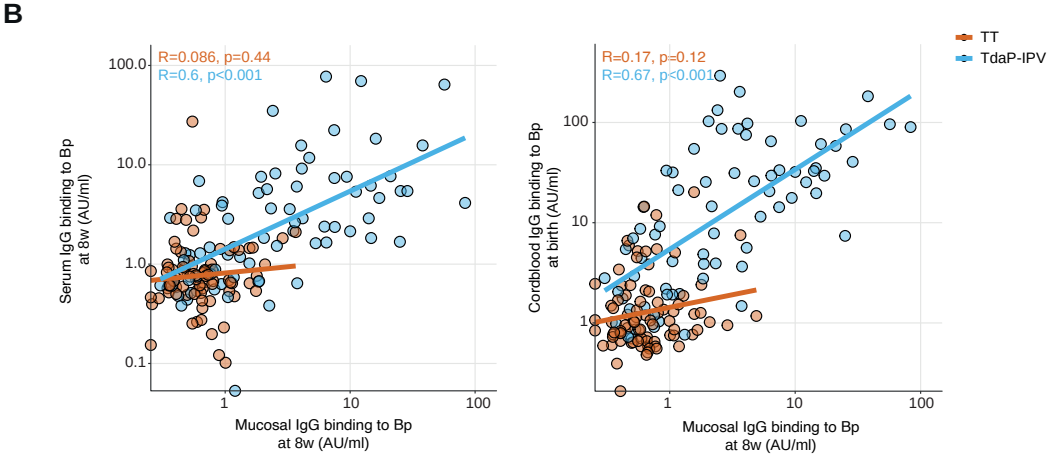
Next, we examined the impact of primary vaccination with DTwP versus DTaP at 20W, i.e., 1-month post-primary vaccination. There was no increase in mucosal *Bp*-binding IgA post-vaccination with no significant differences across the four infant groups, and no correlation between mucosal and serum *Bp*-binding IgA (**Figure S3a-b**). Primary vaccination did, however, induce a weak but significant serum *Bp*-binding IgA response in the wP-vaccinated infants (**Figure S4**), following a similar pattern as observed for serum *Bp*-binding IgG [main GaPs paper under submission].

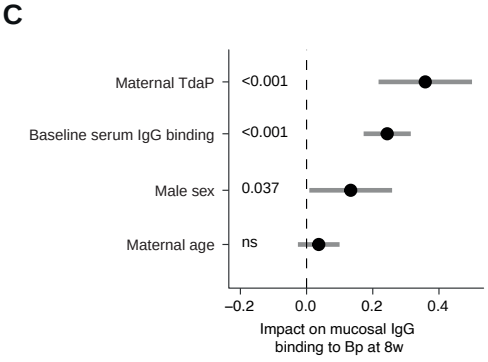
Table 1: Selected baseline characteristics of maternal-infant pairs in the GaPs mucosal substudy; SD, standard deviation)

	Total (n=160)	**Tdap-IPV/hexa-aP (n=33)**	**Tdap-IPV/penta-wP (n=41)**	**TT/hexa-aP (n=45)**	**TT/penta-wP(n=41)**
Maternal characteristics					
Age in years, mean (SD)	27 (5.0)	29 (5.9)	27 (4.5)	27 (4.8)	27 (4.8)
Infant characteristics					
Gestational age at maternal vaccination in weeks, mean (SD)	30 (1.5)	30 (1.4)	30 (1.7)	30 (1.5)	30 (1.5)
Gestational age at birth in weeks, mean (SD)	39.2 (1.5)	39.3 (1.3)	39.4 (1.4)	39.0 (1.2)	39 (1.9)
Time between maternal vaccination and birth in weeks, mean (SD)	9.4 (2.15)	9.5 (1.97)	9.3 (2.33)	9.2 (1.96)	9.4 (2.35)
Weight at birth in kg, mean (SD)	3.0 (0.42)	3.0 (0.34)	3.0 (0.41)	3.0 (0.50)	2.9 (0.39)
Female sex baby, n (%)	84 (52)	18 (54)	18 (44)	26 (58)	22 (54)

Figure 2. a) Effect of vaccination during pregnancy on mucosal Bp-binding IgG at baseline (8 weeks). Baseline nasal mucosal lining fluid samples were included from children born to mothers who received TT (red, n=86) versus TdaP-IPV (blue, n=74) during pregnancy. The GMR is depicted between the boxplots and describes the ratio of GMC in TdaP-IPV versus TT groups. Boxes represent the 25th and 75th percentile, lines inside the boxes represent medians, whiskers represent the upper and lower adjacent values (3/2 times the IQR from the end of the box) as defined by Tukey, and dots represent all participant values (including outliers outside the whiskers). Differences between the groups were calculated using the Wilcoxon rank sum test. The two-fold threshold line is defined by taking the 2-fold of the GMC of the TT group at baseline. The percentage of infants above the cut-off is depicted at the top of the figure. **b)** Correlation of baseline (8 weeks) mucosal Bp-binding IgG and baseline (8 weeks) serum and cord Bp-binding IgG. Spearman regression was performed on both the TT (red, n=86) and the TdaP-IPV (blue (n=74) groups, and R and p-value are depicted in the upper left corner of the figure. **c)** Multivariate model investigating the influence of maternal vaccination, baseline serum deposition levels and participant demographics on baseline log10 transformed mucosal IgG antibody deposition to B1917 Bp. The black circle and grey bars represent the co-efficient and 95% confidence interval of each variable. Variables were included if the univariate model showed a p-value <0.1. AU, arbitrary units; Bp, Bordetella pertussis; GMR, Geometric Mean Ratio; GMC, Geometric Mean Concentration; TdaP-IPV, Tetanusdiphtheria-acellular pertussis-inactivated polio virus; TT, Tetanus Toxoid.


A
11.6%
59.6%
% above threshold
GMR=3.84
p<0.001
Mucosal IgG binding to Bp (AU/ml) at 8w
100
10
1
2-fold threshold
0.66
2.56
GMC
TT
(n=86)
TdaP-IPV
(n=74)

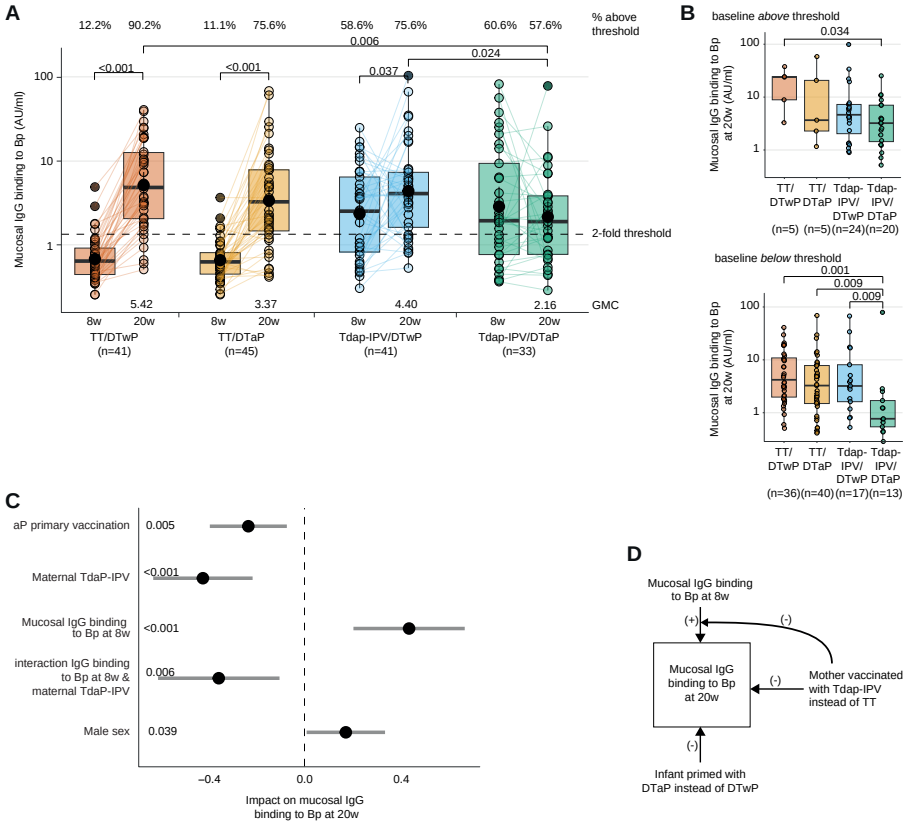

B
R=0.086, p=0.44
R=0.6, p<0.001
Serum IgG binding to Bp at 8w (AU/ml)
Mucosal IgG binding to Bp at 8w (AU/ml)
R=0.17, p=0.12
R=0.67, p<0.001
Cordblood IgG binding to Bp at birth (AU/ml)
Mucosal IgG binding to Bp at 8w (AU/ml)
TT
TdaP-IPV


C
Maternal TdaP
<0.001
Baseline serum IgG binding
<0.001
Male sex
0.037
Maternal age
ns
–0.2
0.0
0.2
0.4
Impact on mucosal IgG binding to Bp at 8w

In contrast to IgA, mucosal *Bp*-binding IgG post-vaccination significantly increased over baseline in all groups, except for DTaP-immunised infants born to TdaP-IPV-vaccinated mothers, who had significantly blunted levels post-vaccination, with the lowest overall *Bp*-binding IgG nasoconversion rate (57.6%). *Bp*-binding IgG in the TdaP-IPV/DTaP group was significantly lower than both DTwP-vaccinated groups, (p=0.006 for TT/DTwP, p=0.024 for TdaP-IPV/DTwP, **Figure 3a**). Of note, this blunting effect was not explained by high pre-immunization IgG levels, given that even TdaP-IPV/DTaP infants with low antibody levels (below nasoconversion threshold) at 8W showed lower binding IgG concentrations at 20W (**Figure 3b**). No clear blunting effect following DTwP was observed (GMC of 5.42 in TT/DTwP vs. 4.40 in TdaP/DTwP, p=0.35 **Figure 3a**). Overall, there was no significant correlation between post-vaccination serum and MLF *Bp*-binding IgG (**Figure S3b**).

To identify factors influencing the pertussis-specific antibody response up to 20W, we applied a multivariate regression model (**Figure 3c**). DTaP infant vaccination and TdaP-IPV vaccination during pregnancy were both significantly associated with lower *Bp*-binding IgG at 20W (p=0.005 and p<0.001, respectively). Conversely, male infants as well as infants who had higher *Bp*-binding IgG levels at 8W tended to

Figure 3. a) Effect of primary vaccination with DTaP or DTwP on mucosal Bp-binding IgG in children born to mothers who received TT (red and yellow, n=86) versus TdaP-IPV (blue and green, n=74) during pregnancy. The post-vaccination GMC is depicted below the 20W boxplot. Boxes represent the 25th and 75th percentile, lines inside the boxes represent medians, whiskers represent the upper and lower adjacent values (3/2 times the IQR from the end of the box) as defined by Tukey, and dots represent all participant values (including outliers outside the whiskers).Paired differences between the two timepoints within a vaccination group are calculated using the paired Wilcoxon and the differences at 20 weeks between the 4 vaccination groups are calculated using the Kruskall Wallis and Dunns post-hoc test with Benjamini-Hochberg correction for multiple testing. The twofold nasoconversion threshold line is defined by taking the 2-fold of the GMC of the TT group at baseline. The percentage of infants above the cut-off is depicted at the top of the figure. **b)** Postvaccination mucosal Bp-binding IgG per vaccination cohort. The infants are split based on whether their baseline mucosal IgG levels were above or below the 2-fold nasoconversion threshold. Boxes represent the 25th and 75th percentile, lines inside the boxes represent medians, whiskers represent the upper and lower adjacent values (3/2 times the IQR from the end of the box) as defined by Tukey, and dots represent all participant values (including outliers outside the whiskers).The differences at 20 weeks between the 4 vaccination groups are calculated using the Kruskall Wallis and Dunns post-hoc test with Benjamini-Hochberg correction for multiple testing. **c)** Multivariate model investigating the influence of maternal vaccination type, primary vaccination type, baseline (8 week) IgG deposition, and infant sex on post-vaccination log10 transformed mucosal IgG antibody deposition to B1917 Bp. The black circle and grey bars represent the coefficient and 95% confidence interval of each variable. Variables were included if the univariate model showed a p-value <0.1. **d)** A schematic overview of the effects found in the multivariate model. The dependent variable is depicted in the square, and the independent variables with their corresponding effects are depicted around the square. AU, arbitrary units; Bp, Bordetella pertussis; GMR, Geometric Mean Ratio; GMC, Geometric Mean Concentration; TdaP-IPV, Tetanus-diphtheriaacellular pertussis-inactivated polio virus; TT, Tetanus Toxoid.



have higher post-vaccination levels (p=0.039 and p<0.001, respectively). Notably, in the model there was a strong negative interaction between baseline *Bp*-binding IgG levels and the TdaP-IPV maternal vaccine, suggesting that the positive effect of baseline binding IgG to post-vaccination binding IgG was significantly diminished for infants born to TdaP-IPV vaccinated mothers (**Figure 3D**).

All infants demonstrated a significant decline in their binding IgG levels from 20W to 9M, regardless of vaccination background (Figure 4A). Although there were no significant differences in the fold-change reduction between the four infant groups (**Figure b**), more infants vaccinated with DTwP remained above the nasoconversion threshold (49% for TT/DTwP and 39% for TdaP-IPV/DTwP) than in the DTaP-vaccinated infants (29% for TT/DTaP and 27% for TdaP-IPV/DTaP) as they had a higher level at 20W. Taken together, the main driver of *Bp*-binding IgG persistence at 9M was the IgG level immediately post-primary vaccination (20W); sex no longer significantly influenced levels by 9M (**Figure 4c**, data from multivariate model not shown).

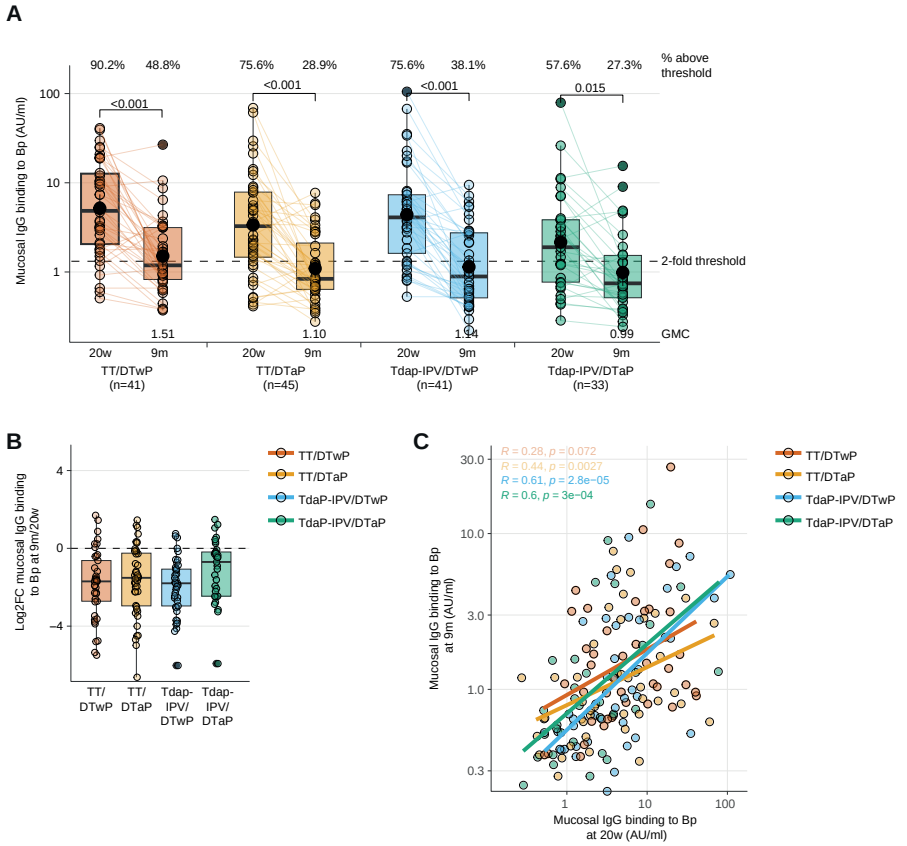


Figure 4. a) Longevity of mucosal Bp-binding IgG in children vaccinated with DTaP or DTwP vaccination and born to mothers who received TT (red and yellow, n=86) or TdaP-IPV (blue and green, n=74) during pregnancy. The 9-month GMC is depicted below the corresponding boxplot. Boxes represent the 25th and 75th percentile, lines inside the boxes represent medians, whiskers represent the upper and lower adjacent values (3/2 times the IQR from the end of the box) as defined by Tukey, and dots represent all participant values (including outliers outside the whiskers). Paired differences between the two timepoints within a vaccination group are calculated using the paired Wilcoxon and the differences at 9 months between the 4 vaccination groups are calculated using the Kruskall Wallis and Dunns post-hoc test with Benjamini-Hochberg correction for multiple testing. The two-fold nasoconversion threshold line is defined by taking the 2-fold of the GMC of the TT group at baseline (8 weeks). The percentage of infants above the cut-off is depicted at the top of the figure. **b)** Log2 fold changes in mucosal Bp-binding IgG (9months/20 weeks) across the 4 vaccination groups. Boxes represent the 25th and 75th percentile, lines inside the boxes represent medians, whiskers represent the upper and lower adjacent values (3/2 times the IQR from the end of the box) as defined by Tukey, and dots represent all participant values (including outliers outside the whiskers). The differences at 5 months between the 4 vaccination groups are calculated using the Kruskall Wallis and Dunns post-hoc test with Benjamini-Hochberg correction for multiple testing, but no significant differences were found. **c)** Correlation of the 20-week mucosal IgG deposition levels with the 9-month mucosal IgG deposition levels, per vaccination group. Spearman correlations were performed, and the R and p-value are depicted at the upper-left corner of the figure. AU, arbitrary units; Bp, Bordetella pertussis; GMR, Geometric Mean Ratio; GMC, Geometric Mean Concentration; TdaP-IPV, Tetanusdiphtheria-acellular pertussis-inactivated polio virus; TT, Tetanus Toxoid.

To investigate the mucosal cellular response to pertussis vaccination, we measured a panel of T-cell associated cytokines. Mucosal cytokine concentrations at 16W were generally low across all vaccination groups (**Figure 5**, **Figure S6a**). Nonetheless, DTwP-vaccinated infants showed the highest concentrations for almost all measured cytokines post-vaccination at 17W regardless of the maternal vaccine group (**Figure 5**, **Figure S6b**). IL-2, IFNy, and IL-1b were significantly higher in TT/DTwP infants and IL-4, TNFa and IL-15 were significantly elevated in TdaP-IPV/DTwP infants, when compared to one or both DTaP-primed groups. IL-10 was significantly elevated in both DTwP-vaccinated compared to DTaP-vaccinated groups (**Figure S6b**). Of note, when grouping cytokines into T-helper (Th) 1, 2 and 17-associated subclasses, higher levels were observed in DTwP-primed groups not only for Th1 and Th17-associated cytokines, but also for Th2. A possible effect of vaccination during pregnancy was also observed in DTwP-vaccinated infants, with infants born to mothers who received TdaP-IPV during pregnancy more skewed towards Th1/Th2, compared to a mixed Th1/Th17 profile in infants born to TT-vaccinated mothers (**Figure 5**).

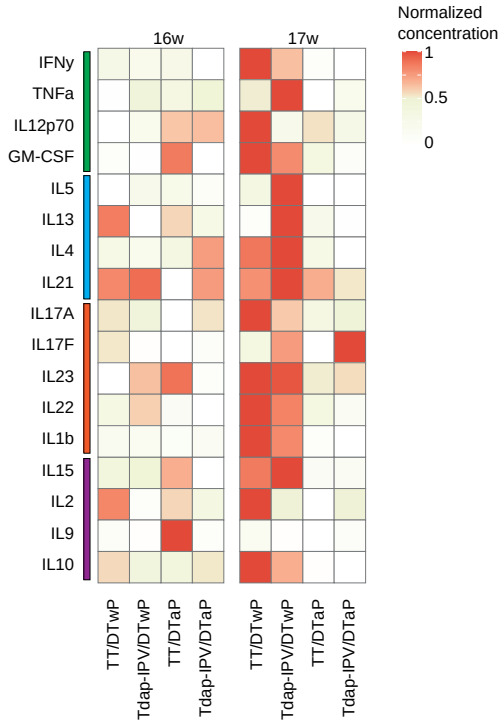


Figure 5. Pre- and post-vaccination cytokine levels per vaccine group. Cytokines were measured just before the 3rd vaccination dose (16 weeks) and 7 days after the 3rd vaccination dose (17 weeks). Geometric mean concentrations were normalized per timepoint and cytokine. The color of the square depicts the normalized values. Cytokines were ordered based on the Th-profile they are associated with (green= Th1, blue= Th2, red= Th17, purple=other).

Discussion

This immunological sub-study of the GaPs trial demonstrates that maternally-derived IgG antibodies cross the mucosal barrier of the upper airways to reach the mucosal lining fluid of the upper respiratory tract in infants, and that these antibodies are able to recognise and bind to *Bp*. Previous epidemiological data and animal models suggest that wP induce more effective mucosal immunity than aP vaccines, thereby preventing colonization and subsequent transmission of *Bp*. Both our antibody and cytokine findings support these hypotheses for the first time in infants, who are most vulnerable to severe pertussis complications and death.

Firstly, we found that while DTaP-vaccination results in detectable nasal IgG responses to *Bp*, DTwP-vaccinated infants showed higher post-vaccination levels at 20W, and nasoconversion rates remained highest in these groups up until 9M. These IgG antibodies are likely systemically-derived, either through exudation or via active transport by the mucosal FcRn receptor, with patterns broadly mirroring post-vaccination pertussis-specific binding IgG responses in the serum which have been described previously [main GaPs paper under submission]. However, while there was a strong positive correlation between *Bp*-specific mucosal and serum/cord IgG at baseline, consistent with maternally-derived antibodies in circulation that cross the mucosal barrier in infants, this association disappeared following primary vaccination. The reasons for this are unclear but may be driven by different transfer-rates of *Bp* IgG subclasses. Although serum *Bp*-binding IgA responses were induced following vaccination with DTwP, neither maternal nor infant vaccination resulted in detectable nasal *Bp*-binding IgA responses. This suggests that vaccine-induced IgA in the circulation was not transferred to the infant upper airway or only at low levels below assay detection. It is expected that a vaccine delivered intranasally would induce a mucosal *Bp*-specific IgA response, important in preventing bacteria binding to the epithelium; this has been described in a recent phase 2b randomized-controlled trial using a live attenuated vaccine.[21,22]

This study is unique because, in addition to mucosal antibodies, we also measured nasal cytokine levels to explore the induction of mucosal cellular responses following infant vaccination. Studies in mice have shown that wP but not aP induces nasal and lung TRMs T-cells, specifically T-helper (Th)1 and Th17 type, which play an important role in bacterial clearance.[11,12] More recently, adults immunised with wP vaccines as children were found to have significantly more pertussis-specific IL-17A and IFN-y-producing TRM cells in their respiratory tissues when compared with aP-primed donors.[23] Results from our study support the notion that wP-

vaccines but not aP-vaccines induce mucosal cellular responses, with DTwP-vaccinated infants showing increased mucosal T-cell associated cytokines compared to DTaP-vaccinated infants. Further studies are needed to investigate the specific cellular source of these cytokines. Interestingly, while the magnitude of the overall nasal cytokine response was dictated by pertussis vaccination type, DTwP cytokine responses were not restricted to Th1 and Th17-associated cytokines, which have been linked with protection to infection in the mouse model, but also clearly showed induction of Th2-associated cytokines.[24] Of note, nasal cytokine responses were overall low, thereby limiting our ability to identify subtle differences between the groups. Although these results should be interpreted with caution, an interesting trend was observed, with Tdap-IPV vaccination during pregnancy potentially skewing the infant mucosal cytokine response to DTwP-vaccination towards Th2. A recent study in mice similarly demonstrated that maternal aP vaccination resulted in significantly prolonged nasal carriage of *Bp* by inhibiting recruitment of IL-17-producing TRMs and subsequent neutrophil influx in the nasal tissue.[25]

Although vaccinating against pertussis in pregnancy is crucial to prevent complications and death in newborns, concerns remain about the short- and longer-term implications of blunting of subsequent infant responses following primary immunization.[15-17] While blunting has been extensively studied in serum, less is known about the mucosal compartment. This is in part due to the practical and ethical challenges of accessing mucosal sites, the low cell yield, and difficulties in ensuring reliable mucosal sampling, standardization and analysis. Our findings suggest that maternally-derived TdaP-IPV-induced antibodies have a significant blunting effect on IgG-mediated binding to *Bp* at the infant's nasal mucosa, particularly following DTaP primary vaccination. This effect was observed in TdaP-IPV/DTaP-vaccinated infants, even when their 8W (maternally-derived) antibody levels were below the nasoconversion threshold and comparable to infants born to TT-vaccinated mothers. The underlying mechanisms driving blunting warrant further investigation but are thought to be primarily due to epitope masking, preventing antigen-binding by infant B-cells, thereby limiting their priming, and inhibition of infant B-cell activation by FcγRIIB-receptor-mediated signalling.[26] DTwP-vaccinated infants were able to overcome the blunting, suggesting that aP-specific antibody binding to inactivated *Bp* bacteria in the DTwP vaccine does not negatively influence antibody responses to other antigens on the same bacterium. As we only followed up vaccinated infants to the age of 9M, it remains unclear whether the reduced immunogenicity observed in TdaP-IPV/DTaP vaccinated infants is short-lived or has longer-term effects. By 9M, there were no longer

significant differences in nasal binding IgG between the study groups. Although this may be partially due to IgG levels waning below detection threshold, a trend remained. Given that vaccination during pregnancy significantly reduces the risk of severe pertussis in the first few months of life, delaying administration of the first primary dose may, therefore, be a possible option to reduce blunting effects, particularly in settings that use aP schedules. Indeed, the Netherlands have delayed (3 instead of 2 months) and reduced (2-1 instead of 3-1 schedule) the priming series in infants whose mothers receive pertussis vaccination in pregnancy. [27]

Interestingly, we found that male babies had both higher baseline as well as higher post-vaccination nasal IgG levels at 20W than female babies. This may be driven by sex differences (possibly hormonally mediated) in IgG production and/or cross-barrier transport. While males are generally more susceptible to infectious diseases, a multi-country meta-epidemiological investigation found that pertussis disease incidence is actually higher in females than in males.[28] Although potentially clinically relevant, these differences had disappeared by 9M so further validation and investigation of the underlying mechanisms is warranted.

There are a few limitations to our sub-study. Firstly, we had a relatively short duration of follow-up and the cohort for this mucosal analysis was based on convenience and availability of nasal MLF samples. Furthermore, our mucosal cytokine analysis was largely exploratory. Cytokines levels were low and measured as a proxy for TRMs. Nonetheless, there was a consistent increase in cytokine levels post-vaccination compared to baseline, which was clearly delineated according to infant vaccination group.

In conclusion, the GaPs trial provided a unique opportunity to systematically assess the impact of different maternal and infant vaccination strategies on infant mucosal immunity, specifically focusing on antibody binding to whole *Bp* bacteria as a potential biomarker of protection against infection, as well as nasal cytokine responses, as a proxy of mucosal cellular activation. Our study demonstrates the successful use of minimally-invasive mucosal sampling in combination with standardized immune assays that may be useful in future vaccine trials involving young infants and pregnant women. Our novel study findings will contribute to evidence-based decision-making, particularly when considering maternal pertussis immunization programmes in LMICs, where currently they largely remain an exception.

Acknowledgements

We would like to sincerely thank the GaPs' field team for all their hard work, effort and resilience throughout the trial, particularly given the obstacles presented by the COVID-19 pandemic in 2020-21. Similarly, we are grateful to the rest of the MRC Unit The Gambia support and administrative staff for enabling the trial to continue despite these obstacles and for their logistical and technical contributions throughout. With thanks also to Dr. Ryan Thwaites (Imperial College London, UK) for his invaluable support, advice and patience during the set-up of this mucosal aspect of the GaPs trial; Professor Marien de Jonge (Radboud University Medical Centre, The Netherlands) for providing feedback and helping to set up the antibody deposition assay; and the whole of the PERISCOPE consortium for all the interesting discussions and helpful contributions. Finally, and above all, we are hugely indebted to the GaPs' mothers, infants and their families, without whom this trial would never have taken place.

References

1. ECDC. Factsheet about pertussis. 28 Nov 2023 2023. https://www.ecdc.europa.eu/en/pertussis/facts (accessed 24 July 2024).
2. ECDC. Increase of pertussis cases in the EU/EEA. 2024.
3. Organization WH. The immunological basis for immunization series: module 4: pertussis: update 2017. 2017.
4. Guiso N, Meade BD, von König CHW. Pertussis vaccines: the first hundred years. *Vaccine* 2020; **38**(5): 1271-6.
5. ECDC. Vaccine schedules in all countries in the EU/EEA. 2024. https://vaccine-schedule.ecdc.europa.eu/ (accessed 24 July 2024).
6. Warfel JM, Zimmerman LI, Merkel TJ. Acellular pertussis vaccines protect against disease but fail to prevent infection and transmission in a nonhuman primate model. *Proceedings of the National Academy of Sciences* 2014; **111**(2): 787-92.
7. Ward JI, Cherry JD, Chang S-J, et al. Efficacy of an acellular pertussis vaccine among adolescents and adults. *New England Journal of Medicine* 2005; **353**(15): 1555-63.
8. Holubová J, Staněk O, Brázdilová L, et al. Acellular pertussis vaccine inhibits Bordetella pertussis clearance from the nasal mucosa of mice. *Vaccines* 2020; **8**(4): 695.
9. Solans L, Locht C. The role of mucosal immunity in pertussis. *Frontiers in immunology* 2019; **9**: 3068.
10. Gill C, Rohani P, Thea DM. The relationship between mucosal immunity, nasopharyngeal carriage, asymptomatic transmission and the resurgence of Bordetella pertussis. *F1000Research* 2017; **6**.
11. Jaffar Z, Ferrini ME, Herritt LA, Roberts K. Cutting edge: lung mucosal Th17-mediated responses induce polymeric Ig receptor expression by the airway epithelium and elevate secretory IgA levels. *The Journal of Immunology* 2009; **182**(8): 4507-11.
12. Schmitt P, Borkner L, Jazayeri SD, McCarthy KN, Mills KH. Nasal vaccines for pertussis. *Current Opinion in Immunology* 2023; **84**: 102355.
13. Amirthalingam G, Andrews N, Campbell H, et al. Effectiveness of maternal pertussis vaccination in England: an observational study. *The Lancet* 2014; **384**(9953): 1521-8.
14. Vygen-Bonnet S, Hellenbrand W, Garbe E, et al. Safety and effectiveness of acellular pertussis vaccination during pregnancy: a systematic review. *BMC infectious diseases* 2020; **20**: 1-22.
15. Havers FP, Skoff TH, Rench MA, et al. Maternal Tetanus Toxoid, Reduced Diphtheria Toxoid, and Acellular Pertussis Vaccination During Pregnancy: Impact on Infant Anti-Pertussis Antibody Concentrations by Maternal Pertussis Priming Series. *Clinical Infectious Diseases* 2023; **76**(3): e1087-e93.
16. Briga M, Goult E, Brett TS, Rohani P, Domenech de Cellès M. Maternal pertussis immunization and the blunting of routine vaccine effectiveness: a meta-analysis and modeling study. *Nature Communications* 2024; **15**(1): 921.
17. Zimmermann P, Perrett KP, Messina NL, et al. The effect of maternal immunisation during pregnancy on infant vaccine responses. *EClinicalMedicine* 2019; **13**: 21-30.
18. Saso A, Roetynck S, Okoye M, et al. The impact of acellular pertussis vaccination in pregnancy on the immunogenicity of acellular versus whole-cell pertussis vaccines in infancy: A protocol for a phase IV randomised, controlled mother-infant pertussis vaccine trial (The Gambian Pertussis Study, GaPs). *Wellcome Open Research* 2024; **9**(487): 487.

19. Thwaites RS, Jarvis HC, Singh N, et al. Absorption of nasal and bronchial fluids: precision sampling of the human respiratory mucosa and laboratory processing of samples. *JoVE (Journal of Visualized Experiments)* 2018; (131): e56413.
20. van Schuppen E, Fröberg J, Venkatasubramanian PB, et al. Prior exposure to B. pertussis shapes the mucosal antibody response to acellular pertussis booster vaccination. *Nature Communications* 2022; **13**(1): 7429.
21. Lin A, Apostolovic D, Jahnmatz M, et al. Live attenuated pertussis vaccine BPZE1 induces a broad antibody response in humans. *The Journal of clinical investigation* 2020; **130**(5): 2332-46.
22. Keech C, Miller VE, Rizzardi B, et al. Immunogenicity and safety of BPZE1, an intranasal live attenuated pertussis vaccine, versus tetanus–diphtheria–acellular pertussis vaccine: a randomised, double-blind, phase 2b trial. *The Lancet* 2023; **401**(10379): 843-55.
23. da Silva Antunes R, Babor M, Carpenter C, et al. Th1/Th17 polarization persists following whole-cell pertussis vaccination despite repeated acellular boosters. *The Journal of clinical investigation* 2018; **128**(9): 3853-65.
24. Ross PJ, Sutton CE, Higgins S, et al. Relative contribution of Th1 and Th17 cells in adaptive immunity to Bordetella pertussis: towards the rational design of an improved acellular pertussis vaccine. *PLoS pathogens* 2013; **9**(4): e1003264.
25. Dubois V, Chatagnon J, Depessemier M, Locht C. Maternal acellular pertussis vaccination in mice impairs cellular immunity to Bordetella pertussis infection in offspring. *JCI insight* 2023; **8**(18).
26. Saso A, Kampmann B. Maternal immunization: nature meets nurture. *Frontiers in Microbiology* 2020; **11**: 1499.
27. Barug D, Pronk I, van Houten MA, et al. Maternal pertussis vaccination and its effects on the immune response of infants aged up to 12 months in the Netherlands: an open-label, parallel, randomised controlled trial. *The Lancet Infectious Diseases* 2019; **19**(4): 392-401.
28. Peer V, Schwartz N, Green MS. A multi-country, multi-year, meta-analytic evaluation of the sex differences in age-specific pertussis incidence rates. *PLoS One* 2020; **15**(4): e0231570.

Supplementary materials

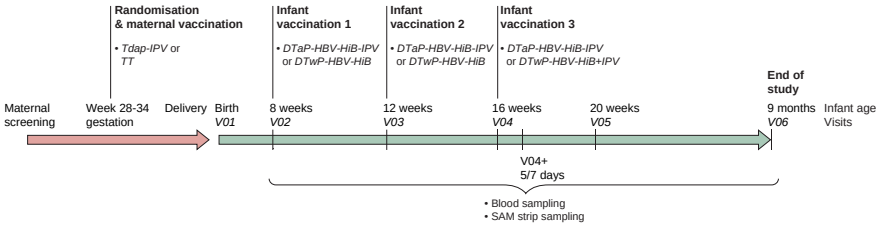
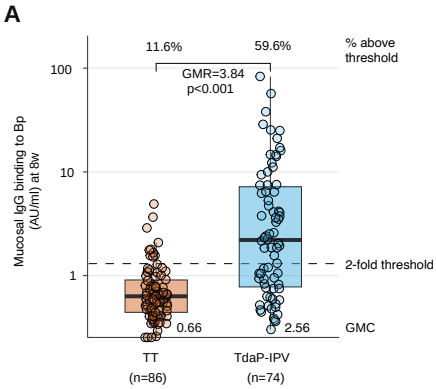
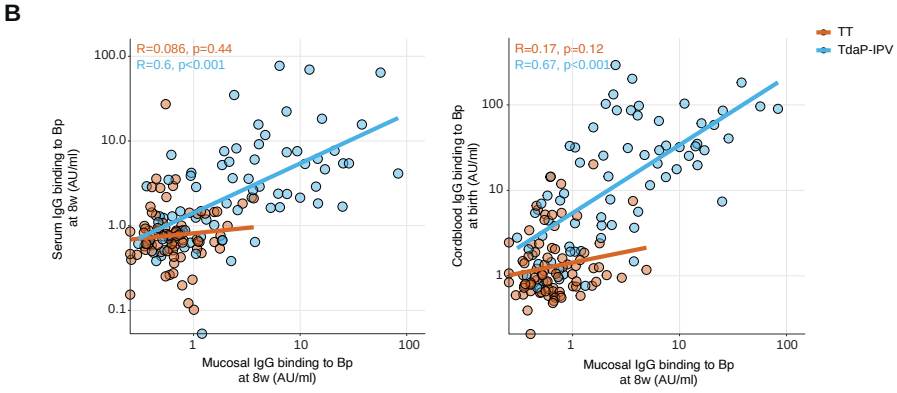
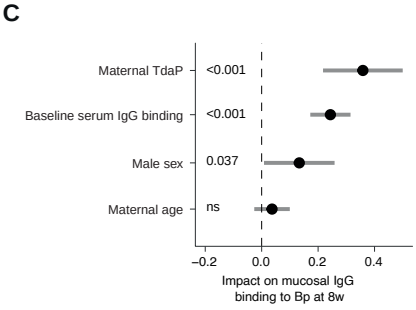


Figure S1. Overview of primary vaccination doses and sampling timepoints relevant to this substudy. All infants were vaccinated with either DTaP or DTwP-containing primary series at 8 weeks, 12 weeks, and 16 weeks of age.

Figure S2. A) Effect of vaccination during pregnancy on baseline (8 weeks) mucosal Bp-binding IgA. Baseline MLF samples were included from children born to mothers who received TT (red, n=86) versus TdaP-IPV (blue, n=74) during pregnancy. The GMR is depicted in the top left corner and describes the ratio of GMC in TdaP-IPV versus TT groups. Boxes represent the 25th and 75th percentile, lines inside the boxes represent medians, whiskers represent the upper and lower adjacent values (3/2 times the IQR from the end of the box) as defined by Tukey, and dots represent all participant values (including outliers outside the whiskers). Differences between the groups are calculated using the Wilcoxon rank sum test. **B)** Correlation of baseline (8 weeks) mucosal Bp-binding IgA and baseline (8 weeks) serum and cord Bp-binding IgA. Spearman regression was performed on both the TT (red, n=86) and the TdaP-IPV (blue (n=74) group, and R and p-value are depicted at the upper left corner of the figure. AU, arbitrary units; Bp, Bordetella pertussis; GMR, Geometric Mean Ratio; GMC, Geometric Mean Concentration; TdaP-IPV, Tetanus-diphtheria-acellular pertussis-inactivated polio virus; TT, Tetanus Toxoid.


A
11.6%
59.6%
% above threshold
GMR=3.84
p<0.001
Mucosal IgG binding to Bp (AU/ml) at 8w
100
10
1
2-fold threshold
0.66
2.56
GMC
TT
(n=86)
TdaP-IPV
(n=74)


B
R=0.086, p=0.44
R=0.6, p<0.001
Serum IgG binding to Bp at 8w (AU/ml)
Mucosal IgG binding to Bp at 8w (AU/ml)
R=0.17, p=0.12
R=0.67, p<0.001
Cordblood IgG binding to Bp at birth (AU/ml)
Mucosal IgG binding to Bp at 8w (AU/ml)
TT
TdaP-IPV


C
Maternal TdaP
<0.001
Baseline serum IgG binding
<0.001
Male sex
0.037
Maternal age
ns
Impact on mucosal IgG binding to Bp at 8w

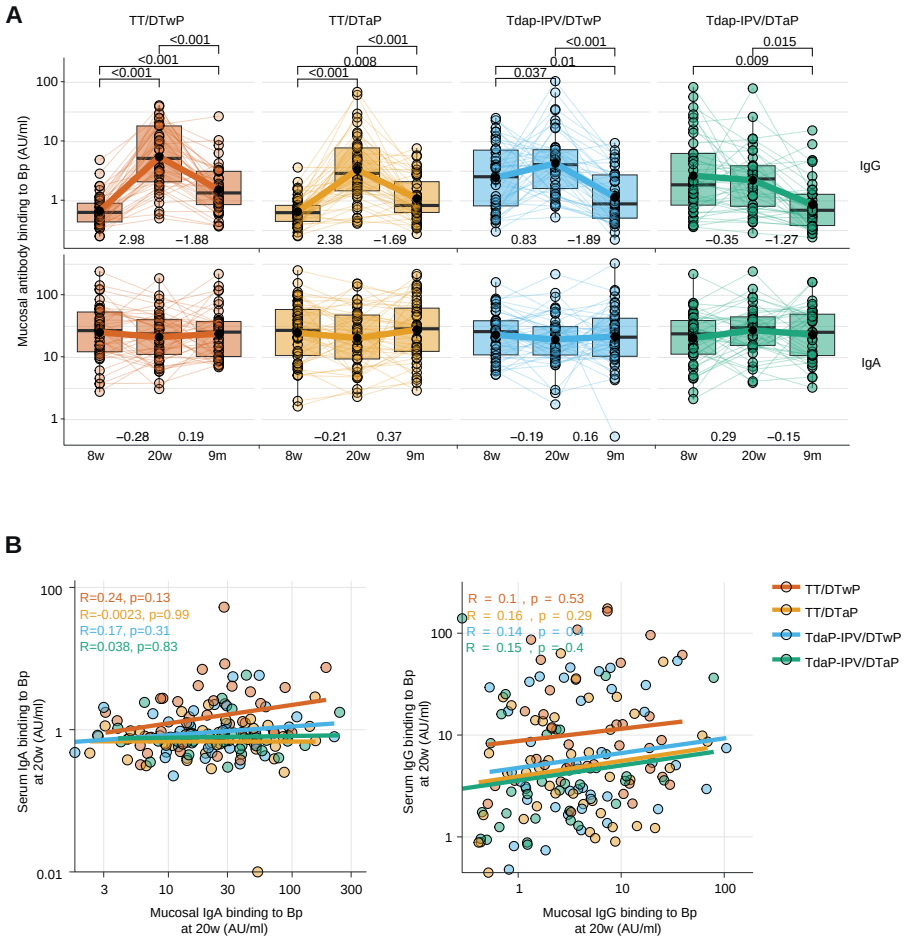


Figure S3. A) Longitudinal changes in mucosal Bp IgG and binding IgA, for each vaccine group. Each line depicts an infant's trajectory, and the solid thicker line depicts the GMC of the vaccine group. Boxes represent the 25th and 75th percentile, lines inside the boxes represent medians, whiskers represent the upper and lower adjacent values (3/2 times the IQR from the end of the box) as defined by Tukey, and dots represent all participant values (including outliers outside the whiskers). Paired differences between the three timepoints within a vaccination group are calculated using Kruskall Wallis and Dunns post-hoc testing. Log2 fold changes are depicted at the bottom of the figure. **B)** Correlation of 20 weeks mucosal Bp IgA or binding IgG and 20 weeks serum Bp IgA or binding IgG. Spearman regression was performed on all three vaccine groups, and R and p-value are depicted at the upper left corner of the figure. AU, arbitrary units; Bp, Bordetella pertussis; GMR, Geometric Mean Ratio; GMC, Geometric Mean Concentration; TdaP-IPV, Tetanus-diphtheria-acellular pertussis-inactivated polio virus; TT, Tetanus Toxoid.

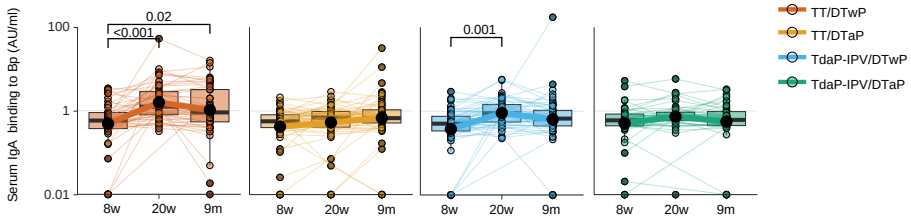


Figure S4. Longitudinal changes in serum Bp-binding IgA, for each vaccine group. Each line depicts an infant's trajectory, and the solid thicker line depicts the GMC of the vaccine group. Boxes represent the 25th and 75th percentile, lines inside the boxes represent medians, whiskers represent the upper and lower adjacent values (3/2 times the IQR from the end of the box) as defined by Tukey, and dots represent all participant values (including outliers outside the whiskers).Paired differences between the three timepoints within a vaccination group are calculated using Kruskall Wallis and Dunns post-hoc testing with Benjamini-Hochberg correction for multiple testing.

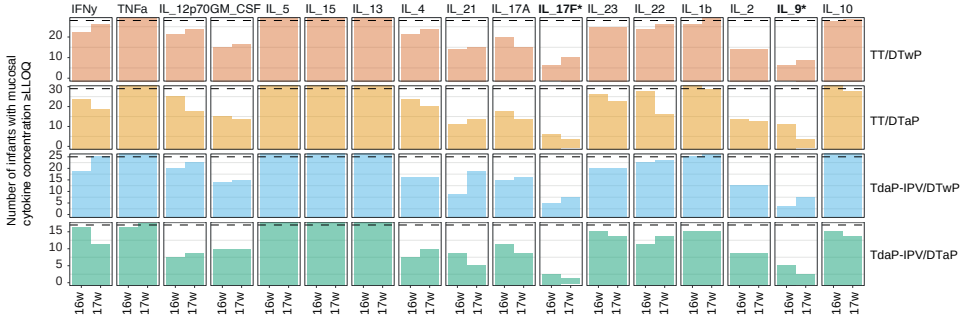


Figure S5. Overview of the number of infants with in-range values at 16 weeks and 17 weeks, for each cytokine. The dashed line depicts the total number of individuals in the vaccine group. *= cytokine was excluded from analysis due to low number of in-range infants.

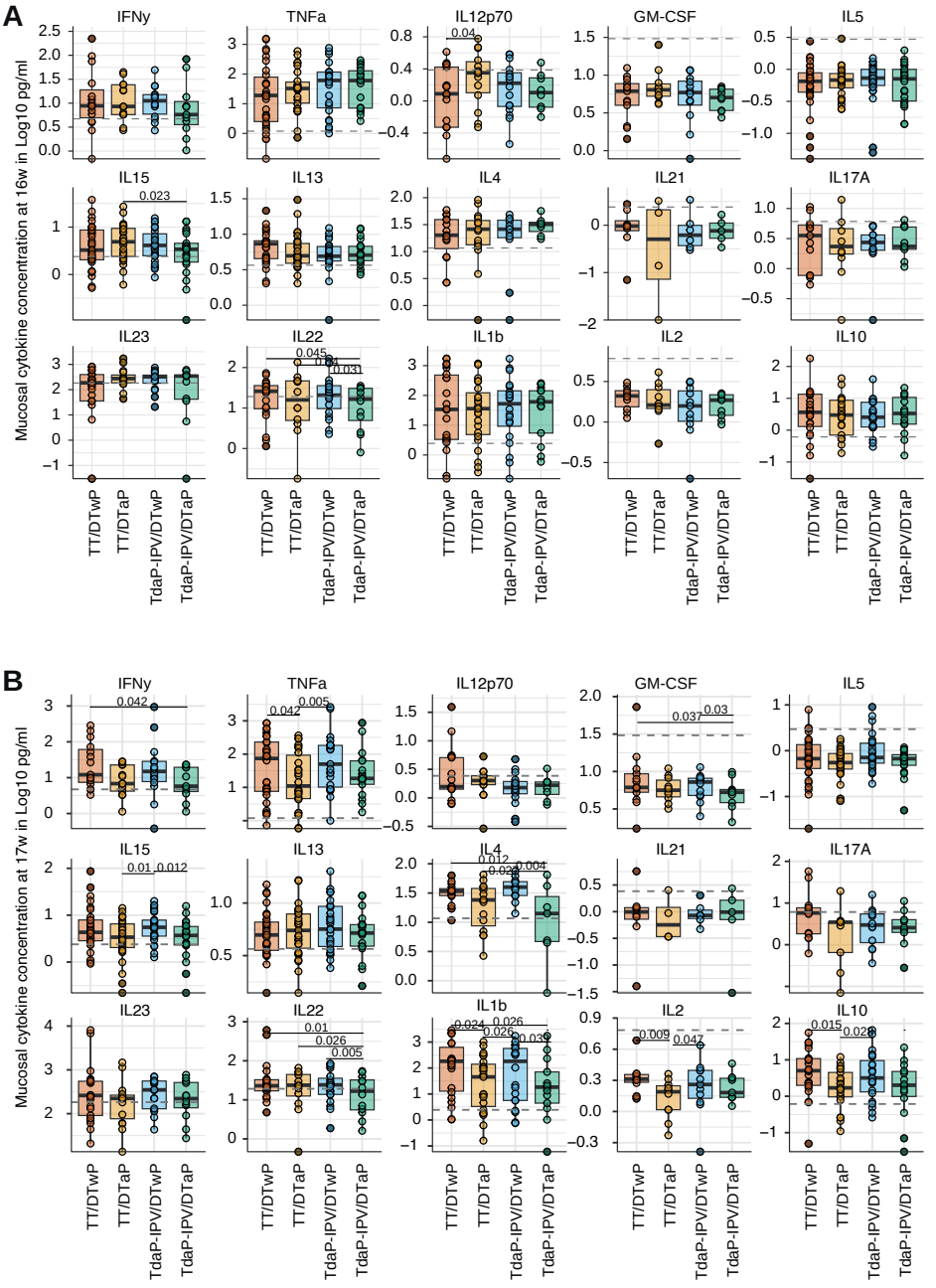
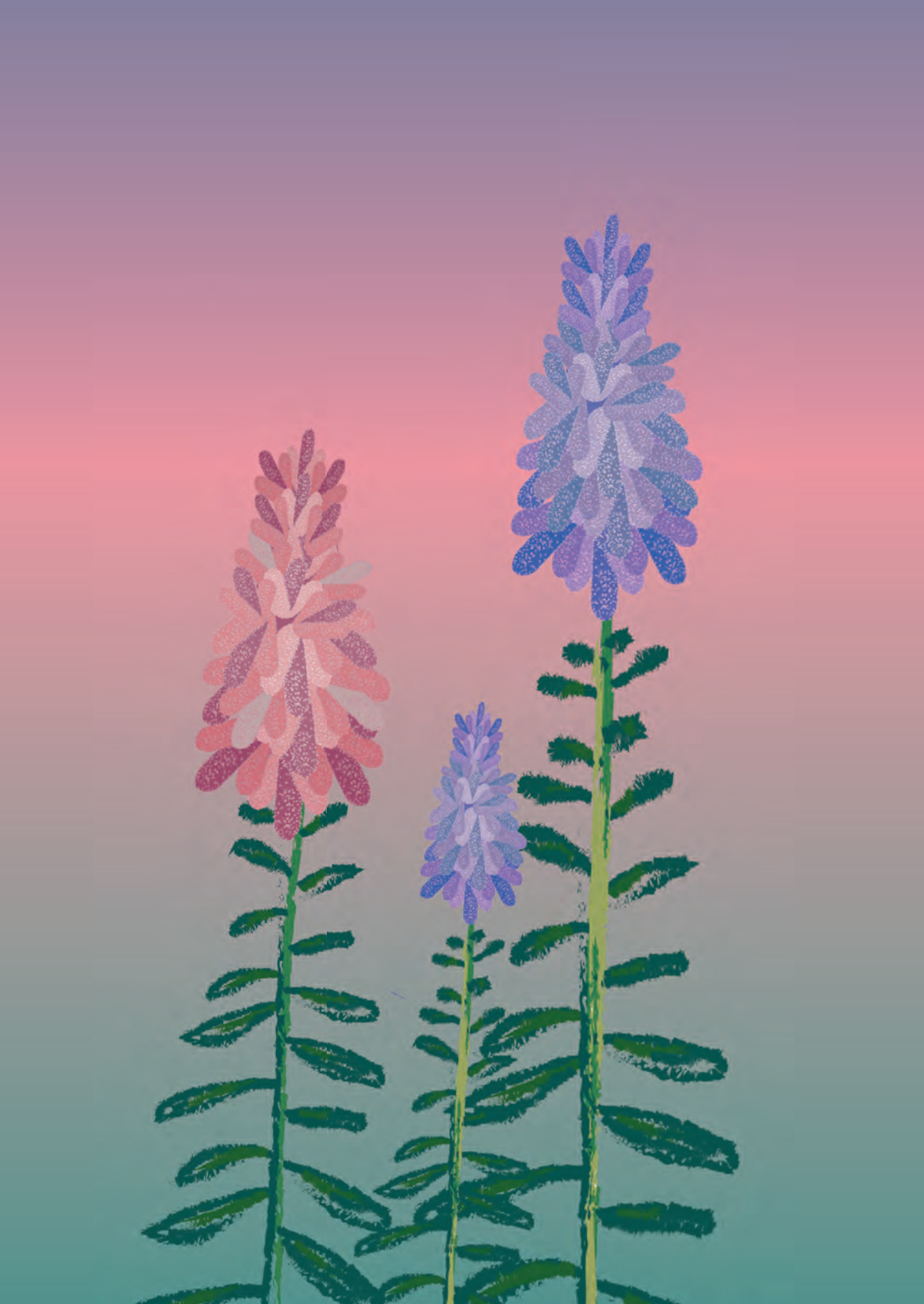


Figure S6. A) Log10 transformed cytokine levels at 16 weeks of age, pre-3rd vaccine dose, for each infant group. B) Log10 transformed cytokine levels post-vaccination at 17 weeks of age, for each infant group. The differences between the 4 vaccination groups in A and B are calculated using the Kruskall Wallis and Dunns post-hoc test.



Chapter 6

Prior exposure to *B. pertussis* shapes the mucosal antibody response to acellular pertussis booster vaccination

Evi van Schuppen[1,2*], Janeri Fröberg[1,2*], Prashanna Balaji Venkatasubramanian[3], Pauline Versteegen[4], Hans de Graaf[5], Jana Holubová[6], Joshua Gillard[1,2], Pieter G.M. van Gageldonk[4], Irma Joosten[1,2], Ronald de Groot[1,2], Peter Šebo[6], Guy A.M. Berbers[4], Robert C. Read[5], Martijn A. Huynen[3], Marien I. de Jonge[1,2], Dimitri A. Diavatopoulos[1,2]
* Contributed equally

[1.] Laboratory of Medical Immunology, Radboud Institute for Molecular Life Sciences, Radboudumc, 6525 GA, Nijmegen, The Netherlands.

[2.] Radboud Center for Infectious Diseases, Radboudumc, 6525 GA, Nijmegen, The Netherlands.

[3.] Center for Molecular and Biomolecular Informatics, Radboud Institute for Molecular Life Sciences, Radboudumc, 6525 GA Nijmegen, The Netherlands.

[4.] National Institute for Public Health and the Environment, Centre for Infectious Disease Control, Bilthoven, 3720 BA, Netherlands.

[5.] Faculty of Medicine and Institute for Life Sciences, University of Southampton, Academic Unit of Clinical Experimental Sciences, National Institute of Health Research (NIHR) Clinical Research Facility and NIHR Southampton Biomedical Research Centre, University Hospital Southampton, Southampton, United Kingdom.

[6.] Institute of Microbiology of the Czech Academy of Sciences, Videnska 1083, 142 20 Prague 4, Czech Republic.

Nat Commun. 2022 Dec 2;13(1):7429. doi: 10.1038/s41467-022-35165-w.

Abstract

Bordetella pertussis (Bp), the causative agent of pertussis, continues to circulate despite widespread vaccination programs. An important question is whether and how (sub)clinical infections shape immune memory to Bp, particularly in populations primed with acellular pertussis vaccines (aP). Here, we examine the prevalence of mucosal antibodies against non-vaccine antigens in aP-primed children and adolescents of the BERT study (NCT03697798), using antibody binding to a Bp mutant strain lacking aP antigens (*Bp_mut*). Our study identifies increased levels of mucosal IgG and IgA binding to *Bp_mut* in older aP-primed individuals, suggesting different Bp exposure between aP-primed birth cohorts, in line with pertussis disease incidence data. To examine whether Bp exposure influences vaccination responses, we measured mucosal antibody responses to aP booster vaccination as a secondary study outcome. Although booster vaccination induces significant increases in mucosal antibodies to Bp in both cohorts, the older age group that had higher baseline antibodies to *Bp_ mut* shows increased persistence of antibodies after vaccination.

Introduction

Pertussis, or whooping cough, is a highly contagious acute respiratory disease and one of the least-controlled vaccine-preventable diseases[1]. Several countries that replaced whole cell pertussis (wP) with acellular pertussis (aP) vaccines have experienced an increase in pertussis epidemics in the last decades, with high disease incidence in infants, children and adolescents[2]. Various studies have shown that the first pertussis vaccine given after birth has long-lasting imprinting effects[3-6] that are maintained even after multiple (aP) booster vaccinations given later in life[7-9].

aP vaccines differ both with regard to their antigen content and formulation. They prevent severe disease and mortality due to pertussis in vulnerable infants, but are considered less effective in reducing circulation of *Bordetella pertussis* in the population[7,10-14]. The true prevalence of *B. pertussis* infections is unknown, since the vast majority of individuals infected with *B. pertussis* never undergo diagnostic testing[15,16]. This is particularly the case for infections occurring in vaccinated individuals, which are generally milder or asymptomatic. A better understanding of *B. pertussis* infections and their effect on immune memory is important for guiding public health policies.

B. pertussis infections are restricted to the respiratory mucosal surfaces, and dissemination beyond the airways is extremely rare[17]. Animal studies have suggested that aP vaccination protects against lower respiratory infection, but is less effective against infection of the upper respiratory tract[18,19]. In this study, we examined the prevalence of nasal antibodies against *B. pertussis* in two aP-primed cohorts, i.e. 7-10y old and 11-15y old. Our study identified increased levels of mucosal IgG and IgA binding to *Bp_mut* in older aP-primed individuals, which suggests increased prior exposure to *B. pertussis*. Higher mucosal antibodies at baseline were associated with better persistence of mucosal antibodies at one year after aP booster vaccination. The effect of *B. pertussis* infection on mucosal antibodies was further studied in a controlled human infection model of *B. pertussis*. Taken together, this study provides insight into the prevalence of *B. pertussis* infection-induced mucosal antibodies and their potential effect on the response to aP booster vaccination.

Results

Baseline differences in mucosal antibody binding to *Bordetella pertussis*

As part of a larger international multicenter clinical study, i.e. the BERT study, we performed a phase IV open-label longitudinal intervention study in the Netherlands in four different cohorts with varying age and primary pertussis vaccination backgrounds[20]. For this study, we focused on participants primed with aP vaccines during infancy, i.e. children born in 2008 and 2009 (N = 32, cohort A), and adolescents born in 2005 and 2006 (N = 22, cohort B) (**Figure 1a** and **Table 1**). Samples were obtained at baseline as well as 28 days and one year after vaccination with Tdap-IPV (**Figure 1b**).

To detect the presence of antibodies against non-aP pertussis antigens, we constructed an isogenic *B. pertussis* mutant strain that lacked the three-component acellular pertussis vaccine (aP3) antigens FHA, PRN, and PT (*Bp_mut*) (**Figure 1c**). We used this mutant strain to measure baseline variation in antibodies binding to *B. pertussis*. **Figure 2** shows the log2-transformed mean fluorescence intensity (MFI) of IgM, IgG, and IgA binding to the *Bp_mut* strain in cohorts A and B. IgG and IgA binding to *Bp_mut* was significantly higher in cohort B compared to cohort A (p = 0.014 for IgG, and p = 0.024 for IgA). Although both three- (aP3) and five-component aP vaccines (aP5) were administered during infancy in both cohorts, aP5 vaccines were more commonly used (**Table 1**). Given that the *Bp_mut* strain still expresses FIM3, participants who received aP5 vaccines, which includes FIM2/3-antigens, may have residual primary vaccination-induced anti-FIM antibodies that could bind to the *Bp_mut* strain. To examine this further, we stratified each cohort into participants who received either only aP3 vaccines (N=2 for both cohorts), or participants who received one or more aP5 vaccines (N=30 for cohort A and N=20 for cohort B) (**Figure S3**). Although the numbers in the aP3-primed group were too low for a formal statistical comparison, antibody binding to the mutant strain at baseline did not seem different between aP3- and aP5-primed subjects within each respective cohort. These results did not change when we categorized study participants who had received three aP3-vaccine doses and one aP5-vaccine dose as aP3-primed (data not shown). Notably, IgG and IgA binding to *Bp_mut* remained significantly higher in older aP5-primed subjects than in younger aP5-primed subjects. We also examined the correlation between the exact age and baseline antibody binding to *Bp_mut* (**Figure S4**), which showed significant positive correlations between age and bacterial binding for all antibody classes, suggesting that older age and not vaccination-induced antibodies to FIM was the major contributing factor to bacterial antibody binding.

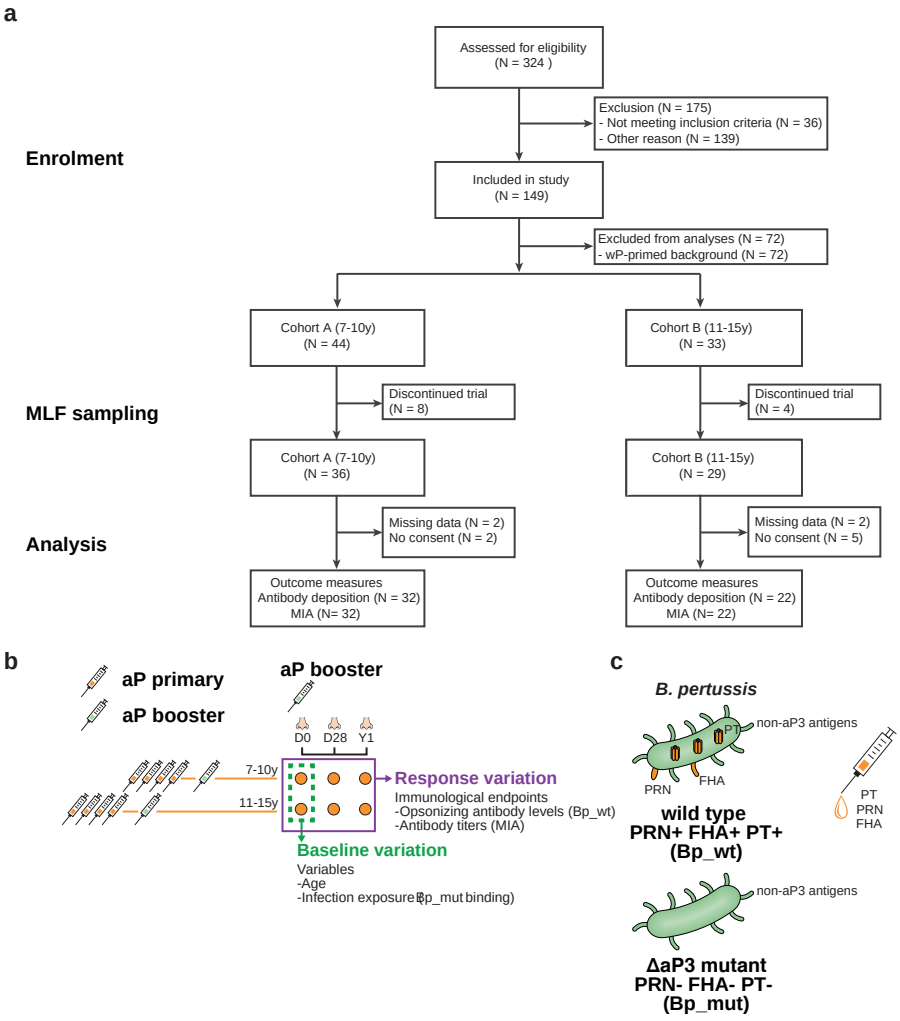


Figure 1. Flow diagram of the booster vaccination study and study procedures. **a)** Flow diagram describing the recruitment of volunteers, sample sizes, and outcome measures. **b)** Overview of the study design, immunological endpoints and framework for analyzing factors contributing to variation in immunological endpoints. Nasal mucosal lining fluid (MLF) was obtained at baseline (D0), and 28 days (D28) and one year (Y1) after vaccination with a dose of the tetanus-diphtheria-acellular pertussis-inactivated polio virus (Tdap- IPV) combination vaccine. Bacterial antibody deposition and antigen-specific antibody concentrations were used as immunological endpoints to measure response variation. Baseline variation in age and mucosal antibody binding to an isogenic mutant B. pertussis strain lacking expression of the aP3 vaccine antigens FHA, PRN, and PT (Bp_mut) were included as variables for the response variation. **c)** For bacterial antibody deposition experiments, the wild type B. pertussis B1917 strain (Bp_wt) and Bp_mut were used.

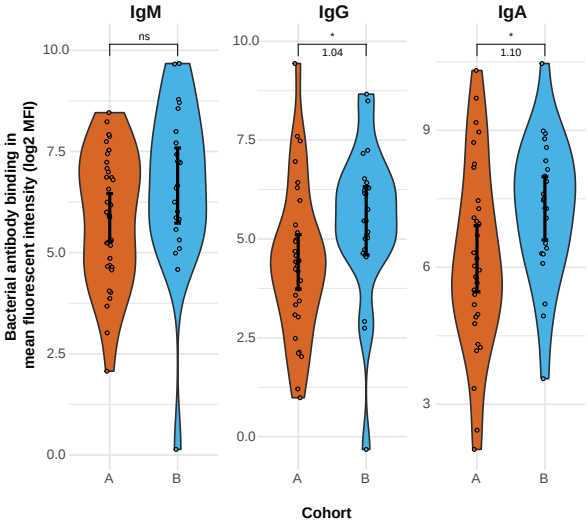


Figure 2. Baseline variation in mucosal antibody binding to Bp_mut. Bp_mut, deficient for FHA, PRN, and PT, was incubated with heat-inactivated MLF, after which antibody binding to the bacteria was measured by flow cytometry. Log2-transformed mean fluorescence intensity (MFI) of IgM, IgG and IgA binding to Bp_mut in the aP-primed cohorts A and B. Data are N = 32 individuals for cohort A and N= 22 individuals for cohort B. Sample means with 95% confidence intervals (solid black point and line) are plotted, and for the significant differences, log2 fold changes are depicted in the figure. Kruskal-Wallis followed by Wilcoxon rank-sum test was used to compare across cohorts. * p≤0.05, p=0.014 for IgG and p=0.024 for IgA.

Infection pressure of *B. pertussis*

The increased prevalence of antibody binding to *Bp_mut* suggests increased exposure to *B. pertussis* infection in the adolescent age group. Even though many, if not most *B. pertussis* infections in vaccinated individuals likely remain undiagnosed, we reasoned that these differences may also be reflected in disease notifications. Because pertussis outbreaks are highly cyclical and regional, we evaluated the cumulative pertussis disease incidence in two birth cohorts, representing cohort A (born in 2008-2009) and cohort B (born in 2005-2006), respectively. Pertussis notifications were examined in the same geographical areas where the booster vaccination study was carried out. Pertussis disease incidence was obtained for each year, covering the period from birth until the respective age at inclusion into our study (**Figure 3a**). Our analysis indicated different pertussis disease incidence trajectories between the two birth cohorts, with adolescents who were born earlier showing a steeper incline than children who were born later, starting approximately from the age of two to three. When looking at the yearly regional pertussis disease incidence, the difference in trajectories seem to be mainly caused by outbreaks in 2012 and 2014, where the older age group was affected more than the younger age

group (**figure S5**). These data show that different birth cohorts may be exposed to different levels of infection, and that the *Bp_mut* antibody binding assay described in this manuscript may be a valid biomarker for *B. pertussis* infection in aP-primed populations.

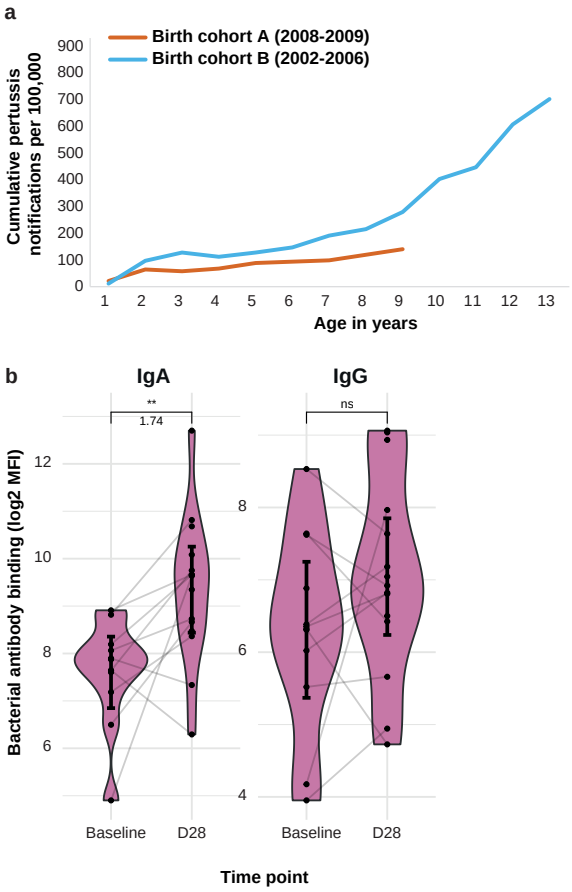


Figure 3. Cumulative B. pertussis disease incidence in different aP-primed birth cohorts over time and impact of asymptomatic infection on mucosal antibody responses. **a)** Cumulative pertussis disease incidence for aP-primed birth cohorts including children born between 2007-2010 (red line) and between 2002-2006 (blue line), representing cohorts A and B, respectively. Pertussis disease incidence was obtained for postcode-matched regions where the vaccination study was conducted, covering the period from birth until the start of the BERT study. The solid lines reflect the cumulative pertussis disease incidence as the pertussis notifications/100,000 inhabitants. **b)** MLF was obtained from adult volunteers prior to (day -7, baseline) and 28 days after intranasal inoculation with B. pertussis. Bp_mut, deficient for FHA, PRN, and PT, was incubated with heat-inactivated MLF and antibody binding was subsequently measured by flow cytometry. Log2-transformed mean fluorescence intensity (MFI) of IgA and IgG binding to Bp_mut. Data are N = 10. Sample means with 95% CI (solid black point and line) are plotted. Kruskal-Wallis followed by Wilcoxon signed-rank test was used to test significant differences over baseline in the controlled human infection study. ** p≤0.01, p=0.0042 for IgA.

Relationship between *B. pertussis* infection and mucosal bacterial surface-binding antibodies

To establish a direct causal relationship between *B. pertussis* infection in humans and (changes in) mucosal antibody deposition, we made use of the recently established controlled human *B. pertussis* infection model (CHIM)[21]. Previously, in an in-patient pilot study, 15 healthy volunteers were intranasally inoculated with 10^5 colony forming units of *B. pertussis*[21]. MLF samples were obtained at baseline as well as 28 days post-challenge, after which antibody deposition was measured as described above. Significant increases in mucosal IgA antibody deposition to *Bp_mut* over baseline were observed at day 28 ($p = 0.004$, **Figure 3b**). A similar trend was observed for IgG, although not significant ($p = 0.17$, **Figure 3b**). These data suggest that asymptomatic infection with *B. pertussis* increases mucosal antibody binding to *Bp_mut*.

Mucosal antibody response to Tdap-IPV vaccination in aP-primed children and adolescents

We examined the mucosal antibody response to a dose of Tdap-IPV across the two age cohorts. Samples were collected at day 0, day 28 and one year after vaccination. To assess vaccine immunogenicity, mucosal antibody binding to *Bp_wt* was measured by flow cytometry, and antibody concentrations against the individual aP vaccine antigens were measured by MIA. Binding to *Bp_mut* was included as a negative control (**Figure S6**). Both cohorts showed significant increases over baseline with regards to mucosal IgG binding to *Bp_wt* 28 days after vaccination ($p < 0.001$) (**Figure 4a**). IgG deposition one year after vaccination remained significantly elevated for cohort B, but not for cohort A (**Figure 4a**). IgA showed a non-significant trend towards increased bacterial binding one month after Tdap-IPV vaccination, and waning after one year in both cohorts (**Figure 4b**). No significant increases were observed for IgM binding to *Bp_wt* in both cohorts (**Figure S7**). Between cohort comparisons per timepoint showed significantly higher IgG and IgA binding levels to *Bp_wt* at all timepoints for cohort B compared to cohort A, but especially for IgG one year after vaccination, suggesting a better IgG persistence in cohort B **(Figure 4c** and **4d**).

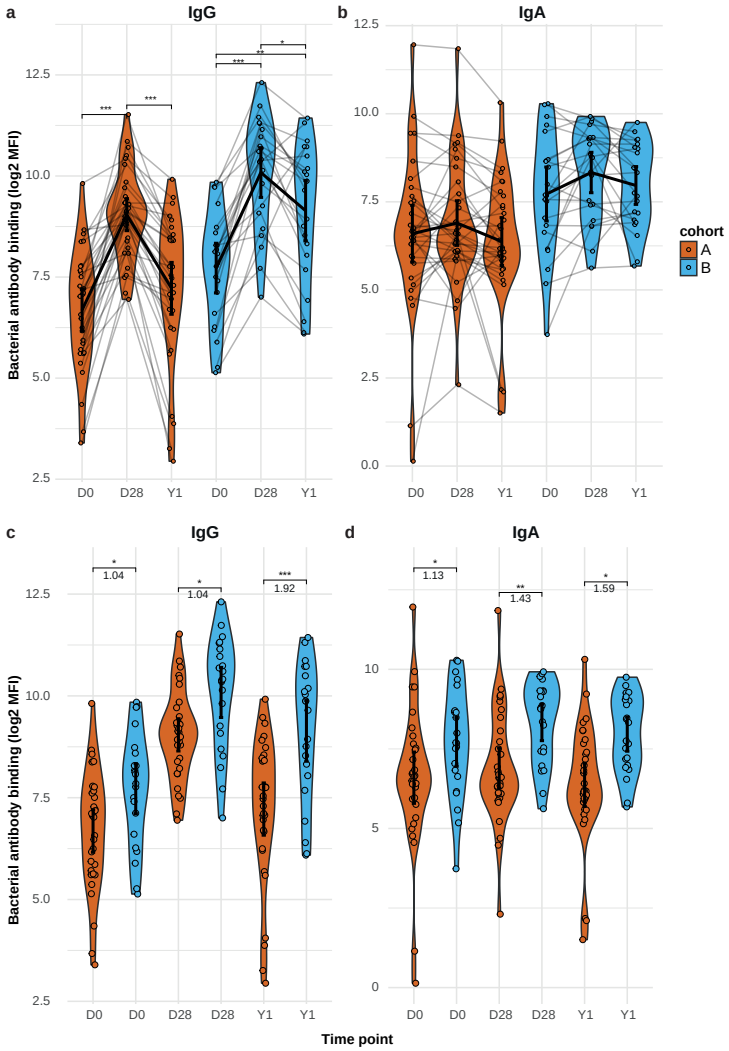


Figure 4. Effect of vaccination on mucosal antibody binding to *B. pertussis***.** MLF was obtained from participants at baseline (D0) and 28 days (D28) and one year (Y1) after a dose of Tdap-IPV. *Bp_wt* was incubated with heat-inactivated MLF and antibody binding to bacteria was subsequently measured by flow cytometry. Log2-transformed mean fluorescence intensity (MFI) of IgG (**a**) and IgA (**b**) binding to *Bp_wt* over time per cohort, and cross-cohort comparison per timepoint for IgG (**c**) and IgA (**d**) binding to *Bp_wt*. Cohort A and cohort B are indicated by color. Data are N = 32 individuals for cohort A and N= 22 individuals for cohort B. Sample means with 95% confidence intervals (solid black point and line) are plotted. The Friedman with Dunn's post-hoc test and Benjamini Hochberg correction for multiple testing was used to compare timepoints within one cohort (a and b), and log2 fold change differences can be found in Figure 6. An unpaired two-sided Wilcoxon rank-sum test was used to compare the levels at each timepoint between the two cohorts (c and d), and log2 fold change differences between the groups are depicted in the figure. * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$. From left to right exact p-values in figure 4a are 4.1e-9, 1.6e-5, 3.0e-6, 2.9e-3, 3.9e-2, in 4c are 7.1e-3, 1.5e-2, 1.2e-4, and in 4d are 8.1e-3, 4.2e-3, and 5.6e-3.

Using MIA, we also determined the concentration of mucosal IgG and IgA against FHA, PRN, PT and FIM2/3. Significant increases in IgG were observed one month post-vaccination compared to baseline for FHA, PRN and PT (**Figure 5a**). The stronger waning of antibody binding to *B. pertussis* seen in younger aP-primed participants (**Figure 4**, cohort A) was also observed for IgG concentrations against the individual vaccine antigens, with larger log2 fold reductions between 1 year and baseline in cohort A for all vaccine antigens. None of the cohorts showed a significant mucosal IgA response to the vaccine antigens, although there was a small but significant decrease of PT IgA from day 28 to one year in cohort A (**Figure 5b**). As expected, no significant vaccine effect was observed for IgG or IgA against FIM2/3, which was not included in the Tdap-IPV vaccine used in this study. Of note, a small but significant decrease in FIM IgG was observed from day 28 to one year in cohort A (**Figure S8a**).

Since we observed higher mucosal IgG *Bp_wt* binding levels 28 days and one year after vaccination in the older aP-primed cohort, we investigated if this was reflected in differences in the response after booster vaccination. We did this by determining the log2-transformed fold changes of IgG and IgA *Bp_wt* binding between day 28 and baseline (FC D28/D0), between one year and day 28 (FC 1Y/D28), and between one year and baseline (FC 1Y/D0). No differences were observed between the responses after booster vaccination in the two aP-primed cohorts, although there was a trend for better persistence after booster vaccination in the older cohort ($p = 0.051$ for IgG FC 1Y/D28 and $p = 0.068$ for IgG FC 1Y/D0, **Figure 6**).

Influence of baseline infection-induced antibodies on the mucosal antibody levels after booster vaccination

Based on the previous analyses, we concluded that there is no difference in the response after booster vaccination between the two cohorts. However, there is a difference in the absolute levels of mucosal Bp-binding antibodies, both at 28 days and one year after vaccination, which are higher in the older aP-primed cohort. To investigate if there was a relation between baseline *Bp_mut* antibody deposition and the subsequent Bp-binding antibodies after Tdap-IPV vaccination in aP-primed individuals, we examined the correlations between mucosal baseline IgA and IgG binding levels to *Bp_mut* and mucosal IgG binding levels to *Bp_wt* at day 28 and one year (**Figure 7a** and **7b**). We did not stratify on cohort, since the fold changes of the responses did not differ between the cohorts. We excluded mucosal IgA against *Bp_wt,* as there was no vaccine response for this antibody. We observed that higher baseline IgA and IgG antibody deposition on the mutant strain was

positively correlated with the IgG antibody levels after aP booster vaccination, at both 28 days and one year post-vaccination.

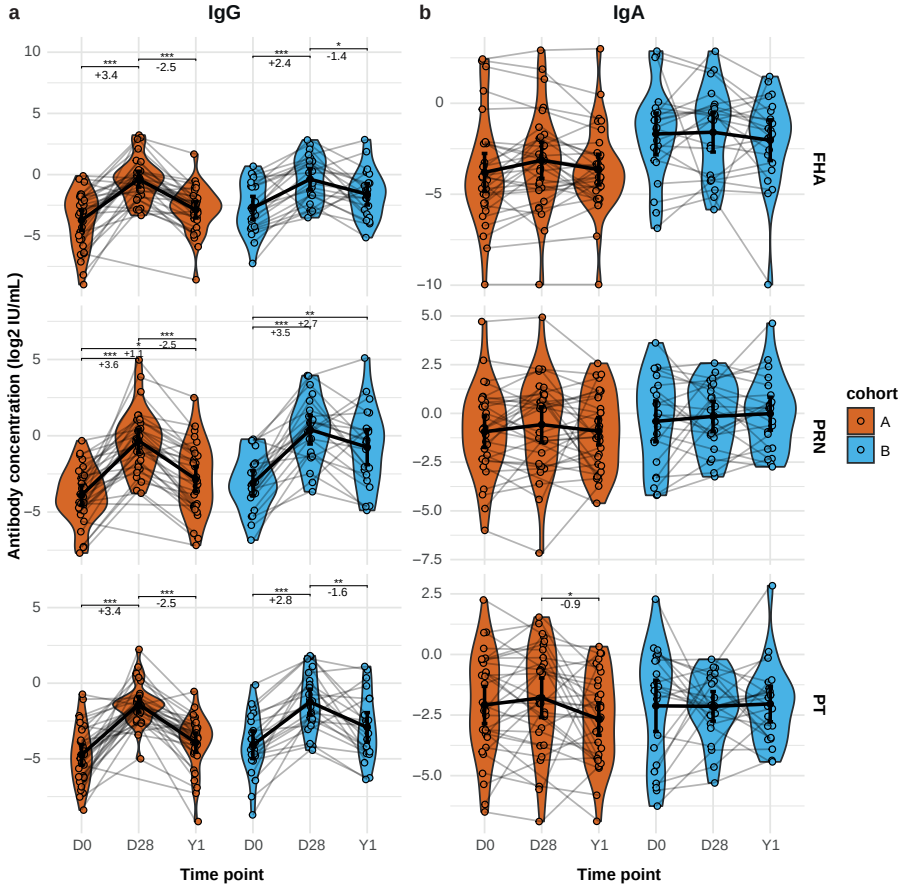


Figure 5. Effect of Tdap-IPV vaccination on aP-antigen-specific mucosal antibody concentrations. MLF was obtained from participants at baseline (D0) and 28 days (D28) and one year (Y1) after a dose of Tdap-IPV. Antibody concentrations in MLF were measured by multiplex immunoassay (MIA). **a.** Log2-transformed concentrations of FHA-, Prn-, and PT-specific IgG in international units (IU)/mL in MLF at the various sample time points. b. Log2-transformed concentrations of FHA-, Prn-, and PTspecific IgA in IU/mL in MLF at the various sample time points. Data are N = 32 individuals for cohort A and N= 22 individuals for cohort **b.** Sample means with 95% confidence intervals (solid black point and line) are plotted. The Friedman with Dunn's posthoc test and Benjamini Hochberg correction for multiple testing was used to compare timepoints within one cohort, and log2 fold change differences for the significant changes are depicted in the figure. * p≤0.05; ** p≤0.01; *** p≤0.001. From left to right exact p-values in figure 5a are 1.3e-8, 3.1e-6, 4.1e-4, and 3.1e-2 for FHA, 5.2e-9, 3.9e-2, 3.3e-5,2.2e-6 and 1.3e-3 for PRN, and 1.6e-10, 5.9e-7, 2.8e-5, and 5.9e-3 for PT. exact p-value in figure 5b is 0.034.

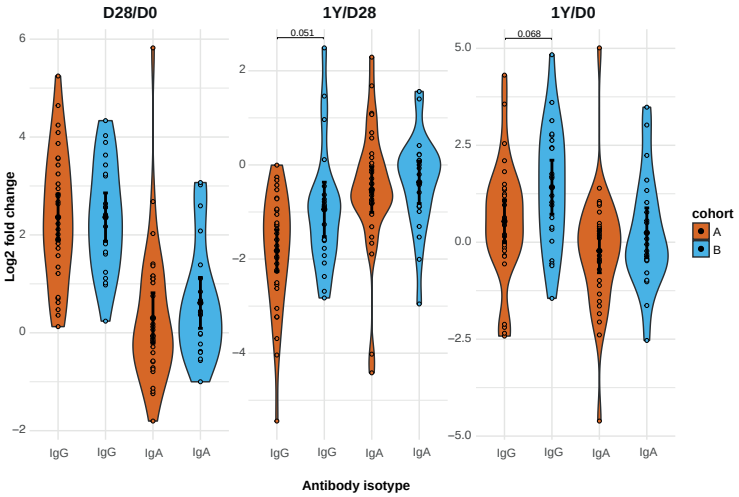


Figure 6. Effect of vaccination on mucosal antibody response to B. pertussis. Longitudinal changes in IgG and IgA Bp_wt mucosal deposition levels (log2-transformed mean fluorescence intensity) at D28 over baseline, Y1 over D28 and Y1 over baseline were calculated as log2 fold changes for each cohort. Sample means with 95% confidence intervals (solid black point and line) are plotted. Data are N = 32 individuals for cohort A and N= 22 individuals for cohort B. An unpaired two-sided Kruskal-Wallis followed by Wilcoxon rank-sum test was used to compare across cohorts.

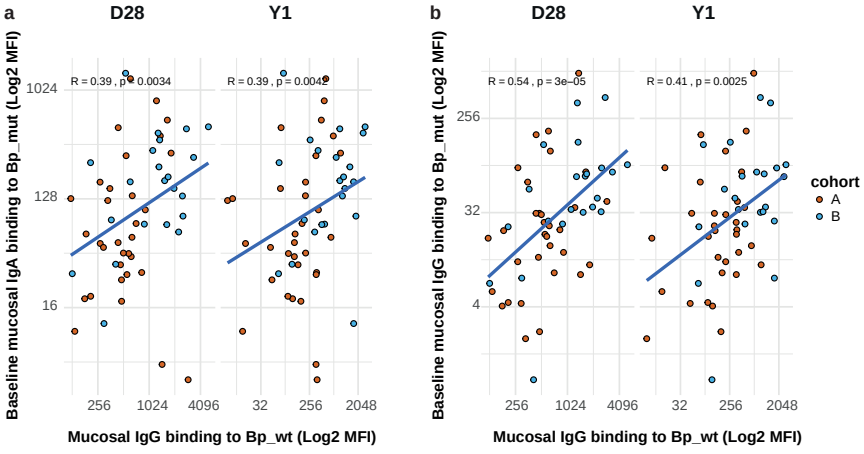


Figure 7. Correlation between baseline Bp_mut antibody deposition and the antibody levels after aP booster vaccination in aP-primed individuals. **a.** Spearman correlation between mucosal IgA binding to Bp_mut and mucosal IgG binding to Bp_wt at day 28 and one year (log2-transformed mean fluorenscence intensity levels). **b.** Spearman correlation between mucosal IgG binding to Bp_mut and mucosal IgG binding to Bp_wt at day 28 and one year (log2-transformed mean fluorenscence intensity levels). For both a and b, a two-sided spearman correlation was performed and the correlation factor R and p-value are depicted in the figure.

Discussion

Epidemiological, animal, and modeling studies suggest that continued circulation of *B. pertussis* in vaccinated populations is an important driver of disease and can lead to outbreaks[18,22,23]. Different primary pertussis vaccines induce distinct functional programs in memory T and B cells that can persist for decades[8,24,25]. An important question is how infection influences the memory response to *B. pertussis*, particularly among aP-primed individuals[13]. Since it is difficult to directly detect *B. pertussis* infection by PCR or culture in epidemiological studies, immunological biomarkers of infection may offer a more complete view of *B. pertussis* infections, which may be particularly relevant for aP-vaccinated populations. Here, we developed a innovative immune assay to measure antibody-mediated recognition of an isogenic mutant *B. pertussis* strain that lacks the PRN, PT and FHA antigens that are present in aP3 vaccines. We used this assay to detect bacterial surface-binding antibodies in nasal mucosal lining fluid. This approach provides evidence for increased prevalence of *B. pertussis* infection-induced antibodies among aP-primed adolescents. At this moment, it remains unknown at what age these infections occurred exactly and how long mucosal antibodies to *B. pertussis* persist. A larger follow-up longitudinal epidemiological study is needed to answer these questions.

The differences that we observed between older and younger aP-primed individuals were independent of antibodies against FIM3, which is included in aP5 vaccines. Notably, these age-dependent differences are in line with municipal health surveillance data, indicating that exposure to *B. pertussis* may vary between birth cohorts primed with the same pertussis vaccines.

Boosting with Tdap-IPV increased mucosal IgG deposition on *Bp_wt*, but not IgA. The absence of a consistent increase in mucosal IgA binding after systemic vaccination is in line with other studies demonstrating that IgA responses are primarily induced by infection rather than vaccination[26,27]. Nonetheless, we recently reported significant age-associated increases in FHA-, PRN- and PT-specific IgA in serum after booster vaccination, although much weaker than IgG[20]. Since antibody concentrations in MLF are significantly lower than in serum, sensitivity of detection may play a role. Alternatively, IgA in serum is predominately monomeric, which is not efficiently transported across the mucosal surface by the polymeric Ig receptor[28]. It is also possible that parenteral booster vaccination does not induce production of secretory dimeric IgA antibodies.

Next, we examined whether *B. pertussis* infection-associated mucosal antibodies influence the antibody response to pertussis booster vaccination in aP-primed individuals. We showed that higher baseline antibody binding to the mutant strain is positively correlated with mucosal antibody levels to aP vaccine antigens following vaccination. Mouse studies have shown that, in contrast to wP vaccines, aP vaccines fail to induce tissue-resident memory cells in the upper respiratory tract[29]. However, since the aP vaccine antigens PT, PRN and FHA are also expressed by *B. pertussis* during infection, infection with *B. pertussis* in aP-primed individuals may not only lead to *de novo* formation of memory B and T cells against non-vaccine antigens, but also activate pre-existing aP antigen-specific memory B and T cells. These aP-antigen specific memory cells may be re-activated following Tdap-IPV vaccination, which could potentially explain the enhanced levels and persistence of mucosal antibodies in aP-primed adolescents one month and one year after vaccination.

Our findings suggest that care must be taken when evaluating booster vaccination responses between different cohorts and regions and that infection status should be taken into account along with vaccination status. Improving our understanding of how infection and vaccination continue to shape immune memory is a key area for further research, not only for pertussis but also for other diseases such as COVID-19.

A limitation of our study is that there was limited age variation among the aP-primed participants within each of the cohorts. Consequently, the effects of infection exposure and age are difficult to separate, and further studies are needed to substantiate these findings. The *Bp_mut* strain is useful to measure the effects of *B. pertussis* infections in aP-primed populations, but may be less suited as a biomarker for *B. pertussis* infection in wP-primed subjects as the *Bp_mut* strain still expresses many *B. pertussis* antigens that are also present in wP vaccines. It should be noted that, due to the overlap in antigens expressed by the *Bp_mut* and *Bp_wt* strains, measurements are not independent. This is also observed in Figure S9a, showing a strong positive correlation between *Bp_mut* and *Bp_wt* IgG binding at baseline. We cannot rule out that exposure to other, non-*Bordetella* bacteria or antigens also results in cross-reactive antibodies that recognize *B. pertussis*. Nonetheless, the results from the controlled human infection study, as well as the absence of increased binding to *Bp_mut* after Tdap booster vaccination, demonstrate that our assay is specific and able to detect transient asymptomatic infections.

Establishing mucosal antibody correlates of protection offers significant advantages over serum-based measures. The highly standardized, well-tolerated and non-

invasive sampling method allows much broader and repetitive sampling of target groups that are normally difficult to sample, including infants and children. Nasal MLF collection in combination with the *Bp_mut* antibody deposition assay allows a quantitative assessment of *B. pertussis* infection exposure in distinct aP-primed populations and geographical regions, independent of variation in (national) disease surveillance systems. This approach may therefore enable evaluation of infection prevalence in the population, not just for *B. pertussis*, but also for other respiratory tract pathogens.

Methods

Study population and design

This immunological sub-study is part of a larger international multi-center booster vaccination study (NCT03697798)[20]. For this sub-study, children born between 2007 and 2010 (N = 32, 7-10 year old: cohort A), and adolescents born between 2002 and 2006 (N = 22, 11-15 year old: cohort B) were included (**Figure 1a**). This interventional, longitudinal, open-label phase IV study in four different age cohorts, i.e. 7-10y (cohort A; aP-primed, N = 32), 11-15y (cohort B; aP- or wP-primed, N = 42), 20-34y (cohort C; wP-primed, N = 20), and 60-70y (cohort D; wP-primed or unvaccinated, N = 22), was designed and conducted in accordance with the provisions of the Declaration of Helsinki (1996) and the International Conference on Harmonisation Guidelines for Good Clinical Practice. The trial was registered at the EU Clinical Trial database (EudraCT number 2016-003678-42) and was approved by the Medical Research Ethics Committees United (MEC-U, NL60807.100.17-R17.039) in the Netherlands. Written informed consent was obtained from all participants older than 12y, as well as from parents or legal guardian of children younger than 16y, at the start of the study.

This manuscript includes data on the following secondary outcome measures[20]: the concentrations of pertussis toxin (PT) specific IgG antibody one year after vaccination with Tdap-IPV, the change from baseline of antigen-specific IgG antibody levels against other pertussis vaccine antigens (FHA, PRN, FIM2/3) to 28 days and 1 year after vaccination with Tdap-IPV, the change from baseline of functional pertussis-specific antibody levels (i.e. bacterial antibody binding) 28 days and 1 year after vaccination with Tdap-IPV. For this immunological sub-analysis, we focused on aP-primed children born between 2007 and 2010 (N = 32, 7-10 year old: cohort A) and aP-primed adolescents born between 2002 and 2006 (N = 22, 11-15 year old: cohort B) (Figure 1a).

The vaccination history of all participants was obtained through the national health institute (RIVM), by reviewing the online records. Children in cohorts A and B received a dose of the diphtheria-tetanus-acellular pertussis-inactivated poliovirus (DTaP-IPV) combination vaccine at 2, 3, 4, and 11 months of age, as recommended by the national immunization program. Each cohort can be further stratified into participants who exclusively received aP3 vaccine doses (defined as aP3-primed), and participants who received exclusively aP5 vaccine doses (defined as aP5-primed). Participants with a mixed aP3/aP5 background who received at least one dose of aP5 vaccine were classified as aP5-primed. Tdap-IPV booster vaccination was given to all participants in cohorts A and B at the age of 4, according to the Dutch national immunization program. Within this study, all participants received one dose of Tdap-IPV vaccine via intramuscular injection in the upper arm (Boostrix™-IPV - GlaxoSmithKline (GSK), Wavre, Belgium). Boostrix®-IPV contains at least 2 international units (IU) of diphtheria toxoid (DT), at least 20 IU of tetanus toxoid (TT), 8 µg of pertussis toxoid (PT), 8 µg filamentous haemagglutinin (FHA) and 2.5 µg pertussis (PRN). It also contains D-antigen units of different polio virus strains: 40 D-antigen units of type 1 (Mahoney strain), 8 D-antigen units of type 2 (MEF-1 strain), and 32 D-antigen units of type 3 (Saukett strain)[20].

For the controlled human *B. pertussis* infection model ($CHIM_{Bp}$), healthy adult volunteers aged 18-45 years primed with wP during infancy were included[21,30]. This trial was registered with ClinicalTrials.gov (NCT03751514; ethical committee reference 17/SC/0006) and was conducted in accordance with the provisions of the Declaration of Helsinki (1996) and the International Conference on Harmonization Guidelines for Good Clinical Practice. Written informed consent was obtained from all participants. Participants had a history of vaccination against pertussis at least five years before enrollment, were nonsmokers, had no use of antibiotics within four weeks of enrollment, and had no contact with people vulnerable to pertussis disease. Participants with anti-PT IgG concentrations higher than 20 IU/L or a positive culture were excluded. Volunteers challenged with a standard dose of 10^5 colony forming units (CFU) of *B. pertussis* B1917 were included in the mucosal antibody analysis. These samples were collected from volunteers that received the final inoculum dose that we previously identified as the standard dose[21]. None of the volunteers developed severe symptoms during the follow-up, only mild symptoms were registered.

Sample collection

Nasal mucosal lining fluid (MLF) samples were obtained from all participants of the vaccination study at baseline, at 28 days (± 4 days) after vaccination, and at one

year (± 4 weeks) after vaccination (**Figure 1b**). MLF samples from the volunteers of the $CHIM_{Bp}$ were obtained seven days before (t = -7 days) and one month (t= 28 days) after challenge. MLF was collected by nasosorption using a synthetic absorptive matrix (SAM, Hunt Developments). SAM strips were gently inserted into the right nostril of the volunteer and placed along the surface of the inferior turbinate. The index finger was lightly pressed to keep the nasosorption device in place and to allow mucosal lining fluid absorption for 60 seconds, after which the nasosorption device was placed back in the protective plastic tube and stored at -80°C until further analyses.

For elution of MLF, 300 µL of elution buffer (PBS/1% BSA/0.05% Tween20/0.05% azide) was pipetted into a 1.5 mL microcentrifuge tube containing a filter cup with cellulose acetate membrane and placed on ice for 30 minutes to ensure that the filter membrane was blocked to prevent nonspecific protein binding. Following thawing, SAM strips were detached from the holder using sterile forceps by applying pressure at the base of the handle. Thereafter, the SAM strip was placed into the buffer-containing filter in the microcentrifuge tube. Samples were then centrifuged for 20 minutes at 16,000 x *g* at 4°C. After centrifugation, the filter cup containing the SAM strip was removed, and the eluate was placed into aliquots and transferred to -80°C until further analyses. All mucosal antibody analyses described were undertaken as exploratory endpoints for both the booster vaccination study as well as the $CHIM_{Bp}$ and have been approved by the medical ethics committee. The primary outcomes for both studies have already been published[21,31].

Bacterial strains

B. pertussis strain B1917 is a fully genotyped representative of current European isolates[32] and has been used as the challenge strain in the recently developed $CHIM_{Bp}$[30]. Wild type B1917 (*Bp_wt*) expresses the aP antigens filamentous hemagglutinin (FHA), pertactin (PRN), pertussis toxin (PT), and fimbriae 3 (FIM3). For this study, we constructed an isogenic triple mutant in B1917 lacking the *fhaB, prn and ptxS1-S3* genes (B1917 Δ*fhaB* Δ*prn* Δ*ptxS1-S3*), respectively (**Table S1**) encoding the aP vaccine antigens FHA, PRN, and PT, by allelic exchange using plasmid pSS4245[33,34] (**Figure 1c**). Briefly, fragments with ~700 bp length of the upstream and downstream sequences of the respective open reading frames (ORFs) to be deleted were PCR-amplified using appropriate primer pairs (listed in **Table S2**) and inserted into the corresponding restriction site of pSS4245 used for deletion of the targeted open reading frames by allelic exchange on bacterial chromosome.

The triple mutant strain was sequentially constructed by first deleting *fhaB,* followed by *prn* and finally a *ptxS1-S3* deletion. The B1917 Δ*fhaB* Δ*prn* Δ*ptxS1-S3* is referred to as *Bp_mut*. Of note, the *Bp_mut* strain still expresses FIM3, which is included in some aP vaccine formulations.

The mutations were verified by restriction analysis and resequencing of the corresponding PCR-amplified fragments. Absence of protein products of the *fha*B, *prn,* and *ptxS1-S3* genes was confirmed by Western blotting of whole bacterial cell lysates using mouse monoclonal antibodies (MAb) specific for FhaB, for S1 subunit of Ptx (Santa Cruz Biotechnology) and a polyclonal rabbit serum raised against the Prn (data not shown).

Bacteria were harvested at mid-log growth phase (OD_{620} 0.5-0.6) and frozen in aliquots in 15% glycerol at -80°C until use in the antibody deposition assays below, as described[19].

Antibody deposition

To measure antibody deposition on the *Bp_wt* and *Bp_mut* strains, $2x10^6$ colony forming units (CFU) of bacteria were incubated with 50% heat-inactivated (30 minutes at 56°C; to inactivate complement and other inhibitory components) MLF in PBS + 2% BSA for 30 minutes at 37°C + 5% CO2 while shaking. Subsequently, the bacteria-antibody complexes were centrifuged and fixed in 2% paraformaldehyde for 20 minutes at room temperature. Bacteria were then centrifuged again and resuspended in PBS + 2% BSA containing 1:500 goat polyclonal anti-human IgM-AF647 (Fc-specific, Jackson ImmunoResearch), 1:500 goat polyclonal anti-human IgG-PE (Fc-specific, Jackson ImmunoResearch), and 1:100 goat polyclonal anti-human IgA-FITC (α-specific, Sigma-Aldrich). After 15 minutes incubation at room temperature, surface-bound IgM, IgG, and IgA was measured by flow cytometry on a FACS LSR-II (BD biosciences, SanJose, CA, USA). Heat-inactivated normal human serum (NHS; GTI diagnostics, PHS-N100, lot nr. 2148U) was included as a positive control on each plate (Figure S1a and S1b) and gating the wild type and the mutant bacterial populations were based on this condition (Figure S2a and S2b). Bacteria alone were measured in all experiments to correct for background antibody binding. The Eight-peak Rainbow bead calibration particles (RCP-30-5A, Spherotech, Lake Forest, IL, USA) were used throughout the study for initial PMT characterization and for setting target Mean Fluorescence Intensities (MFI) values, as well as for daily checks, as described[35]. Data were analyzed using FlowJo Version X (FlowJo, LLC, Ashland, OR, USA) and R.

Antibody concentrations

Mucosal IgG and IgA concentrations before and after booster vaccination against the individual pertussis antigens FHA, PRN, PT, and FIM2/3 were quantified in independent duplicate using a fluorescent bead-based multiplex immunoassay (MIA), as previously described[36]. Antibodies against FIM2/3, which is not included in the Boostrix-IPV vaccine, were also measured. Analysis was performed with Bio-Plex LX200 in combination with Bio-Plex Manager 6.2 (Bio-Rad Laboratories, Hercules, CA). Pertussis standard serum and control sera were included on each plate. For IgG, the in-house standard used was calibrated against the Pertussis Antiserum (human) 1st WHO International Standard (IS) NIBSC 06/140 and values were assigned in international units (IU/mL) for FHA, PRN, and PT. The in-house standard reference for IgG-FIM2/3 was calibrated against the U.S. Reference Pertussis Antiserum (human) lot 3 and arbitrarily set at 100 AU/mL as previously described[37].

For IgA, the Pertussis Antiserum (human) 1st WHO International Standard (IS) NIBSC 06/140 was used and values were assigned in international units (IU/mL) for FHA, PRN, and PT. Since FIM2/3 IgA concentrations have not been reported in the reference standard, the Pertussis Antiserum (human) 1st WHO IS was arbitrarily set at 100 AU/mL. The LLOD for both IgG and IgA was set at 0.001 IU/mL for FHA, PRN, and PT. For FIM2/3-IgG and FIM2/3-IgA this was 0.001 AU/mL.

Pertussis disease incidence

Even though pertussis notifications are an underestimation of the true prevalence of *B. pertussis* infections, they may be indicative of the infection pressure. Since pertussis outbreaks are both cyclical and regional, we determined the regional cumulative pertussis disease incidence in the area where the BERT vaccination study was performed. Pertussis disease notifications were obtained for the birth years of participants in cohorts A and B, respectively, covering the period from 2003 until 2017, i.e. the start of the BERT study. Pertussis notifications were obtained from the Dutch National Institute for Public Health and the Environment (RIVM) from the regions Haarlem, Haarlemmermeer, Heemstede, Lisse, Teylingen, and Noordwijkerhout. The number of inhabitants of these regions per year was obtained from the Statistics Netherlands website[38]. Annual pertussis incidence for each birth cohort was calculated by dividing the number of pertussis notifications by the number of inhabitants within the age cohort per year. To estimate the overall pertussis infection pressure in each birth cohort, cumulative pertussis incidences were calculated by adding the disease incidence of the preceding years to each new year.

Statistical analyses

All statistical analyses were performed using the programming language "R"[39] in the Rstudio environment with libraries 'ggpubr'[40] and 'tidyverse'[41] used for data cleaning and 'ggplot2'[42] used for plotting. MFIs of the opsonization assays were log2-transformed to account for a skewed distribution and data were presented as violin plots with geometric mean concentrations (GMCs) with a 95% confidence interval (CI). Anti-FHA, anti-PRN, anti-PT, and anti-FIM2/3 IgG and IgA levels were log2-transformed and presented as geometric mean concentrations (GMCs) with a 95% confidence interval (CI). A pairwise Wilcoxon signed-rank test was used for comparisons within one cohort between two timepoints, while the differences between two cohorts at one timepoint were analyzed by an unpaired Wilcoxon rank-sum test. Differences between more than one group/timepoint were analyzed by the Friedman with Dunn's post-hoc test. Significance cut-off was set at $p\text{-value} < 0.05$ and all *p-values* in analyses with many comparisons were corrected for multiple testing using the Benjamini-Hochberg method[43].

The *p*-values and R values of correlations were calculated using the 'lm' command in base R[39], and the 'stat_cor' command of the 'ggpubr' package.

Supplementary materials

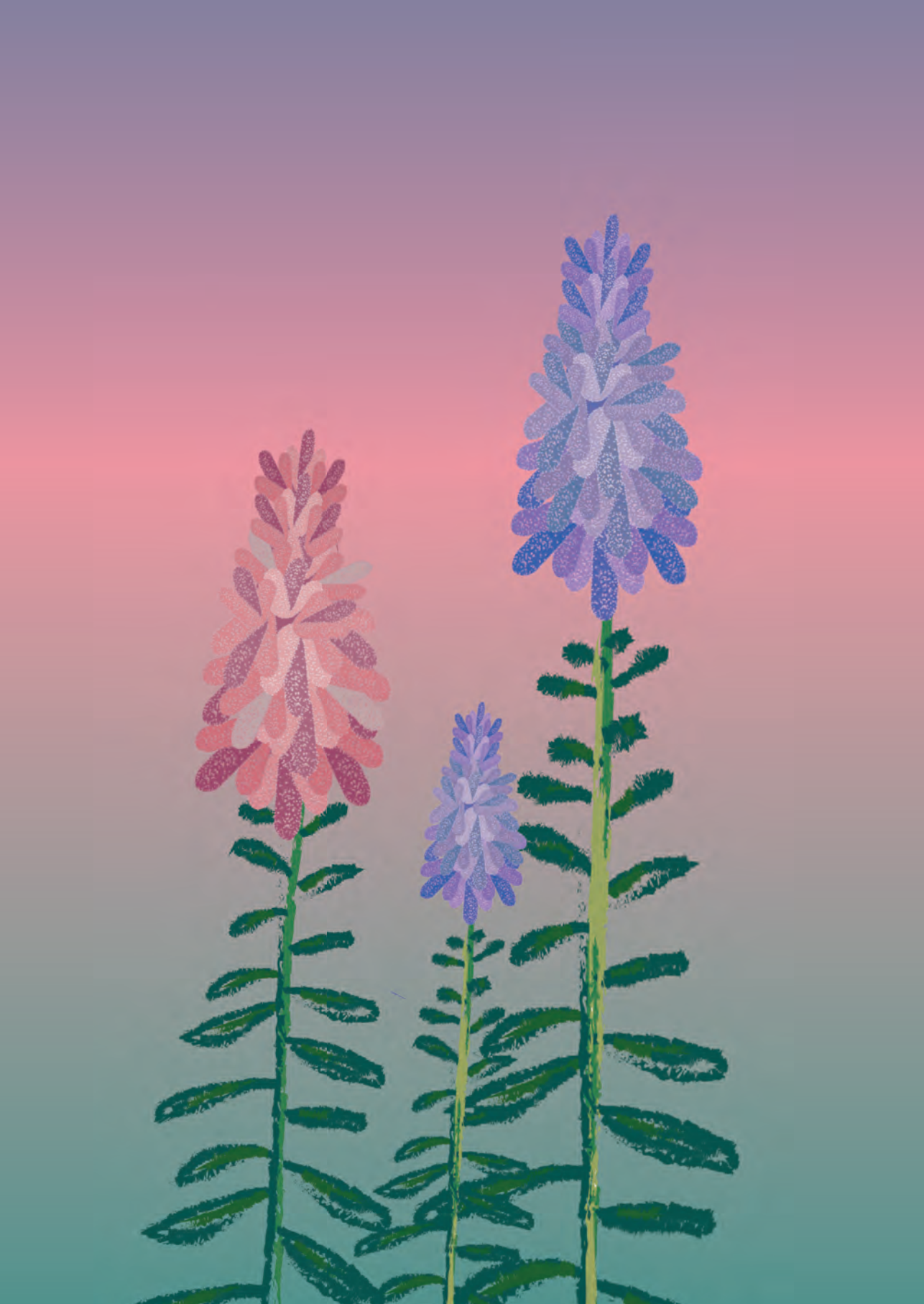
Scan the QR code for the supplementary materials:

References

1. Melvin JA, Scheller EV, Miller JF, Cotter PA. Bordetella pertussis pathogenesis: current and future challenges. *Nat Rev Microbiol* 2014; **12**(4): 274-88.
2. ECDC S. European Centre for Disease Prevention and Control. Pertussis. *Annual Epidemiological Report on Communicable Diseases in Europe* 2016.
3. Sheridan SL, Ware RS, Grimwood K, Lambert SB. Number and order of whole cell pertussis vaccines in infancy and disease protection. *JAMA* 2012; **308**(5): 454-6.
4. Liko J, Robison SG, Cieslak PR. Priming with whole-cell versus acellular pertussis vaccine. *N Engl J Med* 2013; **368**(6): 581-2.
5. Gambhir M, Clark TA, Cauchemez S, Tartof SY, Swerdlow DL, Ferguson NM. A change in vaccine efficacy and duration of protection explains recent rises in pertussis incidence in the United States. *PLoS Comput Biol* 2015; **11**(4): e1004138.
6. Klein NP, Bartlett J, Fireman B, Rowhani-Rahbar A, Baxter R. Comparative effectiveness of acellular versus whole-cell pertussis vaccines in teenagers. *Pediatrics* 2013; **131**(6): e1716-22.
7. Schure RM, Hendrikx LH, de Rond LG, et al. T-cell responses before and after the fifth consecutive acellular pertussis vaccination in 4-year-old Dutch children. *Clin Vaccine Immunol* 2012; **19**(11): 1879-86.
8. van der Lee S, Hendrikx LH, Sanders EAM, Berbers GAM, Buisman AM. Whole-Cell or Acellular Pertussis Primary Immunizations in Infancy Determines Adolescent Cellular Immune Profiles. *Front Immunol* 2018; **9**: 51.
9. da Silva Antunes R, Babor M, Carpenter C, et al. Th1/Th17 polarization persists following whole-cell pertussis vaccination despite repeated acellular boosters. *J Clin Invest* 2018; **128**(9): 3853-65.
10. Chen Z, He Q. Immune persistence after pertussis vaccination. *Hum Vaccin Immunother* 2017; **13**(4): 744-56.
11. Ausiello CM, Cassone A. Acellular pertussis vaccines and pertussis resurgence: revise or replace? *mBio* 2014; **5**(3): e01339-14.
12. Warfel JM, Edwards KM. Pertussis vaccines and the challenge of inducing durable immunity. *Curr Opin Immunol* 2015; **35**: 48-54.
13. de Greeff SC, de Melker HE, van Gageldonk PG, et al. Seroprevalence of pertussis in The Netherlands: evidence for increased circulation of Bordetella pertussis. *PLoS One* 2010; **5**(12): e14183.
14. Gill CJ, Gunning CE, MacLeod W, et al. Asymptomatic Bordetella pertussis infections in young African infants and their mothers identified within a longitudinal cohort. *medRxiv* 2020.
15. Pauline Versteegen GAMB, Gaby Smits, Elisabeth A.M. Sanders, Fiona R.M. van der Klis, Hester E. de Melker, Nicoline A.T. van der Maas. More than 10 years after introduction of an acellular pertussis vaccine in infancy: a cross-sectional serosurvey of pertussis in the Netherlands. *The Lancet Regional Health - Europe* 2021.
16. de Melker HE, Versteegh FG, Schellekens JF, Teunis PF, Kretzschmar M. The incidence of Bordetella pertussis infections estimated in the population from a combination of serological surveys. *J Infect* 2006; **53**(2): 106-13.
17. Janda WM, Santos E, Stevens J, Celig D, Terrile L, Schreckenberger PC. Unexpected Isolation of Bordetella-Pertussis from a Blood Culture. *Journal of Clinical Microbiology* 1994; **32**(11): 2851-3.

18. Warfel JM, Zimmerman LI, Merkel TJ. Acellular pertussis vaccines protect against disease but fail to prevent infection and transmission in a nonhuman primate model. *Proc Natl Acad Sci U S A* 2014; **111**(2): 787-92.
19. Zeddeman A, van Schuppen E, Kok KE, et al. Effect of FHA and Prn on Bordetella pertussis colonization of mice is dependent on vaccine type and anatomical site. *PLoS One* 2020; **15**(8): e0237394.
20. Versteegen P, Valente Pinto M, Barkoff AM, et al. Responses to an acellular pertussis booster vaccination in children, adolescents, and young and older adults: A collaborative study in Finland, the Netherlands, and the United Kingdom. *EBioMedicine* 2021; **65**: 103247.
21. de Graaf H, Ibrahim M, Hill AR, et al. Controlled human infection with Bordetella pertussis induces asymptomatic, immunising colonisation. *Clin Infect Dis* 2019.
22. Craig R, Kunkel E, Crowcroft NS, et al. Asymptomatic Infection and Transmission of Pertussis in Households: A Systematic Review. *Clin Infect Dis* 2020; **70**(1): 152-61.
23. Althouse BM, Scarpino SV. Asymptomatic transmission and the resurgence of Bordetella pertussis. *BMC Med* 2015; **13**: 146.
24. Smits K, Pottier G, Smet J, et al. Different T cell memory in preadolescents after whole-cell or acellular pertussis vaccination. *Vaccine* 2013; **32**(1): 111-8.
25. Hendrikx LH, de Rond LG, Ozturk K, et al. Impact of infant and preschool pertussis vaccinations on memory B-cell responses in children at 4 years of age. *Vaccine* 2011; **29**(34): 5725-30.
26. Deen JL, Mink CA, Cherry JD, et al. Household contact study of Bordetella pertussis infections. *Clin Infect Dis* 1995; **21**(5): 1211-9.
27. Deville JG, Cherry JD, Christenson PD, et al. Frequency of unrecognized Bordetella pertussis infections in adults. *Clin Infect Dis* 1995; **21**(3): 639-42.
28. Turula H, Wobus CE. The Role of the Polymeric Immunoglobulin Receptor and Secretory Immunoglobulins during Mucosal Infection and Immunity. *Viruses* 2018; **10**(5).
29. Wilk MM, Borkner L, Misiak A, Curham L, Allen AC, Mills KHG. Immunization with whole cell but not acellular pertussis vaccines primes CD4 TRM cells that sustain protective immunity against nasal colonization with Bordetella pertussis. *Emerg Microbes Infect* 2019; **8**(1): 169-85.
30. de Graaf H, Gbesemete D, Gorringe AR, et al. Investigating Bordetella pertussis colonisation and immunity: protocol for an inpatient controlled human infection model. *BMJ Open* 2017; **7**(10): e018594.
31. Versteegen P, Pinto MV, Barkoff AM, et al. Responses to an acellular pertussis booster vaccination in children, adolescents, and young and older adults: A collaborative study in Finland, the Netherlands, and the United Kingdom. *Ebiomedicine* 2021; **65**.
32. Bart MJ, van der Heide HG, Zeddeman A, Heuvelman K, van Gent M, Mooi FR. Complete Genome Sequences of 11 Bordetella pertussis Strains Representing the Pandemic ptxP3 Lineage. *Genome Announc* 2015; **3**(6).
33. Stibitz S, Black W, Falkow S. The Construction of a Cloning Vector Designed for Gene Replacement in Bordetella-Pertussis. *Gene* 1986; **50**(1-3): 133-40.
34. Inatsuka CS, Xu QA, Vujkovic-Cvijin I, et al. Pertactin Is Required for Bordetella Species To Resist Neutrophil-Mediated Clearance. *Infection and Immunity* 2010; **78**(7): 2901-9.
35. Kalina T, Flores-Montero J, van der Velden VH, et al. EuroFlow standardization of flow cytometer instrument settings and immunophenotyping protocols. *Leukemia* 2012; **26**(9): 1986-2010.
36. van Gageldonk PG, van Schaijk FG, van der Klis FR, Berbers GA. Development and validation of a multiplex immunoassay for the simultaneous determination of serum antibodies to Bordetella pertussis, diphtheria and tetanus. *J Immunol Methods* 2008; **335**(1-2): 79-89.

37. Hendrikx LH, Berbers GAM, Veenhoven RH, Sanders EAM, Buisman AM. IgG responses after booster vaccination with different pertussis vaccines in Dutch children 4 years of age: Effect of vaccine antigen content. *Vaccine* 2009; **27**(47): 6530-6.
38. CBS. https://www.cbs.nl/nl-nl/visualisaties/bevolkingsteller.
39. Team RC. R: A language and environment for statistical computing. *Foundation for Statistical Computing, Vienna, Austria* 2019; **https://www.R-project.org/**.
40. Kassambara A. Package 'ggpubr'. *R package version 0.1 6* 2020.
41. Wickham H. tidyverse: Easily Install and Load the 'Tidyverse'.. *R package version 121 https://CRANR-projectorg/package=tidyverse* 2017.
42. Wickham H. ggplot2: Elegant Graphics for Data Analysis. *Use R* 2009: 1-212.
43. Benjamini Y, Hochberg Y. Controlling the False Discovery Rate - a Practical and Powerful Approach to Multiple Testing. *J R Stat Soc B* 1995; **57**(1): 289-300.



Chapter 7

Serum IgA binding to *Bordetella pertussis* reveals early and widespread subclinical infection with *B. pertussis* in aP-primed children living in the Netherlands

J. Fröberg[1,2], M. Eleveld[1,2], K. Baalman[1,2], J. de Groot[1,2], G. Smits[3], F. van der Klis[3], C. van Els[3], N. Rots[3], D. Kelly[4], B. Kampmann[5,6], H. de Graaf[1,7], R.C. Read[7], M.I. de Jonge[1,2], G. den Hartog[1,3], M.A. Huynen[8], D.A. Diavatopoulos[1,2]

1. Laboratory of Medical Immunology, Radboudumc, 6525 GA, Nijmegen, The Netherlands
2. Radboud Center for Infectious Diseases, Radboudumc, 6525 GA, Nijmegen, The Netherlands
3. National Institute for Public Health and the Environment, Centre for Infectious Disease Control, Bilthoven, 3720 BA, The Netherlands
4. Oxford Vaccine Group, Department of Paediatrics, University of Oxford and the National Institute for Health Research Oxford Biomedical Research Centre, Oxford, United Kingdom
5. Vaccines & Immunity Theme, MRC Unit The Gambia at the London School of Hygiene and Tropical Medicine, Fajara, The Gambia.
6. Faculty of Infectious and Tropical Diseases, London School of Hygiene & Tropical Medicine, London, United Kingdom
7. Faculty of Medicine and Institute for Life Sciences, University of Southampton, Academic Unit of Clinical Experimental Sciences, National Institute of Health Research (NIHR) Clinical Research Facility and NIHR Southampton Biomedical Research Centre, University Hospital Southampton, Southampton, United Kingdom
8. Center for Molecular and Biomolecular Informatics, Radboudumc, 6525 GA Nijmegen, The Netherlands.

In preparation

Introduction

Bordetella pertussis (Bp), the causative agent of pertussis, is one of the most contagious infectious diseases known to humans, with an estimated R_0 of 12-17, and regional, cyclical outbreaks that are observed every 3-5 years in HICs[1,2]. Routine childhood vaccination is universally recommended to reduce pertussis mortality in children under the age of 1, and in the last decade many countries have adopted a maternal pertussis vaccine to protect infants in their first months of life[3,4]. Acellular pertussis vaccination (aP), primarily used in high-income countries (HICs), is 80-90% effective against disease in the first year post-vaccination, providing essential protection to infants[4,5]. However, aP vaccine effectiveness (VE) wanes relatively fast, particularly after repeated booster vaccination[2]. Additionally, vaccine escape has been described, with an emergence of Prn deficient strains in aP-vaccinating countries[6,7]. Importantly, aP vaccination is thought to have limited effects on subclinical infection, thereby allowing sustained subclinical infections and onward transmission of Bp in the population[6,8]. These factors are important contributing factors to the persistence of pertussis infections in aP-vaccinating countries[1,9]. Indeed, following a period of very low pertussis notifications during the COVID-19 pandemic, significant pertussis outbreaks were observed in many HICs in 2023-2024, with over 32,000 clinical cases reported in Europe alone between January and March 2024[10], indicating high circulation of Bp in aP-vaccinated populations. This study aims to provide more insight into the magnitude of subclinical Bp infections in the aP era.

As mentioned before, there is increasing recognition that infection with Bp may remain subclinical or subclinical with atypical symptoms, particularly in vaccinated individuals, resulting in further underestimation of Bp infection[9,11]. Moreover, PCR or culture-based microbial surveillance of Bp is difficult as patients often only present themselves to the general practitioner 2-3 weeks after the onset of coughing, at which time the sensitivity of Bp detection in clinical samples is generally low[12]. Additionally, PCR testing is typically only performed in at-risk patient groups such as young infants or unvaccinated children[13], resulting in testing-bias and under-ascertainment of Bp infection.

A method less susceptible to symptom-initiated clinical presentation or under-ascertainment is assessing the sero-prevalence to estimate Bp infection. The gold standard for serologically identifying pertussis cases is serum anti-pertussis toxin (PT) IgG, and population level PT seropositivity rates of 5-20% have been reported across the world[14,15]. PT is uniquely expressed by Bp and antibodies against PT wane

relatively fast post-infection, making it a specific and sensitive marker of infection[16]. Conversely, PT is also used in all licensed aP vaccines and therefore, anti-PT IgG is not infection-specific within 1-2 years post-vaccination[16]. Previous studies have suggested that IgA may be a useful biomarker for studying Bp circulation [17-19]. Importantly, multiple clinical studies have shown that aP vaccination induces serum IgG responses but not IgA[18].

In the current study, we systematically assessed serum antibody responses to whole Bp bacteria across multiple cohorts, ranging from infants to adolescents. Specifically, we compared antibody profiles in infants following primary aP vaccination, convalescent pertussis patients, and healthy adults participating in a controlled human infection study. Next, we examined antibody binding in a large cross-sectional cohort of children living in the Netherlands between the age of 1-12 years, who were either unvaccinated or primed with aP vaccines during infancy. Our study identifies early and widespread subclinical circulation of Bp in both aP-primed and unvaccinated children. Together, we show that Bp-binding IgA is a promising biomarker for Bp infection in the context of aP-vaccination.

Methods

Study cohorts

To investigate the specificity of antibody binding to Bp in vaccinated individuals, we assessed antibody binding in three infant cohorts before and after primary aP vaccination, and after aP booster vaccination at the age of 1. The three cohorts varied with respect to geographical area, study period, maternal vaccination status and vaccination schedule (see Figure 1b). The 'UK' cohort includes a subset of infants from the AWARE study (n=49, ISRCTN17271364). Infants were born to mothers who received a tetanus-diphtheria- acellular pertussis (TdaP) vaccine between 16-32 weeks of pregnancy, as per the recommended UK vaccination schedule at that time. At 2, 4 and 12 months of age, infants received a dose of the hexavalent vaccine against diphtheria (D), tetanus (T), pertussis (aP), polio (IPV), hepatitis B virus (HBV) and *Haemophilus influenzae* type B (HiB), i.e. DTaP-HBV-HiB (Infanrix-Hexa, GSK). The 'Netherlands' cohort includes a subset of infants participating in the MIKI-study (n=20, EudraCT 2012-004006-9)[20]. Infants were born to mothers who did not receive aP-containing vaccines during pregnancy. Infants received a dose of DTaP-HBV-HiB (Infanrix-Hexa) at 3-5-11 months of age. The 'Gambia' cohort includes infants who were randomized to two study arms of the GaPs study (n=108, NCT03606096). As part of the GaPs study design, mothers were recruited

to receive either a tetanus toxoid (TT) vaccine or a Tdap-IPV vaccine between 28-34 weeks' gestation. Infants were then vaccinated with DTaP-HBV-HiB (Infanrix-Hexa) at 2-3-4 months of age. From all studies, serum samples were collected and used for analysis as indicated in Figure 1b.

To evaluate the specificity and sensitivity of the assay to detect Bp infection, we included two cohorts. First, we included individuals participating in the IMMFACT-study. In this study, Dutch patients with a laboratory-confirmed, clinical Bp infection were included (n=26, CCMO nr: NL46795.094.13), and serum samples were collected at approximately three and 12 months post-diagnosis. No pre-infection samples were available from this cohort. Because of the large variation in age and primary vaccination background within this cohort, we stratified the group into four age categories: 8-12 years (n=7), 13-25 years (n=6), 25-60 years (n=6) and 60+ years (n=13). These categories also roughly corresponded with the vaccination background of the individuals, with the youngest age-group being fully aP primed, and the oldest age group being either unvaccinated or wP primed (table S1). We also included individuals participating in a controlled human Bp infection study (n=50, NCT03751514). In this study, healthy, 18-50y old participants, primed with wP during infancy, were inoculated intranasally with $1x10^5$ colony forming units (CFU) of Bp strain B1917. Participants were followed up for safety, immunogenicity and microbiological analysis. At 14 days post-inoculation, all participants were treated with azithromycin. Individuals were categorized as infected or uninfected, based on whether they had culturable Bp in any of their nasal wash or pernasal swab samples collected between inoculation and 14 days post-inoculation. For infected individuals, total bacterial burden was calculated by taking the area under the curve (AUC) of all culture timepoints. For antibody measurements, we analysed pre-inoculation (n=50), 28 days post inoculation (n=50) and 3 months post inoculation (n=25) serum samples. Of note, none of the individuals developed symptoms related to whooping cough.

Finally, to assess the prevalence of Bp infection in the Netherlands, we measured serum antibody binding to Bp in a large population cohort. This cohort included a subset of participants from the PIENTER-3 study, a cross-sectional, sero-epidemiological study performed in 2016-2017 in the Netherlands (n=779)[21]. We selected participants born after 2005, i.e. after aP vaccination was introduced in the Dutch National Immunization Programme in 2005. Participants either received all pertussis vaccinations as per national recommendation at that time, i.e. four primary doses at 2, 3, 4 and 11 months, and a 5^{th} booster dose at 4 years, n=720), or were unvaccinated (n=59). Of note, maternal vaccination had not yet been introduced at the time of this study.

Antibody measurements

Antibody binding to whole B. pertussis bacteria was assessed by flow cytometry. B1917 bacteria were cultured according to standardized conditions19, harvested at mid-log growth phase, fluorescently labelled with Oregon Green according to the manufacturer's instruction (Thermo Fisher, catnr: O6149), and cryopreserved in single-use aliquots at −80°C until use. After thawing, 2x106 colony forming units (CFU) of fluorescently-labelled bacteria were incubated for 30 min with heat-inactivated serum (1:200 in PBS+2% BSA) in 96-wells plates at 37°C with 5% CO2 while shaking. Following incubation, bacteria were centrifuged and fixed in 2% paraformaldehyde for 20 minutes in the dark at room temperature. Next, bacteria were centrifuged and stained with a secondary antibody mix, containing FACS buffer (PBS with 2% BSA (w/v)) with 1:500 Alexa Fluor® 647 AffiniPure Goat Anti-Human Serum IgA, α-chain specific (Jackson immunoResearch, Lot. 162504) and 1:500 DyLight™ 405 AffiniPure Goat Anti-Human IgG, Fcγ fragment specific (Jackson immunoResearch, Lot. 145455). After incubation for 15 minutes at room temperature, surface bound IgA and IgG antibodies were quantified by flow cytometry on a CytoFLEX® (Beckman Coulter).

Bacteria incubated with FACS buffer was used to correct for background fluorescence. As a standard curve, we included serial dilutions from a plasma pool of convalescent and vaccinated pertussis patients, provided by the Dutch National Institute of Health and the Environment (RIVM). On each plate, three plasma samples from convalescent pertussis patients were used as quality control. Following acquisition on the flow cytometer, flow cytometry data was exported as FCS files and analysed using FlowJo™ 10.8. After gating on single bacteria, based on Oregon Green 488 signal and FSC vs SSC parameters, mean fluorescent intensity (MFI) of IgG and IgA signal was exported and analysed in GraphPad Prism 9. Using the standard, MFI from experimental samples were then interpolated to predetermined arbitrary units (AU) using five-parameter logistic regression, before data analysis in R.

Anti-pertussis toxin (PT) IgG antibodies were quantified using a fluorescent-bead-based multiplex immunoassay (MIA), as described before[19,22]. Analysis was performed with Bio-Plex LX200 in combination with Bio-Plex Manager 6.2 (Bio-Rad Laboratories, Hercules, CA). The Dutch vaccination cohort did not have anti-PT IgG measurements available, and was excluded from this analysis.

Statistical analysis

Log2 transformed IgG/IgA levels were compared both within each vaccination-group (longitudinal) and between each intervention-group (cross-sectional). Longitudinal analyses were performed using Wilcox signed-rank (2 time-points) or Friedman (≥3 time points) testing. Cross-sectional analyses were performed using Wilcox rank-sum (Mann Whitney U, 2 groups) or Kruskal Wallis (≥3 groups) testing, with Dunn's post hoc testing and Benjamin-Hochberg correction for multiple testing. The number of participants for each measurement is depicted in the figures.

To determine a threshold value to classify individuals as infected, an ROC analysis was performed. Post-vaccination samples (1 month post-booster) of the infant vaccination cohorts were used as the negative group and samples from convalescent pertussis patients (3 months post-diagnosis) were used as the positive group, respectively. This analysis resulted in a cut-off level of 3.33 AU/mL, which was subsequently used to classify individuals as infected or non-infected in the Dutch sero-prevalence cohort.

All analyses were conducted in R-studio 2022.02.1, using R-version 4.1.3.

Results

The effect of primary vaccination on the levels of IgA and IgG binding to Bp

First, we assessed the specificity of serum antibody binding to whole bacteria to distinguish between primary aP vaccination and infection with Bp. We analysed three different primary aP infant vaccination cohorts, representing different time periods, vaccine schedules, and geographical areas, i.e. the UK (n=55, 2019-2024), the Netherlands (n=39, 2013-2020), and the Gambia (n=116, 2019-2022), respectively (**Figure 1a,b**). Our data consistently demonstrates that primary aP vaccination does not increase Bp-binding serum IgA levels (Figure 2a). Of note, a small but significant increase in IgA binding is observed post booster vaccination. Conversely, significant increases in Bp-binding serum IgG, post-primary vaccination, are seen for the Netherlands and the Gambia TT/DTaP cohorts, but not in the UK and Gambia Tdap-IPV/DTaP cohorts (Figure 2b). Booster vaccination at 11/12 months of age also results in a strong IgG increase (Figure 2b). A clear effect of maternal immunization is observed in the UK and Gambia Tdap-IPV/DTaP cohorts (**Figure 2a**). Assuming that most infants are not infected with Bp at this early age,

this analysis establishes a serological 'baseline' for further analysis, and showed little to no effect of aP vaccination on Bp-binding IgA levels (**Figure 2c**).

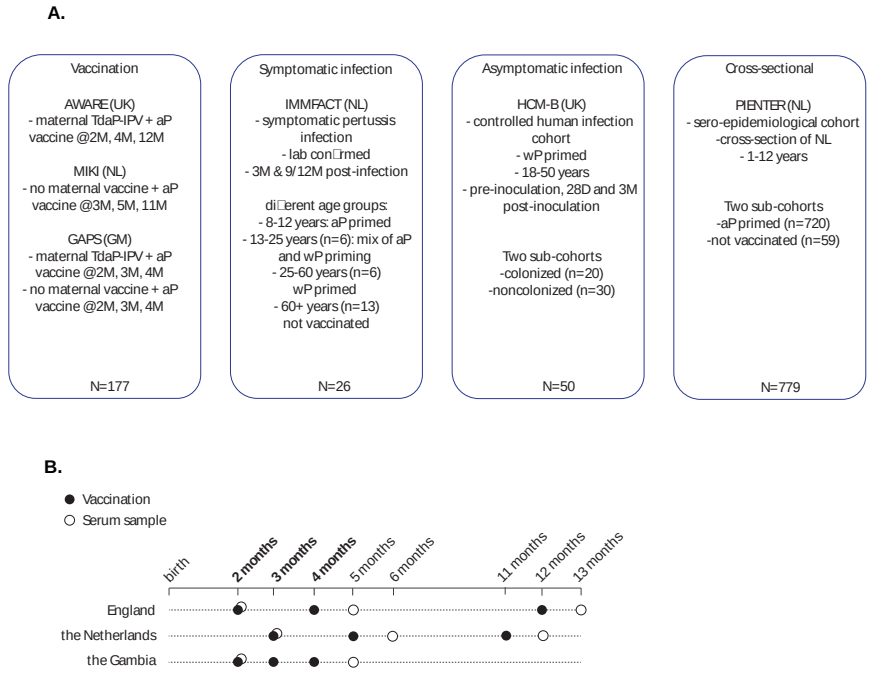


Figure 1. a) Cohort descriptions **b)** time schedule of vaccination and serum sampling of the vaccination cohorts.

The effect of clinical Bp infection on IgA and IgG binding

Next, we investigated the effect of clinical Bp infection on serum antibody binding to Bp. We compared the previously described post-booster vaccination levels of Bp-binding IgA and IgG serum antibody to those of a cohort of laboratory-confirmed clinical pertussis cases (clinical pertussis cohort, n=26). In this cohort, we analysed antibody binding at three and 12 months after diagnosis of clinical pertussis. **Table S1** shows the vaccination background and age of the patients, with older individuals (>25 years) either not vaccinated or vaccinated with a wP vaccine, and younger individuals vaccinated with only aP or mixed doses of wP/aP vaccines during primary vaccination. To correct for possible demographic confounders, we fitted a uni- and multivariable model to the Bp-binding IgA levels. This model showed that, when corrected for vaccination history and age, the duration of pertussis disease influenced Bp-binding IgA levels, with higher levels in individuals that reported pertussis disease of 4-8 weeks, compared to <4 weeks (**Table S2**).

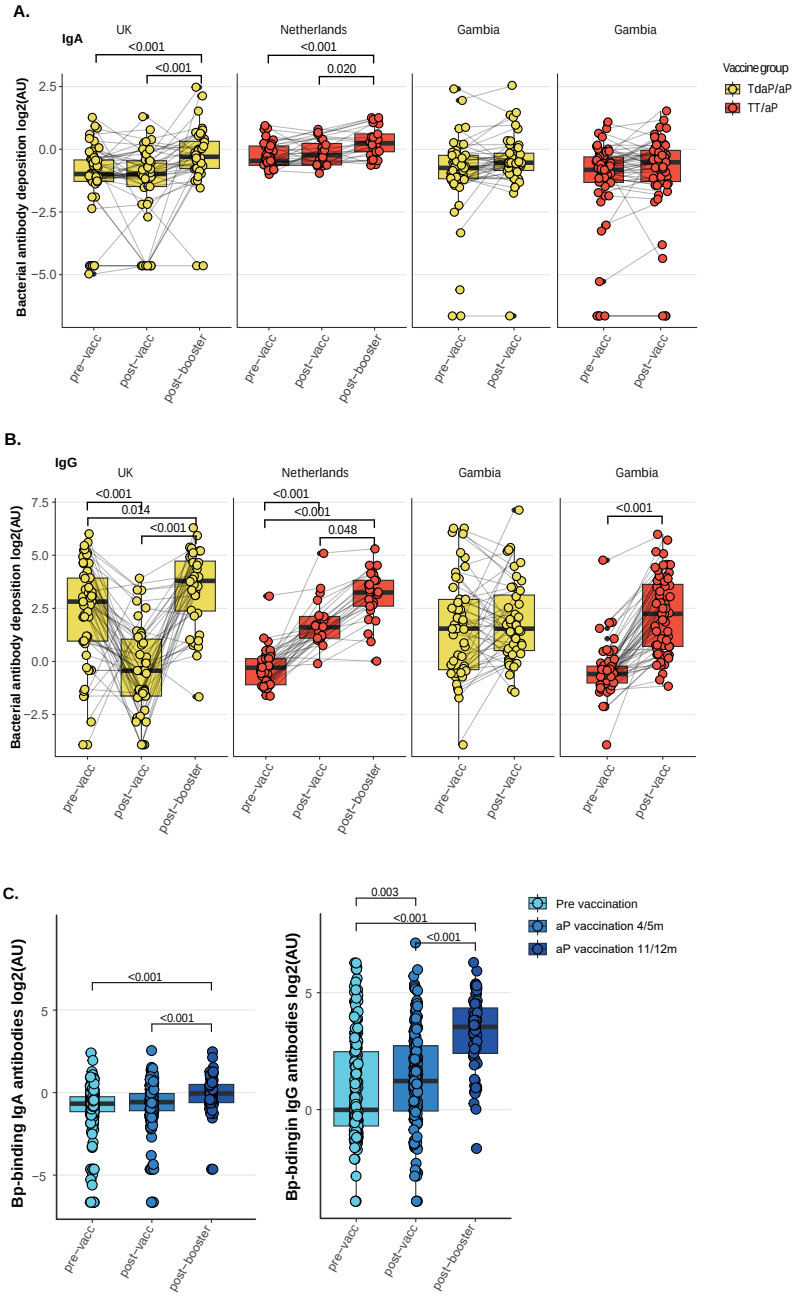


Figure 2 a) Bp-binding IgA antibody levels pre-vaccination, post primary series and post-'booster' vaccination **b)** Bp-binding IgG antibody levels pre-vaccination, post primary series and post-'booster' vaccination. **c)** Bp-binding IgA and IgG levels, pre-vaccination, post primary series and post-'booster' vaccination, with all vaccine groups together.

Bp-binding IgG was not influenced by age, time since symptom onset, duration of pertussis disease, or vaccination history (data not shown).

At three months post-diagnosis, IgA binding to Bp was significantly elevated in the clinical infection cohort, compared to the post-booster levels (**Figure 3a**). This difference remained significant when we restricted the analysis to the aP primed individuals (n=7) (**Figure S1**). No significant differences were observed for IgG binding between clinical cases and post-booster vaccination (**Figure 3b**). Although no pre-infection baseline samples were available from the clinical cases, these data suggest that clinical infection with Bp elevates serum IgA antibodies to Bp.

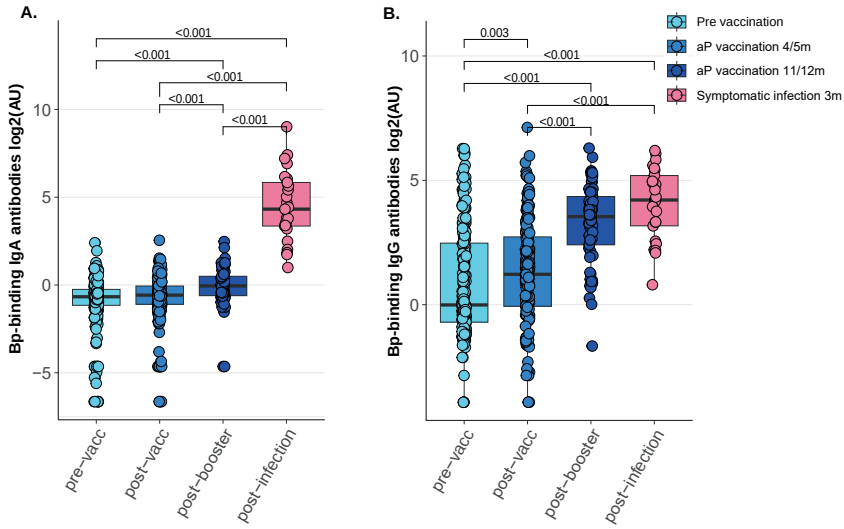


Figure 3. a) Bp-binding IgA levels pre-vaccination, post-primary vaccination (4-5mo of age) and post-'booster' vaccination (11-12mo of age), compared to the levels 3 months post a clinical Bp infection. **b)** Bp-binding IgG levels pre-vaccination, post-primary vaccination (4-5mo of age) and post-'booster' vaccination (11-12mo of age), compared to the levels 3 months post a clinical Bp infection.

Bp-binding IgA and IgG measured in sub-clinically infected individuals

One of the proposed explanations for the pertussis epidemiology in countries using aP vaccines is the high prevalence of subclinical infections with Bp[9,11,23]. To investigate the effect of subclinical Bp infections on the Bp-binding antibody levels, we included data from a controlled human infection (CHIM) study. In this study, 50 adult volunteers were intranasally inoculated with Bp, of whom 20 became colonized. Serum IgA and IgG binding to Bp was assessed at pre-inoculation baseline, as well as at 28 days and three months post-inoculation. We found a

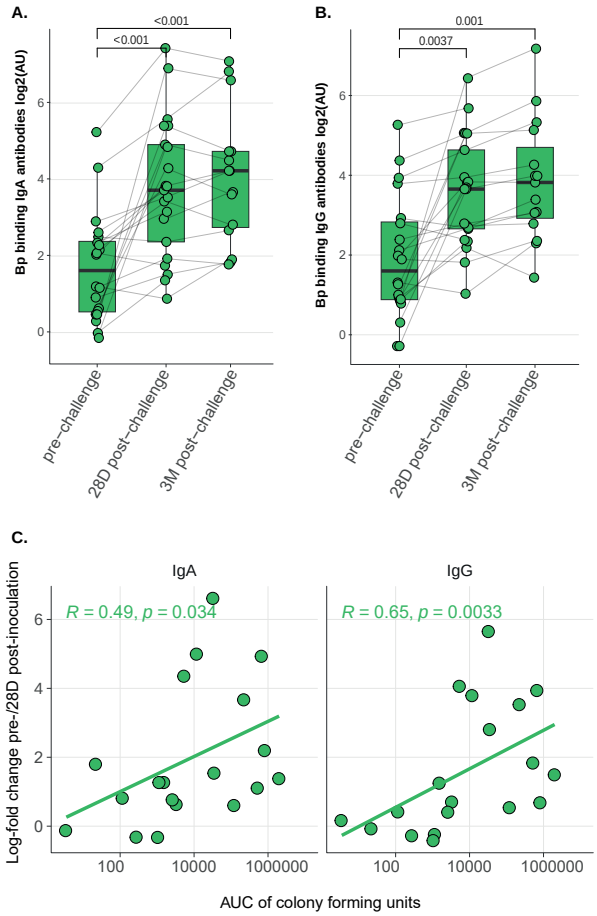


Figure 4. a) Bp-binding IgA levels pre-inoculation, 28 days post-inoculation and 3 months post-inoculation, of the colonized volunteers. **b)** Bp-binding IgG levels preinoculation, 28 days post-inoculation and 3 months post-inoculation, of the colonized volunteers. **c)** Spearman correlation of the Log2 fold change of Bp-binding IgA and I gG levels from 28 days post-inoculation over baseline, with the Area Under the Curve (AUC) of the colony forming units (CFU) of Bp in the colonized individuals.

significant increase in Bp-binding serum IgA and IgG in colonized participants (**Figure 4a,b**). Moreover, the magnitude of the antibody response, defined as log2-fold change over baseline, was positively correlated with total bacterial burden (Figure 4c). Conversely, no change in IgA or IgG binding was observed for non-colonised volunteers, suggesting that replication of *B. pertussis* is required to initiate a detectable serum antibody response (**Figure S2**). Moreover, at 3 months post-inoculation, serum IgA levels of colonized participants were similar to clinical infection cases. Conversely, non-colonized individuals showed significantly lower serum IgA levels post-inoculation than clinical pertussis cases (**Figure 5a**). For serum IgG binding, post-inoculation levels (3 months) did not significantly differ

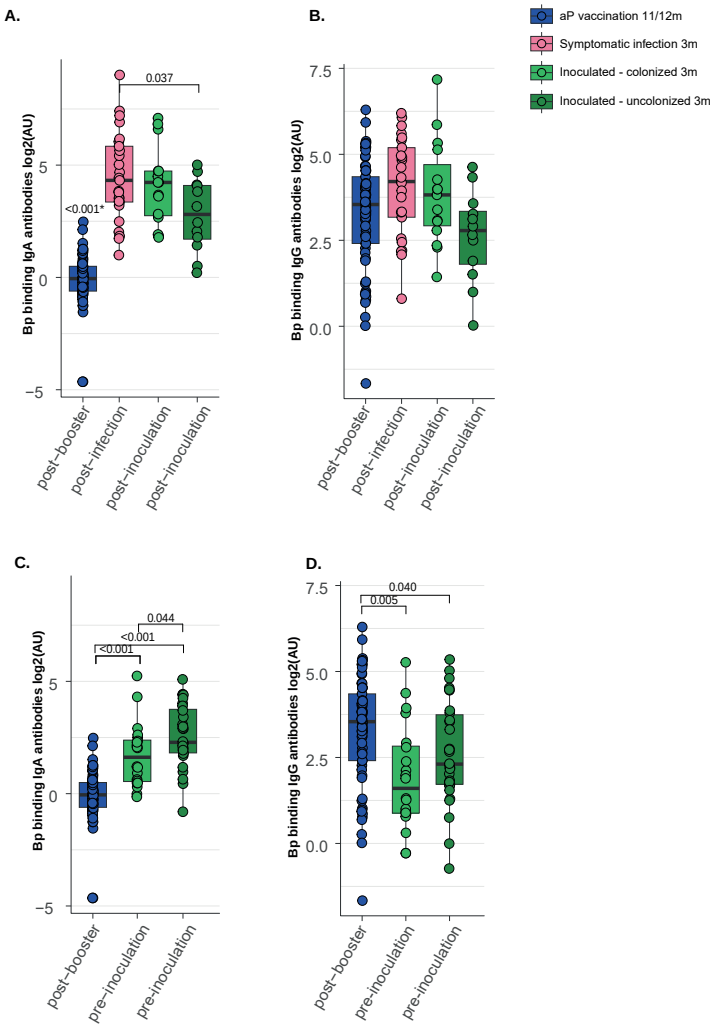


Figure 5. a) Bp-binding IgA and IgG **(b)** levels post-'booster' vaccination (11-12mo of age), compared to the levels 3 months post a clinical Bp infection, the 3 month levels in inoculated and colonized individuals, and the levels in inoculated and noncolonized individuals. **c)** Bp-binding IgA and IgG **(d)** levels post-'booster' vaccination (11-12mo of age), compared to te levels pre-inoculation of the colonized and noncolonized individuals.

from clinical pertussis cases, regardless of the colonization status of the inoculated volunteers (**Figure 5b**), although a trend was observed for lower IgG in the non-colonized volunteers (p=0.056 after correction for multiple testing). At baseline (pre-inoculation), serum IgA binding of non-colonized individuals was significantly higher than levels observed in colonized participants (**Figure 5c**). Conversely, no significant differences in Bp-binding IgG levels were detected between colonized

and non-colonized individuals at baseline (**Figure 5d**). These results demonstrate that serum IgA binding to Bp increases following subclinical Bp infection, but only in individuals in whom bacterial replication can be detected. Together with the observation that Bp-binding serum IgA levels do not increase post-aP vaccination, these findings support the use of serum IgA, but not IgG binding as a specific biomarker for detecting Bp infection in aP-primed individuals.

Comparison of Bp binding IgA and PT IgG

Since anti-PT IgG (PT-IgG) is the current immunological benchmark for detecting recent Bp infections, we also analysed serum PT-IgG levels of the cohorts for which we had PT-IgG data available (UK & Gambia vaccination, clinical infection and subclinical infection cohorts). When we compared PT-IgG concentrations post-booster vaccination and post-infection, we found very similar patterns as observed for IgG binding to Bp, although the clinical and subclinical Bp infection cohorts showed a nearly significant trend towards higher levels compared to the post-booster vaccination and non-colonized CHIM cohorts (**Figure S3**). To put these data into context, we incorporated the widely used cut-off level of 100 IU/mL for recent Bp infection (**Figure S3**, dashed red line). While both clinical and subclinical cases were -as expected- above the PT-IgG cut-off, specificity was poor, with almost 50% of non-colonized individuals and 48% of post-booster vaccination above 100 IU/mL.

Bp-binding serum IgA cut-off for Bp infection

The large differences in IgA Bp binding between the post-vaccination and post-infection cohorts, supported our hypothesis that we could use these Bp-binding antibody levels as a biomarker for (subclinical) infection. To establish a threshold value for Bp infection based on serum Bp-binding, we created a receiver operating curve (ROC), and calculated the optimal cut-off with a maximized sum of specificity and sensitivity, for IgA and IgG separately. We used the post-booster vaccination cohort as the negative population (n=65) and the 3 months post-diagnosis timepoint of the clinical infection cohort as the positive population (n=26). As a sensitivity analysis, only the aP primed individuals within the infection cohort (n=7), and/or the post-primary vaccination timepoint within the vaccination cohort were selected (n=168).

Comparing the area under the curve (AUC) of IgG and IgA binding to Bp, Bp-binding IgA was superior in predicting Bp infection, with a maximum AUC of 99.1% (sensitivity of 96%, specificity of 97%), at a cut-off level of 3.33 AU/mL (**Figure S4**, **Table S2**). Conversely, Bp-IgG binding showed poor performance, with a maximum AUC of 63.7%, (sensitivity of 56.0%, specificity of 73.8%). The sensitivity analysis

did not change this conclusion, although the use of the post-primary vaccination timepoint did improve the sensitivity of Bp-binding IgG (**table S3**). Thus, an IgA cut-off value of 3.33 AU/mL was able to categorize an individual into "infected" and "uninfected" in the aP vaccinated population.

Assessment of waning of Bp-binding IgA and IgG

To estimate waning of serum Bp-IgA and Bp-IgG binding, we fitted a binomial antibody decay model to the serum antibody data from the clinical infection cohort (**figure S5**, n=26). A decreasing pattern was observed from 3 to 9-12 months post-diagnosis in 24 clinical cases. The average time to reach the pre-defined serum Bp-IgA binding cut-off level of 3.33 AU/mL was 1,000 days, although significant inter-individual variation was observed between clinical cases (**Figure 6a**). Next, we compared antibody decay rates between Bp-IgA, Bp-IgG and PT-IgG (**Figure 6b**). We did not observe a significant difference between Bp-IgA and Bp-IgG waning, with a half-life of 406 and 391 days, respectively. PT-IgG showed faster waning, with a half-life of 231 days, and ~250 days to decrease below the 100 IU/mL cut-off, which is in line with previous research performed in clinical infection cohorts (**Figure 6b**)[24-26]. Thus, sero-positivity for Bp-IgA indicates Bp infection up to three years ago.

Population-based analysis of Bp-binding IgA

Finally, we examined serum Bp-IgA binding in the PIENTER-3 study cohort (n=759), a large cross-sectional sero-epidemiological study in the Netherlands that was performed in 2016-2017[21]. For this investigation, we selected a subset of participants who were born after 2005, i.e. following the switch from wP to aP vaccination. We included a total of 759 children between the age of 1-12, of whom 720 were fully vaccinated according to the Dutch National Immunization Program (DTaP at 2, 3, 4 and 11 months of age, with or without TdaP booster dose at 4y of age), and 59 children who were not vaccinated (**Table S4**). To assess the percentage of individuals that would have been infected following our Bp-binding IgA cut-off, we included a cut-off line and the percentage to the figure. Age-stratified analysis showed that serum IgA binding to Bp in the youngest age group (0-2y) was already significantly higher than the post-vaccination levels at approximately 1 year (p=0.006, **Figure 7**). Although an age-related increase in serum IgA binding was observed across the whole PIENTER-3 cohort (**Figure 7**), there were pronounced differences between the vaccinated and unvaccinated groups. Unvaccinated children reached higher IgA binding levels at an earlier age compared to vaccinated children, peaking around the age of 7-8y with a slow downward trend towards 11-12y (**Figure 7b**). Conversely, vaccinated children showed a significant increase in IgA binding until the age of 9-10y, when around 50% of all children had Bp-binding

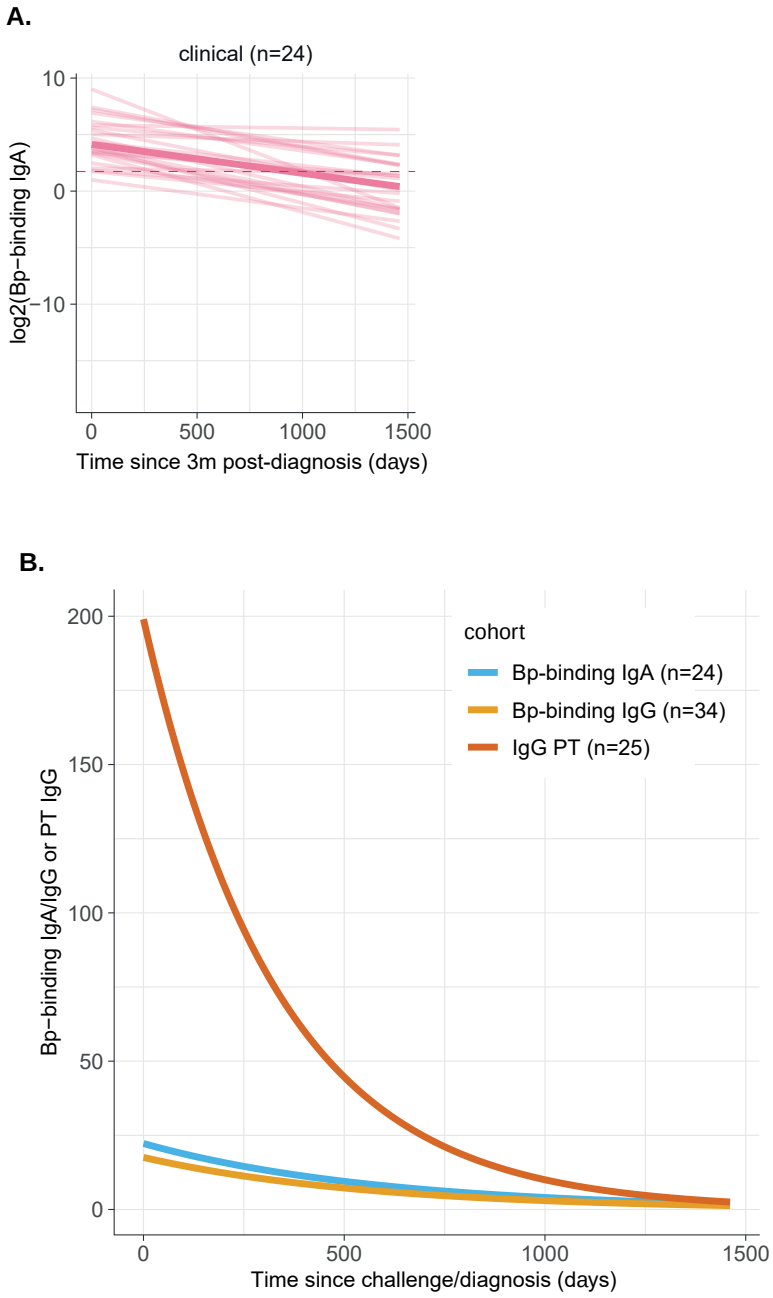


Figure 6. a) Decrease of log2 Bp-binding IgA levels in the clinical infection cohort, modeled with a binomial antibody decay model. The thick solid line is the general decay of the whole cohort, and each light line are the individual antibody decay patterns. Decay was calculated from 3 months post-diagnosis. **b)** The comparison of Bp-binding IgA, Bp-binding IgG and PT-specific IgG decay. The same method as described for A was used. For visualization purposes, the y-axis is not log2-tranformed.

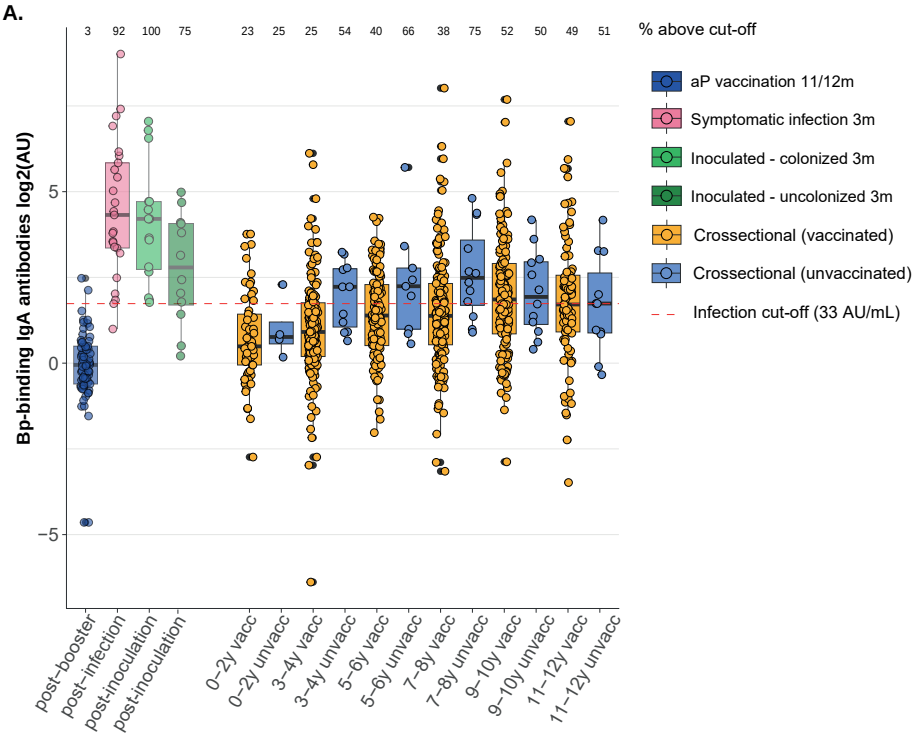


Figure 7. Bp-binding IgA levels in a Dutch aP-primed cohort. The cohort is depicted per 2 year age group, and per vaccination status (vaccinated vs. unvaccinated). Levels were compared to the post-vaccination and post-infection levels. An infection-threshold is depicted with a red dashed line in the figure, and the percentage of individuals above this cut-off is depicted at the top of the figure, for each group.

IgA levels above the infection cut-off. At the age of 9-10y or older, there was no difference anymore between vaccinated and unvaccinated children.

Next, we constructed a uni- and multivariate model to adjust for factors that could influence serum antibody binding levels, such as sex and reported coughing symptoms in the last 12 months, as collected by questionnaire. Age and vaccination status remained significant in this model, while sex and reported coughing did not influence serum Bp-IgA binding levels (table S5). Together, these results show important age- and vaccination-related differences in Bp-IgA seroprevalence in children, strongly suggestive of early and widespread circulation of Bp in the Dutch population.

A different pattern was observed for Bp-IgG, with a strong early increase in serum IgG binding observed between 0-2y and 3-4y, presumably related to the pre-school aP booster dose that is given in the fourth year of life (**Figure S6**). In sharp contrast to IgA, serum IgG binding then gradually declined, stabilizing around the age of 7-8y (**Figure S6**).

Discussion

In this study, we investigated the use of Bp-binding IgA and IgG levels to assess the prevalence of (subclinical) Bp infection in an aP primed cohort of the Netherlands. Where IgG was not able to distinguish well between vaccination and infection, we found that aP vaccination minimally affected Bp-binding IgA. Furthermore, Bp-binding IgA levels were high in clinical cases, and increased in individuals sub-clinically infected with Bp, significantly correlating with their total bacterial burden. In the Dutch aP cohort, serum Bp-binding IgA levels were elevated at an early age compared to immediately post-primary aP vaccination, and gradually increased until the age of 9-10years, after which more than 50% of the children had Bp-binding IgA levels above the infection threshold. Several publications have described that IgA against Bp-antigens increases in older age groups, and it has previously been suggested as a biomarker for infection[17,18]. However, to the best of our knowledge, this study is the first to assess IgA binding to the whole *B. pertussis* bacterium, instead of focusing on individual antigens. Moreover, our study includes a comprehensive serological analysis of complementary cohorts, enabling systematic comparison between primary aP vaccination, clinical and subclinical infection.

Similar to previous reports, we observed that serum Bp-binding IgA increases in older age groups[17,18]. However, studies performed before the switch from wP to aP vaccines reported elevated levels of IgA in individuals aged 20 years or older[17], while our study suggests that Bps infection occurs much earlier. The switch from wP to aP vaccines has been associated with an increase in subclinical infections, which may explain the differences in age-patterns. A recent modelling study, focused on the contribution of asymptomatic transmission, supports this role of subclinical transmission in the aP era[9]. Additionally. a recent prospective observational cohort study performed in South Africa showed that ~70% of the individuals infected with Bp did not develop any disease symptoms, and within households with Bp-transmission, 39% of the index-cases had a subclinical infection[23].

Various studies have described that primary aP vaccination elicit an immune response that, while effective against pertussis disease, may not be optimal for bacterial clearance[27-29], resulting in reduced protection against infection of the upper airways[30,31]. Studies in baboons have investigated the effect of repeated Bp infections in aP-primed baboons on the immune response and protection against subsequent challenge[32]. These studies demonstrate that multiple subclinical Bp infections are required to build up sufficient (mucosal) immunity to protect against subsequent challenge. In our aP-primed cross-sectional cohort, we observed early and high serum Bp-IgA binding, which we attribute to *B. pertussis* infection. Interestingly, we found that unvaccinated children showed much earlier and significantly higher Bp-IgA binding than age-matched children who were vaccinated with aP. There are several factors that may contribute to this difference. For instance, the total Bp bacterial load and severity of infection in infected unvaccinated children is very likely higher than in infected aP-vaccinated children. For other acute infections such as SARS-CoV-2, it has been shown that antigenic load and disease severity during infection is directly associated with the strength of both cellular and humoral immune responses, including antibody responses[33]. Similarly, we found that the serum IgA response following controlled human Bp infection correlated significantly with bacterial burden. An alternative explanation is that unvaccinated children may have simply experienced more Bp infections than vaccinated children. These observations provide important clues for understanding how vaccination and subsequent infections shapes immune status, which is critical for optimizing vaccine strategies.

The results from the controlled human Bp infection study showed that pre-challenge serum Bp-IgA binding was higher in individuals who were not colonized after challenge. Serum IgA responses also increased post-challenge, but only in infected individuals, suggesting that bacterial replication is required to activate B-cells and initiate serum IgA production. Studies in mice have shown that IgA alone may not be sufficient for protection against Bp infection [34]. However, most animal studies to date have systematically underestimated the effect of antibodies on prevention of Bp infection, as the experimental infectious dose is often high and likely does not accurately reflect natural exposure settings. Consequently, such studies typically rely on cellular responses to *clear* Bp infection, rather than *prevent* it. For future research, a prospective cohort study with frequent immunological and microbiological sampling would give more information on the role of IgA in protection against Bp infection.

Currently, we have calculated the infection cut-off by categorizing the post "booster" vaccination at 11/12 months of age as the negative cohort, and the complete clinical infection cohort as the positive cohort. In a sensitivity analysis, we replaced the post-booster timepoint with the post-primary (4 to 5 months of age) timepoint, or replaced the complete clinical infection cohort with only the aP-primed subset. Both replacements lowered the infection cut-off, while only slightly changing the sensitivity or specificity. As most aP-vaccination schedules include a 11/12 month "booster-vaccination" as part of the primary series, we reasoned that the cut-off value of 3.33 AU/mL was the most realistic. Furthermore, although we already see slight responses after the "booster" vaccination at 11/12 months of age, we predict that IgA responses after subsequent booster vaccinations will be higher, as there will be a higher chance that an individual has infection-induced IgA-producing memory B-cells. Including a cohort of individuals that received their 4-year or later booster-vaccination into the ROC-analysis would be interesting, and could give us a more realistic infection cut-off.

Finally, we have analysed the longevity of Bp-binding IgA by modelling the decay rate of the clinical infection cohort, and found that it takes approximately 3 years before levels are below the infection cut-off. However, we did not include the subclinical-infection cohort in this analysis, as only seven of the 20 colonized CHIM participants showed a decrease in Bp binding IgA levels from 28 days to 3 months post-challenge (**Figure 4a**). Thus, although we can say that elevated Bp-binding IgA levels could indicate a clinical infection up to three years ago, we cannot state how long Bp-IgA antibodies stay elevated after a subclinical infection.

In conclusion, our study describes that the binding of IgA to *B. pertussis* could be a promising biomarker for Bp infection-monitoring in aP-vaccinated populations. Using this biomarker, we revealed high rates of (subclinical) Bp infections in a Dutch cohort, that support the hypothesis that aP vaccination can lead to high rates of undiagnosed infection with possible onward transmission.

References

1. Torres RS, Santos TZ, Torres RA, et al. Resurgence of pertussis at the age of vaccination: clinical, epidemiological, and molecular aspects. *Jornal de pediatria* 2015; **91**(4): 333-8.
2. Broutin H, Viboud C, Grenfell B, Miller M, Rohani P. Impact of vaccination and birth rate on the epidemiology of pertussis: a comparative study in 64 countries. *Proceedings of the Royal Society B: Biological Sciences* 2010; **277**(1698): 3239-45.
3. Forsyth K, Plotkin S, Tan T, Wirsing von König CH. Strategies to decrease pertussis transmission to infants. *Pediatrics* 2015; **135**(6): e1475-e82.
4. Guiso N, Meade BD, von König CHW. Pertussis vaccines: the first hundred years. *Vaccine* 2020; **38**(5): 1271-6.
5. Pluijmakers A, de Melker H. The National Immunisation Programme in the Netherlands. Surveillance and developments in 2022-2023. 2023.
6. Ma L, Caulfield A, Dewan KK, Harvill ET. Pertactin-deficient Bordetella pertussis, vaccine-driven evolution, and reemergence of pertussis. *Emerging infectious diseases* 2021; **27**(6): 1561.
7. Barkoff A-M, Mertsola J, Pierard D, et al. Pertactin-deficient Bordetella pertussis isolates: evidence of increased circulation in Europe, 1998 to 2015. *Eurosurveillance* 2019; **24**(7): 1700832.
8. Domenech de Cellès M, Rohani P. Pertussis vaccines, epidemiology and evolution. *Nature Reviews Microbiology* 2024: 1-14.
9. Althouse BM, Scarpino SV. Asymptomatic transmission and the resurgence of Bordetella pertussis. *BMC medicine* 2015; **13**: 1-12.
10. Khalil A, Samara A, Campbell H, Ladhani SN, Amirthalingam G. Recent increase in infant pertussis cases in Europe and the critical importance of antenatal immunizations: We must do better… now. *International Journal of Infectious Diseases* 2024; **146**: 107148.
11. Gill C, Rohani P, Thea DM. The relationship between mucosal immunity, nasopharyngeal carriage, asymptomatic transmission and the resurgence of Bordetella pertussis. *F1000Research* 2017; **6**.
12. Cherry JD, Tan T, Wirsing von König C-H, et al. Clinical definitions of pertussis: Summary of a Global Pertussis Initiative roundtable meeting, February 2011. *Clinical Infectious Diseases* 2012; **54**(12): 1756-64.
13. MacIntyre CR, de Sousa JC, Heininger U, et al. Public health management of pertussis in adults: Practical challenges and future strategies. *Human Vaccines & Immunotherapeutics* 2024; **20**(1): 2377904.
14. Osborne K, Weinberg J, Miller E. The European sero-epidemiology network. *Eurosurveillance* 1997; **2**(4): 29-31.
15. Barkoff A-M, Gröndahl-Yli-Hannuksela K, He Q. Seroprevalence studies of pertussis: what have we learned from different immunized populations. *Pathogens and Disease* 2015; **73**(7): ftv050.
16. de Melker H, Versteegh F, Conyn-van Spaendonck M, et al. Specificity and sensitivity of high levels of immunoglobulin G antibodies against pertussis toxin in a single serum sample for diagnosis of infection with Bordetella pertussis. *Journal of clinical microbiology* 2000; **38**(2): 800-6.
17. Nagel J, Poot-Scholtens E. Serum IgA antibody to Bordetella pertussis as an indicator of infection. *Journal of medical microbiology* 1983; **16**(4): 417-26.
18. Hendrikx LH, Öztürk K, de Rond LG, et al. Serum IgA responses against pertussis proteins in infected and Dutch wP or aP vaccinated children: an additional role in pertussis diagnostics. *PLoS One* 2011; **6**(11): e27681.

19. van Schuppen E, Fröberg J, Venkatasubramanian PB, et al. Prior exposure to B. pertussis shapes the mucosal antibody response to acellular pertussis booster vaccination. *Nature Communications* 2022; **13**(1): 7429.
20. Barug D, Pronk I, van Houten MA, et al. Maternal pertussis vaccination and its effects on the immune response of infants aged up to 12 months in the Netherlands: an open-label, parallel, randomised controlled trial. *The Lancet Infectious Diseases* 2019; **19**(4): 392-401.
21. Verberk JDM, Vos RA, Mollema L, et al. Third national biobank for population-based seroprevalence studies in the Netherlands, including the Caribbean Netherlands. *BMC infectious diseases* 2019; **19**: 1-12.
22. van Gageldonk PG, van Schaijk FG, van der Klis FR, Berbers GA. Development and validation of a multiplex immunoassay for the simultaneous determination of serum antibodies to Bordetella pertussis, diphtheria and tetanus. *Journal of immunological methods* 2008; **335**(1-2): 79-89.
23. Moosa F, Tempia S, Kleynhans J, et al. Incidence and Transmission Dynamics of Bordetella pertussis Infection in Rural and Urban Communities, South Africa, 2016-2018. *Emerg Infect Dis* 2023; **29**(2): 294-303.
24. Dalby T, Petersen JW, Harboe ZB, Krogfelt KA. Antibody responses to pertussis toxin display different kinetics after clinical Bordetella pertussis infection than after vaccination with an acellular pertussis vaccine. *Journal of medical microbiology* 2010; **59**(9): 1029-36.
25. May ML, Doi SA, King D, Evans J, Robson JM. Prospective evaluation of an Australian pertussis toxin IgG and IgA enzyme immunoassay. *Clinical and Vaccine Immunology* 2012; **19**(2): 190-7.
26. Versteegh F, Mertens P, De Melker H, Roord J, Schellekens J, Teunis P. Age-specific long-term course of IgG antibodies to pertussis toxin after symptomatic infection with Bordetella pertussis. *Epidemiology & Infection* 2005; **133**(4): 737-48.
27. da Silva Antunes R, Babor M, Carpenter C, et al. Th1/Th17 polarization persists following whole-cell pertussis vaccination despite repeated acellular boosters. *The Journal of clinical investigation* 2018; **128**(9): 3853-65.
28. Ross PJ, Sutton CE, Higgins S, et al. Relative contribution of Th1 and Th17 cells in adaptive immunity to Bordetella pertussis: towards the rational design of an improved acellular pertussis vaccine. *PLoS pathogens* 2013; **9**(4): e1003264.
29. Higgs R, Higgins S, Ross P, Mills K. Immunity to the respiratory pathogen Bordetella pertussis. *Mucosal immunology* 2012; **5**(5): 485-500.
30. Warfel JM, Zimmerman LI, Merkel TJ. Acellular pertussis vaccines protect against disease but fail to prevent infection and transmission in a nonhuman primate model. *Proceedings of the National Academy of Sciences* 2014; **111**(2): 787-92.
31. Kapil P, Merkel TJ. Pertussis vaccines and protective immunity. *Current opinion in immunology* 2019; **59**: 72-8.
32. Kapil P, Wang Y, Zimmerman L, Gaykema M, Merkel TJ. Repeated bordetella pertussis infections are required to reprogram acellular pertussis vaccine–primed host responses in the baboon model. *The Journal of Infectious Diseases* 2024; **229**(2): 376-83.
33. Lynch KL, Whitman JD, Lacanienta NP, et al. Magnitude and kinetics of anti–severe acute respiratory syndrome coronavirus 2 antibody responses and their relationship to disease severity. *Clinical Infectious Diseases* 2021; **72**(2): 301-8.
34. Solans L, Debrie A-S, Borkner L, et al. IL-17-dependent SIgA-mediated protection against nasal Bordetella pertussis infection by live attenuated BPZE1 vaccine. *Mucosal immunology* 2018; **11**(6): 1753-62.

Supplementary tables

Table S1. Vaccination background of the clinical infection cohort, per age group.

Vaccine background	8-12y	13-25y	25-60y	>60y
aP + aP booster	6	1		
aP/wP + aP booster		3		
wP + aP booster		2		
wP			5	6
None / unknown	1		1	7

Table S2. Uni- and multivariate regression model of influence of demographic variables on log2-transformed Bp-binding IgA levels of the clinical infection cohort.

	Univariate analysis			**Multivariate analysis**		
	Estimate	***SE***	***p-value***	***Estimate***	***SE***	***p-value***
Age in years	0.04	0.01	***0.006***	-0.01	0.04	0.78
Time since symptom onset	0.01	0.01	0.24	-	-	-
Number of symptoms	0.12	0.10	0.24	-	-	-
Duration of pertussis disease						
< 4 weeks	(ref)	-		-	-	
4-8 weeks	2.07	0.82	-	1.45	0.81	-
>8 weeks	0.92	1.12	*0.02*0.42	0.45	1.21	*0.09*0.72
Vaccination history						
aP + aP booster	(ref)	-		(ref)	-	-
aP/wP + aP booster	1.18	1.18		1.22	1.22	0.33
wP + aP booster	0.56	1.37	-	0.28	1.50	0.85
wP	2.61	0.92	0.33	2.67	1.73	0.14
none	3.34	1.08	0.69	3.55	2.66	0.20
unknown	1.82	1.37	*0.010.006*0.20	1.82	1.40	0.21

Table S3. ROC-analysis output, with Area Under the Curve (AUC), Sensitivity and Specificity metrics for the optimal cut-off level. Method 1: post-booster vaccination (negative) & complete clinical infection cohort (positive); method 2: post-primary vaccination (negative) & complete clinical infection cohort (positive); method 3: post-primary vaccination (negative) & aP primed subset of clinical infection cohort (positive); method 4: post-booster vaccination (negative) & aP primed subset of clinical infection cohort (positive). Optimal cut-off levels were calculated using the maximize metric method in the "cutpointr" package.

	AUC	Sensitivity	Specificity	Cut-off level
Method 1 IgA	99.1	96	97.0	3.33
Method 1 IgG	63.7	56	73.8	18.05
Method 2 IgA	99.7	100	95.8	1.99
Method 2 IgG	85.5	96	65.6	4.24
Method 3 IgA	99.2	100	95.8	1.99
Method 3 IgG	83.3	71.4	89.8	18.05
Method 4 IgA	97.1	100	86.2	1.99
Method 4 IgG	65.7	71.4	73.8	18.05

Table S4. Demographics of the cross-sectional Dutch cohort of the PIENTER study.

	Total n= 779	0-2y n=61	3-4y n=158	5-6y n=123	7-8y n=189	9-10y n=152	11-12y n=96
Vaccinated n (%)	720 (92)	57	147	114	177	140	85
Female n (%)	372 (48)	27	75	59	80	83	48
Coughing symptoms in the past 12 months n (%)	247 (32)	31	65	42	53	37	19
Diagnosed with pertussis	6 (<1)	1	0	0	3	2	0

Table S5. Uni- and multivariate regression model of influence of demographic variables on log2-transformed Bp-binding IgA levels in the aP-primed PIENTER cohort.

	Univariate analysis			Multivariate analysis		
	Estimate	***SE***	***p-value***	***Estimate***	***SE***	***p-value***
Age in years	0.11	0.02	***<0.001***	0.11	0.02	***0.009***
Female sex	-0.05	0.12	0.63	-	-	-
Coughing in the past 12 months	0.06	0.12	0.62	-	-	-
Vaccinated following NIP						
yes	(ref)	-	-	-	-	-
no	0.62	0.22	***0.004***	0.55	0.21	***<0.001***

Chapter 8

Discussion

This thesis investigated the dynamics of mucosal immune responses in the context of infection and vaccination against SARS-CoV-2 and *Bordetella pertussis* (Bp). Here, I discuss the results of this thesis, and put them into a broader perspective.

Using immunological markers as an epidemiological tool

Traditionally, with the notable exception of sero-epidemiology[1], immunology and epidemiology have traditionally been distinct fields of infectious disease research[1]. Most studies focus either on the epidemiology of infection and disease, tracking incidence via symptom reporting or pathogen detection, or on the immunological responses such as antibody or cytokine levels. This separation is mainly because collecting both epidemiological and immunological data requires a complex, time-consuming, and expensive study design, and may also represent a high burden to study participants. However, treating these fields of research as separate entities can lead to incomplete or skewed interpretations of disease dynamics[2]. As illustrated in **Figure 1**, when we want to monitor the incidence of infection, symptom-based monitoring will underestimate this incidence in the case of subclinical or atypical infections, as often seen for SARS-CoV-2 and *B. pertussis*. Similarly, infection surveillance through pathogen detection alone will miss infections due to limited sensitivity or testing bias[3]. Conversely, relying solely on immunological biomarkers might overestimate the infection pressure if those markers cannot differentiate between symptomatic or subclinical infection and vaccination. In this thesis, I aimed to use immunological biomarkers of infection to explain epidemiological patterns, and *vice versa*.

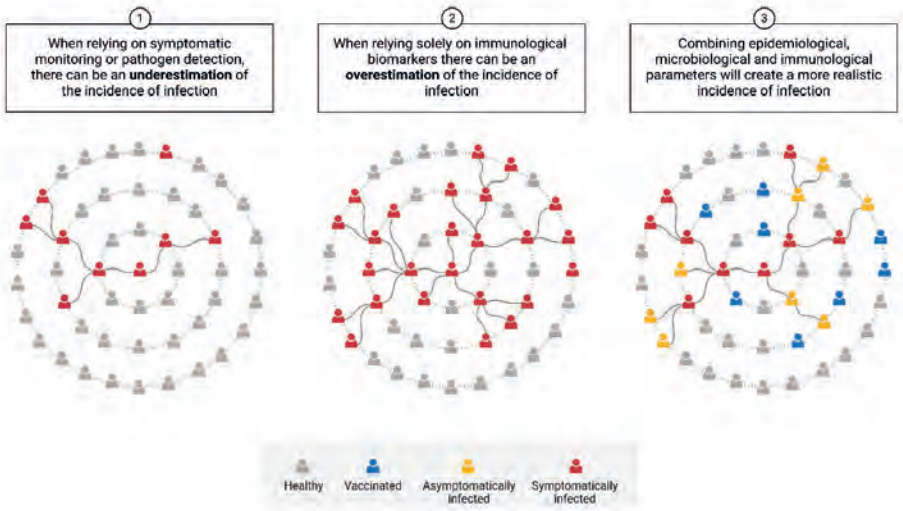


Figure 1. An illustration of how combining epidemiological data with microbiological and immunological data can enhance infection monitoring, by creating a more detailed picture of pathogen dynamics. **1)** Relying on symptomatic monitoring or pathogen detection (PCR or rapid antigen testing) can lead to an underestimation of the incidence of infection. **2)** Relying solely on immunological biomarkers can lead to an overestimation of the incidence of infection. **3)** Combining 1 and 2 will create a more realistic view of the incidence of infection.

In **Chapter 3**, we used the level of IgG antibodies in the serum and mucosal lining fluid (MLF) to identify additional SARS-CoV-2 cases among household contacts, revealing a high percentage of possible infections not captured by PCR testing or symptom-based reporting. Especially at a young age symptoms were often not reported, a unique characteristic of SARS-CoV-2 that has since been widely described[4]. This study showed the potential of using immunological biomarkers to enhance epidemiological research. In **Chapter 6**, pertussis notification data was used to support an immunological observation. We measured antibodies in the MLF that bound to a Bp bacterium that does not express the aP-vaccine antigens (mutant Bp). Older aP-primed children (11-15 years) had higher mucosal IgA binding to this mutant Bp compared to younger children (7-10 years) with the same vaccination history. We hypothesized that this difference was due to differences in exposure rates. We could strengthen this hypothesis by linking the regional pertussis notifications, that suggested greater exposure to *B. pertussis* in the older cohort. This could be attributed to several pertussis outbreaks that occurred before the birth of the younger age group, or possibly to different attack rates between the age groups, e.g. due to different contact patterns[5]. This integration of epidemiological and immunological data provided the basis for our hypothesis

that Bp infection history shapes population immunity, with increased levels of IgA post-infection.

In **Chapter 7**, we further built on the integration of epidemiological and immunological data, and tested the above hypothesis by using Bp-binding serum IgA levels as an immunological biomarker to estimate the Bp infection-rate in the Dutch aP-primed population. In comparison to post-vaccination levels, serum IgA binding to Bp after both clinical and subclinical Bp infection was elevated, providing supportive evidence for the use of IgA as an infection-specific biomarker. Measuring Bp-binding IgA in a large Dutch cohort of aP-primed children between the age of 1-12 years revealed elevated levels from a very young age, with further increases in older groups. These findings suggest early and widespread *B. pertussis* exposure in the aP-primed population of the Netherlands, with many infections presumably asymptomatic or subclinical. Our study aligns with previous studies, such as the studies of Nagel et al. and Hendrikx et al., who identified IgA as a possible biomarker for infection[6,7]. However, where Nagel's stud observed elevated IgA levels only in individuals aged 20 years or older, our study suggested earlier exposure, potentially linked to the transition from whole-cell (wP) to acellular (aP) pertussis vaccines. This vaccine switch has been associated with an increase in asymptomatic infections, that contributed to the pertussis resurgence[8-10]. A modelling study exploring asymptomatic transmission supports this role of asymptomatic transmission in the aP era[11]. Indeed, a recent prospective cohort study performed in South Africa showed that ~70% of individuals colonized with Bp were asymptomatic, and within households with confirmed Bp transmission, ~39% of the index cases did not report any symptoms[12].

While these findings highlight the potential of IgA as an infection-specific biomarker, at least in aP-primed populations, several challenges remain in validating its broader use for diagnosing asymptomatic infections. To serve as a reliable biomarker for asymptomatic infections, Bp-binding IgA should be pathogen-specific and wane relatively quickly following infection. Currently, pertussis toxin (PT)-specific IgG remains the gold standard for diagnosing recent pertussis infection; it shows high increases after infection and decreases relatively fast post-infection[13,14], and is very specific to *B. pertussis*[15,16]. However, its increase following pertussis vaccination complicates its utility in diagnosing cases in a vaccinated population[15]. Similarly, Bp-binding serum IgA shows promise but requires further evaluation, because despite high serum IgA-binding to Bp post-infection[7], the decline of IgA is relatively slow[6], as we observed in **Chapter 7**, which may affect its specificity in detecting

recent infection. Additionally, the pathogen specificity of Bp-binding IgA remains to be fully tested, particularly against closely related species like *B. parapertussis*.

In summary, this thesis illustrates how the integration of immunological and epidemiological data provides a more comprehensive view of disease incidence, especially for pathogens characterized by high rates of asymptomatic infections, such as SARS-CoV-2 and *B. pertussis*. By bridging these research fields, we can uncover patterns and insights that may remain hidden when focusing on one approach alone. Our findings suggest that Bp-binding IgA could be used to assess infection-derived immunity in populations, aiding vaccine effectiveness studies and refining incidence estimates. However, further research is needed to establish IgA's specificity and longevity to support its potential as a biomarker for recent asymptomatic infections.

Mucosal immunity and its implications for vaccine strategies

In the introduction of this thesis, the differences between infection- and vaccination-induced immunity were introduced, with a focus on the differential activation of the mucosal immune system. This difference is important, as it can affect immune responses after subsequent infection/vaccination. For instance, in **Chapter 6** we show that older individuals, who demonstrated a mucosal immunity reflective of higher pertussis exposure, responded more strongly to aP booster vaccination than younger individuals who showed evidence of less pertussis exposure. Additionally, in **Chapter 7**, age-matched unvaccinated children had higher Bp-binding IgA levels than aP-vaccinated children, particularly early in life, which could indicate differential immune activation in vaccinated vs. unvaccinated individuals. Since the COVID-19 pandemic, a lot of attention has been drawn to the differences between infection- and vaccination-induced immunity, and the effects of hybrid immunity as a result of both vaccinations and infections[17-19].

Importantly, in **Chapter 4** we show that the type of primary exposure influences the type of mucosal immune response. We compared mucosal antibody levels after primary infection or vaccination, and found that mucosal IgA production was stronger after a primary infection than after a primary vaccination, while primary vaccination elicited a stronger mucosal IgG response. Although we did not analyse whether the immune responses of the individuals who were primed through infection differed from those who were primed through vaccination upon

subsequent infection, multiple studies have shown that mucosal IgA induced by SARS-CoV-2 infection is associated with a lower risk of reinfection[20-22]. In contrast, post-vaccination IgG showed no correlation with protection against reinfection after adjusting for IgA levels [23]. In **Chapter 3,** we identified a similar possible protective role of mucosal IgA in SARS-CoV-2 infection: mucosal IgA correlates with a lower viral load and faster symptom recovery during primary SARS-CoV-2 infection. These observations align with other studies that investigated the role of nasal and saliva antibodies in primary infection[24]. Thus, our data on SARS-CoV-2 mucosal immunity supports the hypothesis that mucosal immunity can be influenced by the method of immune priming, and highlights an important role for mucosal IgA in limiting SARS-CoV-2 infection.

Similar immunological effects of the type of primary exposure have been described for *B. pertussis*, especially in the context of pertussis resurgence after the switch from whole-cell vaccination to aP vaccination. Studies in animal models comparing aP and wP vaccinations showed that wP vaccines elicit an infection-like cellular immune response, with Th-1/Th-17 T-cell responses that are important for bacterial clearance[25-27]. Contrastingly, acellular vaccines generated Th-2 T-cell responses which are considered less effective in clearing the Bp infection, leading to persistence of Bp in the upper respiratory tract and onward transmission[28]. In **Chapter 5**, we investigated how maternal TdaP-IPV vaccination and primary vaccination of infants with aP or wP influences the mucosal immune profile of infants. Our findings showed that infant wP vaccination elicited nasal cytokine levels reflective of T-cell activation, that were less pronounced in aP-vaccinated infants. These findings align with animal studies, which have shown that wP but not aP vaccination activates tissue-resident memory responses in the respiratory tract, particularly Th-1 and Th-17 T-cell responses[29,30]. Moreover, studies in baboons have shown that, upon Bp-infection, aP-primed baboons mounted a weaker Th-17 response than naïve baboons. Only after repeated *B. pertussis* did these aP-primed baboons develop immune responses comparable to those seen after primary infection[31], indicating that the immune priming could be reversed.

Although further validation is required, we also found that the nasal cytokine profile may vary depending on the maternal vaccine; wP-vaccinated infants born to Tdap-IPV vaccinated mothers exhibited nasal cytokine profile reflective of Th-2 T-cell activation, with a trend of higher levels of IL-5, IL-13, IL-4 and IL-21 compared to those born to TT-vaccinated mothers, and lower IL-17 levels. Interestingly, mouse studies showed that maternal aP vaccination reduces recruitment of IL-17 producing T-cells in the offspring, which was associated with prolonged nasal carriage of *B. pertussis* following experimental challenge[32].

Collectively, evidence from these animal studies combined with the results as described in **Chapter 5** suggest that aP vaccination, including maternal aP vaccination, results in a cellular immune profile characterized by low or even no Th-1/Th-17 activation. This diminished activation may impair mucosal immunity, and it may take (multiple) natural infections or possibly new vaccine formulations to "reprogram" the immune response for more effective bacterial clearance. Importantly, Th-17 has been shown to play an important role in mucosal IgA production and secretion[33,34]. Preclinical studies have shown that Th-17 activation is related to enhanced mucosal IgA production during subsequent infections, improving protection against e.g. *Streptococcus pyogenes*[35]. Recent advancements in the development of mucosal vaccines such as the live-attenuated *B. pertussis* vaccine (BPZE1), which is administered via the nose, further underscore the importance of both Th-17 and IgA in protection against infection, with preclinical models demonstrating that both IgA- and Th-17-dependent mechanisms contribute to protection against nasal colonization with Bp[36]. Moreover, whilst BPZE1 induced a mucosal IgA response and protected against colonization in a clinical study, aP vaccination neither induced mucosal IgA nor protected against secondary challenge with BPZE1, highlighting the potential of targeting mucosal immunity[37].

In summary, while aP vaccines effectively protect against severe disease, they may confer weak protection against colonization due to insufficient induction of mucosal IgA. Moreover, decreased activation of Th-17 T-cells may lower the immune system's ability to clear the infection when the bacterial load is too high for IgA to neutralize the infection. These insights provide a foundation for understanding how vaccination shapes mucosal immunity, which is critical for optimizing vaccine strategies. Candidate mucosal vaccines like BPZE1 offer a promising alternative by enhancing mucosal immunity through local Th-17 activation and IgA production. Future research should prioritize exploring the impact of (maternal) vaccination on Th-profiles, mucosal IgA responses, and transmission, as well as assessing the significance of the immune shift from Th-1/Th-17 to Th-2 activation observed in aP-primed individuals. A deeper understanding of these mechanisms can inform the development of vaccination strategies to not only prevent disease but also limit colonization and transmission.

Future perspectives

While this thesis shows the importance of integrating epidemiological and immunological research to generate new hypotheses, we have only begun to scratch the surface by ad-hoc combining these two fields and investigating indirect associations. Instead, to generate strong hypotheses about causality, particularly regarding protection against infection, large-scale epidemiological studies that integrate immunological, microbiological and clinical parameters are needed. The PIENTER sero-epidemiological study that was used in **Chapter 7** primarily focused on serum antibody levels against vaccine preventable diseases, with a cross-sectional study-design. Adding a longitudinal component and expanding the dataset to include complex (mucosal) immunological markers and pathogen detection data could enable more precise comparisons of infection- and vaccination-induced immunity. Moreover, a large-scale prospective study-design that integrates pathogen detection with systemic and mucosal immune response measurements could clarify the role of asymptomatic infections, especially when analysed using transmission modelling[2], or when integrated in a household transmission study[12].

Next to the population studies mentioned above, controlled human infection models (CHIMs) offer promising opportunities to study infection and immunity dynamics. Although performed in a cohort that is not fully representative of the population of interest (healthy, vaccinated adults), the controlled study-design does offer the unique opportunity to test (early) infection responses. In **Chapter 7**, colonization with B. pertussis following intranasal challenge resulted in increased levels of serum IgA, similar to those observed after clinical infection. Additional unpublished data suggest that individuals who were protected against colonization had higher pre-challenge levels of Bp-specific serum antibodies, Th17 responses, and mucosal IgA, supporting findings from previous animal studies. Building on this, we have recently initiated a CHIM study to investigate the protective efficacy against colonization of an acellular pertussis (aP) booster vaccination in whole-cell pertussis (wP)-primed individuals. This study aims to establish baseline vaccine effectiveness that can be used in future vaccine studies, as well as establish an infrastructure for pertussis CHIMs in the Netherlands.

While CHIM studies provide valuable insights into immune mechanisms and vaccine performance, broader questions remain about how vaccination schedules affect immunity across different population groups. Exploring these effects is crucial for optimizing immunisation strategies and understanding how vaccination

and infection history shapes responses to subsequent interventions. This thesis provides early evidence that vaccination background shapes immune responses and demonstrates in **Chapter 5** how maternal pertussis vaccination impacts subsequent infant vaccine responses.

Currently, much attention is being drawn to maternal Respiratory syncytial virus (RSV) vaccination, which is being implemented in many countries[38]. This vaccination aims to protect vulnerable infants from severe disease, much like the maternal pertussis vaccine, which has been effective in reducing pertussis-related infant mortality[39]. However, our findings in **Chapter 5** along with evidence from the literature, highlight possible challenges such as the blunting effect of maternal antibodies on infant immune responses and the induction of differential cytokine profiles that could influence long-term protection. These potential effects of the maternal vaccine could have broader epidemiological consequences, particularly if they reduce the long-term efficacy of infant immunization programs. For example, while no notable increase or shift in pertussis cases has been reported in England[40]—one of the first countries to implement the maternal pertussis vaccination—it remains possible that subclinical infections and underreporting could mask true changes in population immunity. While the goal of the maternal RSV vaccine is to prevent severe infection currently no RSV childhood vaccinations are implemented, the impact of the vaccine on a population-level needs to be monitored carefully.

Furthermore, co-administration of the maternal RSV and maternal pertussis vaccine could result in reduced responses to one of the vaccines, something that was observed for pertussis antigens in two phase II trials[38]. Addressing these concerns requires longitudinal studies with extended follow-up periods and systematic comparisons of vaccination schedules to fully understand the clinical and epidemiological trade-offs. Integrating immunological markers, such as those investigated in this thesis, with large-scale epidemiological data will be essential for refining maternal and infant vaccination strategies.

Another important consideration when evaluating the impact of vaccination is the quality of the immune response they elicit, particularly in terms of antibody functionality. As discussed in the introduction, IgA antibodies act mainly on the preventative side of protection, binding to the pathogen or its secretions (e.g. pertussis toxin). Due to its multimeric form in mucosal excretions, IgA can bind multiple microbial antigens, thereby effectively blocking interactions with epithelial cells[41]. On the other hand, IgG antibodies act more inflammatory, marking

the pathogen to be taken up by other immune cells, or activating the complement system to kill the bacteria by inducing the formation of a Membrane Attack Complex[42]. While this thesis has explored how vaccination schedules influence immunity, as seen with the effects of maternal vaccination in **Chapter 5**, the quality of antibodies generated after vaccination or infection also plays a critical role in protection. For example, in **Chapter 4** we demonstrate that mucosal antigen-specific antibody responses differ significantly after primary infection versus primary vaccination, predominately inducing IgA vs IgG, respectively. Despite these differences, the functional outcome -ACE-2 binding inhibition- was similar, underscoring the importance of looking beyond total antibody levels.

Additionally, both IgG and IgA are composed of multiple subclasses – IgG1-4 and IgA1-2 - that differ in structure and functionality. For example, while IgG1 and IgG3 are very effective in activating the complement system, IgG4 is unable to do so[42]. For SARS-CoV-2, having broader antibody isotype diversity has been associated with higher serum neutralization capacity[43]. A study investigating antibody subclasses in saliva between COVID-19 vaccinated and recovered individuals showed that repeated mRNA vaccination led to higher IgG4 levels in saliva and plasma[44]. Similarly, *B. pertussis*, studies have shown that repeated aP vaccination induces a relative IgG subclass shift towards IgG4[45]. Individuals primed with aP vaccines also showed elevated IgG4 levels post aP-booster vaccination compared to subjects who were primed with wP vaccines, suggesting that subclass distribution is affected by priming[46]. Thus, it is important to investigate the composition of antibody subclasses, their role in protection against (re)infection, and what type of antibodies are produced after vaccination or infection.

Next to antibody subclass analysis, cross-reactivity of antibodies may represent an important feature of protection. For instance, pre-existing cross-reactive antibodies, induced by prior infections with seasonal coronaviruses, have been related to protection against SARS-CoV-2, with milder COVID-19 symptoms and less intensive care admissions. In **Chapters 3 and 4** we demonstrated the presence of pre-existing mucosal antibodies against the nucleocapsid of SARS-CoV-2. Cross-reactive antibodies against SARS-CoV-2 are interesting because of the frequent mutations that occur in the SARS-CoV-2 viral genome, resulting in new variants that could evade immunity built against previous strains. A recent study investigating cross-reactivity of SARS-CoV-2 antibodies in hybrid immunity showed that, whilst both infection and vaccination generated antibodies against homologous antigens (i.e. those of the virus-variant that was in the vaccine/infection), individuals who got vaccinated after having been primed by infection generated a mucosal IgA

response that displayed a broader range of cross-reactivity against new variants of concern than individuals who were only vaccinated[44]. Conversely, as summarized in a recent review[47], other studies found that pre-existing immunity against the ancestral SARS-CoV-2 strain may also limit responses against the Omicron variant, something that has also been described for influenza[48]. Understanding this intricate interplay of infection and vaccination is therefore of major importance for understanding population immunity, and might guide the most effective booster vaccination strategies.

Regarding *B. pertussis*, cross-reactive antibodies against other closely related *Bordetella* species could influence subsequent immune responses. For instance, *B. parapertussis* expresses many antigens that shares homology to *B. pertussis* and can also infect humans. As mentioned above, the bacterial binding antibody levels measured in this thesis have been shown to bind to a wild-type Bp strain as well as an isogenic Bp strain that lacks the aP antigens. At this point, we have not yet evaluated the specificity of this assay against other *Bordetella* species. Obtaining more insight into the cross-reactivity of post-infection or post-vaccination mucosal antibodies could help with finding better vaccine candidates. New vaccines that provide cross-protective mucosal antibodies may reduce the burden of pertussis-like illness, as suggested by others studying new vaccine antigens[49]. This is especially important knowing that in the Netherlands, there has been an increase in the fraction of pertussis cases due to *B. parapertussis,* although overall numbers remain low[50].

In conclusion, this thesis underscores the important role of mucosal immunity in infection and disease prevention, and suggests that IgA could serve as a candidate biomarker of protection for SARS-CoV-2 and *B. pertussis*. While prevention of severe disease has traditionally been the focus of vaccine development, this thesis suggests that subclinical infections are prevalent and possibly play an important role in disease/transmission. The distinct antibody profiles after infection versus vaccination and the dynamics of mucosal versus systemic responses highlight the need for a broader perspective in vaccine development. For both pathogens, mucosal IgA plays a role in preventing colonization and transmission. Additionally, because of the ability of IgA to cross-react with related antigens it might also contribute to broader immunity and protection. These insights were derived by linking controlled immunological studies to epidemiological data, illustrating the central theme of this thesis: integrating immunological and epidemiological approaches to unravel the complexity of immunity in the real-world context. By combining insights from population-based studies with controlled experimental

models, we can better address critical questions about the interplay of infection, vaccination, and immune memory. Future vaccine development should prioritize strategies that enhance mucosal immunity, aiming not only to prevent disease but also to reduce infection and onward transmission—thereby shifting the paradigm towards controlling epidemic cycles more effectively.

References

1. Osborne K, Weinberg J, Miller E. The European sero-epidemiology network. *Eurosurveillance* 1997; **2**(4): 29-31.
2. Domenech de Cellès M, Rohani P. Pertussis vaccines, epidemiology and evolution. *Nature Reviews Microbiology* 2024: 1-14.
3. Wagenhäuser I, Knies K, Pscheidl T, et al. SARS-CoV-2 antigen rapid detection tests: test performance during the COVID-19 pandemic and the impact of COVID-19 vaccination. *eBioMedicine* 2024; **109**.
4. Abel L, Casanova J-L. Human determinants of age-dependent patterns of death from infection. *Immunity* 2024; **57**(7): 1457-65.
5. Rohani P, Zhong X, King AA. Contact network structure explains the changing epidemiology of pertussis. *Science* 2010; **330**(6006): 982-5.
6. Nagel J, Poot-Scholtens E. Serum IgA antibody to Bordetella pertussis as an indicator of infection. *Journal of medical microbiology* 1983; **16**(4): 417-26.
7. Hendrikx LH, Öztürk K, de Rond LG, et al. Serum IgA responses against pertussis proteins in infected and Dutch wP or aP vaccinated children: an additional role in pertussis diagnostics. *PLoS One* 2011; **6**(11): e27681.
8. Kemmeren J, de Melker H. The National Immunisation Programme in the Netherlands. Developments in 2009. 2010.
9. Schurink-van't Klooster T, De Melker H. The National Immunisation Programme in the Netherlands: Surveillance and developments in 2018-2019. 2019.
10. van Gent M, de Greeff SC, van der Heide HG, Mooi FR. An investigation into the cause of the 1983 whooping cough epidemic in the Netherlands. *Vaccine* 2009; **27**(13): 1898-903.
11. Althouse BM, Scarpino SV. Asymptomatic transmission and the resurgence of Bordetella pertussis. *BMC medicine* 2015; **13**: 1-12.
12. Moosa F, Tempia S, Kleynhans J, et al. Incidence and Transmission Dynamics of Bordetella pertussis Infection in Rural and Urban Communities, South Africa, 2016–2018. *Emerg Infect Dis* 2023; **29**(2): 294-303.
13. Cherry JD, Tan T, Wirsing von König C-H, et al. Clinical definitions of pertussis: Summary of a Global Pertussis Initiative roundtable meeting, February 2011. *Clinical Infectious Diseases* 2012; **54**(12): 1756-64.
14. Versteegh F, Mertens P, De Melker H, Roord J, Schellekens J, Teunis P. Age-specific long-term course of IgG antibodies to pertussis toxin after symptomatic infection with Bordetella pertussis. *Epidemiology & Infection* 2005; **133**(4): 737-48.
15. de Melker H, Versteegh F, Conyn-van Spaendonck M, et al. Specificity and sensitivity of high levels of immunoglobulin G antibodies against pertussis toxin in a single serum sample for diagnosis of infection with Bordetella pertussis. *Journal of clinical microbiology* 2000; **38**(2): 800-6.
16. Guiso N, Berbers G, Fry NK, et al. What to do and what not to do in serological diagnosis of pertussis: recommendations from EU reference laboratories. *European journal of clinical microbiology & infectious diseases* 2011; **30**: 307-12.
17. Lapuente D, Winkler TH, Tenbusch M. B-cell and antibody responses to SARS-CoV-2: infection, vaccination, and hybrid immunity. *Cellular & Molecular Immunology* 2024; **21**(2): 144-58.
18. Reynolds CJ, Pade C, Gibbons JM, et al. Immune boosting by B. 1.1. 529 (Omicron) depends on previous SARS-CoV-2 exposure. *Science* 2022; **377**(6603): eabq1841.

19. Cagigi A, Yu M, Österberg B, et al. Airway antibodies emerge according to COVID-19 severity and wane rapidly but reappear after SARS-CoV-2 vaccination. *JCI insight* 2021; **6**(22).
20. Marking U, Bladh O, Havervall S, et al. Mucosal IgA protects against BQ. 1 and BQ. 1.1 infection. *The Lancet Infectious Diseases* 2023; **23**(8): e272-e3.
21. Marking U, Bladh O, Havervall S, et al. 7-month duration of SARS-CoV-2 mucosal immunoglobulin-A responses and protection. *The Lancet Infectious Diseases* 2023; **23**(2): 150-2.
22. Havervall S, Marking U, Svensson J, et al. Anti-spike mucosal IgA protection against SARS-CoV-2 omicron infection. *New England Journal of Medicine* 2022; **387**(14): 1333-6.
23. Marking U, Havervall S, Norin NG, et al. Correlates of protection and viral load trajectories in omicron breakthrough infections in triple vaccinated healthcare workers. *Nature Communications* 2023; **14**(1): 1577.
24. Pisanic N, Antar AA, Hetrich MK, et al. Early, robust mucosal secretory IgA but not IgG response to SARS-CoV-2 spike in oral fluid is associated with faster viral clearance and COVID-19 symptom resolution. *The Journal of Infectious Diseases* 2024: jiae447.
25. da Silva Antunes R, Babor M, Carpenter C, et al. Th1/Th17 polarization persists following whole-cell pertussis vaccination despite repeated acellular boosters. *The Journal of clinical investigation* 2018; **128**(9): 3853-65.
26. Ross PJ, Sutton CE, Higgins S, et al. Relative contribution of Th1 and Th17 cells in adaptive immunity to Bordetella pertussis: towards the rational design of an improved acellular pertussis vaccine. *PLoS pathogens* 2013; **9**(4): e1003264.
27. Higgs R, Higgins S, Ross P, Mills K. Immunity to the respiratory pathogen Bordetella pertussis. *Mucosal immunology* 2012; **5**(5): 485-500.
28. Warfel JM, Zimmerman LI, Merkel TJ. Acellular pertussis vaccines protect against disease but fail to prevent infection and transmission in a nonhuman primate model. *Proceedings of the National Academy of Sciences* 2014; **111**(2): 787-92.
29. Dubois V, Chatagnon J, Depessemier M, Locht C. Maternal acellular pertussis vaccination in mice impairs cellular immunity to Bordetella pertussis infection in offspring. *JCI insight* 2023; **8**(18).
30. Dubois V, Chatagnon J, Thiriard A, et al. Suppression of mucosal Th17 memory responses by acellular pertussis vaccines enhances nasal Bordetella pertussis carriage. *Npj Vaccines* 2021; **6**(1): 6.
31. Kapil P, Wang Y, Zimmerman L, Gaykema M, Merkel TJ. Repeated bordetella pertussis infections are required to reprogram acellular pertussis vaccine–primed host responses in the baboon model. *The Journal of Infectious Diseases* 2024; **229**(2): 376-83.
32. Borkner L, Curham LM, Wilk MM, Moran B, Mills KH. IL-17 mediates protective immunity against nasal infection with Bordetella pertussis by mobilizing neutrophils, especially Siglec-F+ neutrophils. *Mucosal immunology* 2021; **14**(5): 1183-202.
33. Jaffar Z, Ferrini ME, Herritt LA, Roberts K. Cutting edge: lung mucosal Th17-mediated responses induce polymeric Ig receptor expression by the airway epithelium and elevate secretory IgA levels. *The Journal of Immunology* 2009; **182**(8): 4507-11.
34. Christensen D, Mortensen R, Rosenkrands I, Dietrich J, Andersen P. Vaccine-induced Th17 cells are established as resident memory cells in the lung and promote local IgA responses. *Mucosal immunology* 2017; **10**(1): 260-70.
35. Mortensen R, Christensen D, Hansen LB, Christensen JP, Andersen P, Dietrich J. Local Th17/IgA immunity correlate with protection against intranasal infection with Streptococcus pyogenes. *PLoS One* 2017; **12**(4): e0175707.

36. Solans L, Debrie A-S, Borkner L, et al. IL-17-dependent SIgA-mediated protection against nasal Bordetella pertussis infection by live attenuated BPZE1 vaccine. *Mucosal immunology* 2018; **11**(6): 1753-62.

37. Keech C, Miller VE, Rizzardi B, et al. Immunogenicity and safety of BPZE1, an intranasal live attenuated pertussis vaccine, versus tetanus–diphtheria–acellular pertussis vaccine: a randomised, double-blind, phase 2b trial. *The Lancet* 2023; **401**(10379): 843-55.

38. Papazisis G, Topalidou X. Maternal Vaccination for the Prevention of Infantile RSV Disease: An Overview of the Authorized, In-Progress, and Rejected Vaccine Candidates. *Vaccines* 2024; **12**(9): 980.

39. Amirthalingam G, Andrews N, Campbell H, et al. Effectiveness of maternal pertussis vaccination in England: an observational study. *The Lancet* 2014; **384**(9953): 1521-8.

40. Amirthalingam G, Campbell H, Ribeiro S, et al. Optimization of timing of Maternal Pertussis Immunization from 6 years of postimplementation surveillance data in England. *Clinical Infectious Diseases* 2023; **76**(3): e1129-e39.

41. Mestecky J, Lamm ME, Ogra PL, et al. Mucosal immunology: Elsevier; 2005.

42. Burton D, Gregory L, Jefferis R. Aspects of the molecular structure of IgG subclasses. *Monographs in allergy* 1986; **19**: 7-35.

43. Noval MG, Kaczmarek ME, Koide A, et al. Antibody isotype diversity against SARS-CoV-2 is associated with differential serum neutralization capacities. *Scientific Reports* 2021; **11**(1): 5538.

44. Selva KJ, Ramanathan P, Haycroft ER, et al. Preexisting immunity restricts mucosal antibody recognition of SARS-CoV-2 and Fc profiles during breakthrough infections. *JCI insight* 2023; **8**(18).

45. van Twillert I, Bonačić Marinović AA, Kuipers B, van Gaans-van den Brink JA, Sanders EA, van Els CA. Impact of age and vaccination history on long-term serological responses after symptomatic B. pertussis infection, a high dimensional data analysis. *Scientific reports* 2017; **7**(1): 40328.

46. Schure R-M, Hendrikx LH, de Rond LG, et al. Differential T-and B-cell responses to pertussis in acellular vaccine-primed versus whole-cell vaccine-primed children 2 years after preschool acellular booster vaccination. *Clinical and Vaccine Immunology* 2013; **20**(9): 1388-95.

47. Murray SM, Ansari AM, Frater J, et al. The impact of pre-existing cross-reactive immunity on SARS-CoV-2 infection and vaccine responses. *Nature Reviews Immunology* 2023; **23**(5): 304-16.

48. Krammer F. The human antibody response to influenza A virus infection and vaccination. *Nature Reviews Immunology* 2019; **19**(6): 383-97.

49. Natrajan MS, Hall JM, Weigand MR, et al. Genome-based prediction of cross-protective, HLA-DR-presented epitopes as putative vaccine antigens for multiple Bordetella species. *Microbiology spectrum* 2024; **12**(1): e03527-23.

50. Pluijmakers A, de Melker H. The National Immunisation Programme in the Netherlands. Surveillance and developments in 2022-2023. 2023.

Chapter 9

Nederlandse Samenvatting
English Summary
Acknowledgements
Curriculum Vitae
Research Data Management
Graduate School Portfolio

Nederlandse Samenvatting

Luchtweginfecties zijn wereldwijd de meest voorkomende infectieziekten en veroorzaken jaarlijks miljoenen sterfgevallen, vooral bij kinderen en kwetsbare groepen. Deze infecties kunnen ontstaan door virussen zoals SARS-CoV-2 (COVID-19) en influenza (griep), of bacteriën zoals *Bordetella pertussis* (kinkhoest). Niet elke blootstelling aan een ziekteverwekker leidt tot ziekte. Als je bijvoorbeeld maar kortdurend wordt blootgesteld, is de kans dat je ziek wordt kleiner dan als je langer wordt blootgesteld. Ook het type ziekteverwekker is hierin belangrijk; van sommige "beestjes" word je sneller ziek dan van andere. Ten slotte speelt je eigen immuunsysteem -ook wel afweersysteem genoemd- een cruciale rol: het kan ziekteverwekkers elimineren voordat er klachten ontstaan, of ervoor zorgen dat de ziekteverwekker snel wordt opgeruimd, waardoor de klachten mild blijven. Bij een niet goed functionerend immuunsysteem kunnen er sneller ernstige klachten ontstaan, of kan het voorkomen dat je vaker dan anderen ziek wordt. Mijn proefschrift focust zich op de beschermende rol van het immuunsysteem, met een speciale focus op het immuunsysteem van de bovenste luchtwegen; de neus- en keelholte.

De luchtwegen zijn namelijk vaak de eerste plek waar een ziekteverwekker in aanraking komt met het lichaam: je ademt continu lucht in waar ziekteverwekkers in kunnen zitten. Het immuunsysteem van de luchtwegen bestrijdt ziekteverwekkers op drie verschillende, complementaire manieren:

- **De fysieke barrière:** Neusslijm vangt ziekteverwekkers uit de ingeademde lucht, en trilharen verwijderen ze uit de luchtwegen.
- **Het aangeboren immuunsysteem**: Als de ziekteverwekker het lichaam binnendringt, voorkomt het aangeboren immuunsysteem verdere infectie door snelle, niet-specifieke reacties. De productie van antibacteriële stoffen kan de ziekteverwekker doden. Ook trekken deze stoffen andere immuuncellen aan; cellen die zijn gespecialiseerd in het opruimen van ziekteverwekkers.
- **Het verworven immuunsysteem**: Als het aangeboren immuunsysteem niet genoeg is om de ziekteverwekker op te ruimen, start het lichaam een gerichte immuunreactie. Hierbij werken antigeen-presenterende cellen, T- en B-cellen samen om de ziekteverwekker gericht aan te vallen. Antigeen-presenterende cellen presenteren deeltjes van de ziekteverwekker aan de T-cel, zodat deze geactiveerd wordt. De T-cel kan de bacterie dan doden, of een B-cel activeren. B-cellen maken antistoffen aan. Het verworven immuunsysteem zorgt ook voor immunologisch geheugen, waardoor het lichaam adequater kan reageren, wanneer het lichaam opnieuw in aanraking komt met dezelfde ziekteverwekker.

Mijn proefschrift richt zich vooral op een deel van het verworven immuunsysteem, namelijk de antistoffen, ook wel antilichamen of immunoglobulines genoemd. Antistoffen zijn microscopisch kleine deeltjes, die worden geproduceerd door geactiveerde B-cellen. Ze spelen een sleutelrol bij de bestrijding van onder andere luchtweginfecties. Er zijn 5 types antistoffen: IgG, IgA, IgM, IgE en IgD, die allen een andere functie hebben. In dit proefschrift focus ik me op IgG en IgA. IgG is de meest voorkomende antistof in het bloed, en helpt vooral bij het opruimen van ziekteverwekkers tijdens een infectie: het kan binden aan de ziekteverwekker, waardoor andere immuuncellen, zoals macrofagen, de ziekteverwekker kunnen opruimen. IgA wordt ook wel de antistof van de mucosa (het slijmvlies) genoemd: IgA is in grote hoeveelheden aanwezig in de slijmvlieslaag van de luchtwegen, en biedt hier lokale verdediging tegen virussen en bacteriën. IgA is vooral erg goed in het blokkeren van ziekteverwekkers, waardoor ze geen schade kunnen veroorzaken. Bij infecties wordt vaak zowel een lokale (een immuunrespons in het slijmvlies) als een systemische reactie van het immuunsysteem (een immuunrespons in onder andere het bloed) opgewekt, met een toename van IgG en IgA.

Om ziekte en sterfte door infecties te voorkomen, zijn vaccinaties van groot belang. Wereldwijd hebben vaccinaties miljoenen levens gered. Vaccinaties zorgen, net als natuurlijke infecties, voor een activatie van het verworven immuunsysteem en het aanmaken van immunologisch geheugen. De meeste vaccins worden echter toegediend via injecties (intramusculair) en stimuleren daardoor vooral de afweer in het bloed, en dus met name IgG-antistoffen. Hoewel dit effectief is tegen ernstige ziekte, bieden vaccinaties in sommige gevallen onvoldoende bescherming tegen milde klachten en verspreiding van ziekteverwekkers via de slijmvliezen. Dit is bijvoorbeeld te zien bij de ziekteverwekkers SARS-CoV-2 en *B. pertussis*. Individuen die zijn gevaccineerd tegen deze ziekteverwekkers, kunnen soms nog steeds besmet raken en anderen infecteren. In dit proefschrift focus ik me op de antistofrespons na infectie en de antistofrespons na vaccinatie tegen deze twee ziekteverwekkers.

SARS-CoV-2 is een virus dat de ziekte COVID-19 veroorzaakt. Het virus werd eind 2019 voor het eerst gevonden in China. Het virus behoort tot de coronavirussen en verspreidt zich voornamelijk via druppeltjes in de lucht. COVID-19 kan veel verschillende symptomen geven, variërend van asymptomatische infectie (geen symptomen) of milde klachten zoals koorts en hoesten, tot ernstige longontstekingen en ademhalingsproblemen. Hoewel er inmiddels effectieve vaccins beschikbaar zijn, waren deze nog niet beschikbaar toen ik mijn onderzoek begon. De meeste mensen waren op dat moment ook nog niet blootgesteld

aan SARS-CoV-2. Hierdoor had ik in mijn proefschrift de unieke kans om de antistofrespons in de bovenste luchtwegen na een eerste infectie te onderzoeken, en deze te vergelijken met de respons na een eerste vaccinatie.

Bordetella pertussis is de bacterie die de ziekte kinkhoest veroorzaakt, een luchtweginfectie die bekend staat om de langdurige, heftige hoestbuien ("de 100-dagen hoest"). De ziekte is vooral gevaarlijk voor jonge baby's, die nog niet volledig gevaccineerd zijn, en kan leiden tot ziekenhuisopnames en sterfte. Ondanks uitgebreide vaccinatieprogramma's en een hoge vaccinatiegraad blijft kinkhoest een wereldwijd probleem. Dit heeft verschillende oorzaken. Allereerst is kinkhoest een heel besmettelijke ziekte: een geïnfecteerd individu kan 10 tot 15 andere mensen besmetten. Daarnaast wordt een toename van het aantal kinkhoestbesmettingen gezien, sinds het type vaccin is veranderd. Het huidige kinkhoestvaccin is namelijk erg effectief in het voorkomen van ziekte, maar is minder effectief in het klaren van de bacterie: gevaccineerde individuen zullen minder ziek worden, maar kunnen de kinkhoestbacterie nog steeds bij zich dragen en verspreiden. In mijn proefschrift heb ik de immuunrespons na kinkhoestvaccinatie en -infectie onderzocht.

De afgelopen 5 jaar ben ik samen met andere wetenschappers druk bezig geweest met onderzoek doen, waarvan de resultaten in dit proefschrift te lezen zijn. Het hoofddoel van dit proefschrift was het onderzoeken hoe infectie en vaccinatie de antistofrespons beïnvloeden, met een focus op de antistofrespons in de bovenste luchtwegen. Door vergelijkingen tussen antistofrespons op infectie en vaccinatie, kunnen we beter begrijpen hoe we de bescherming tegen luchtweginfecties kunnen verbeteren. Niet alleen tegen ernstige ziekte maar ook tegen verspreiding. Dit is belangrijk voor de ontwikkeling van betere vaccins.

Het eerste deel van dit proefschrift (Hoofdstukken 2, 3, en 4) gaat over de lokale immuunrespons na SARS-CoV-2-infectie en -vaccinatie. Allereerst wordt in hoofdstuk 2 alle informatie over de rol van het lokale immuunsysteem bij een SARS-CoV-2 infectie samengevat. Daarna onderzochten we in hoofdstuk 3 wat er veranderde in het immuunsysteem van de neus, na een eerste infectie met SARS-CoV-2. We lieten zien dat er een duidelijke toename was van antistoffen in de neus, als iemand besmet was met SARS-CoV-2. We toonden daarnaast een verband aan tussen een snelle IgA-reactie en een lagere hoeveelheid virusdeeltjes in de neus. De lokale antistofrespons in de neus, vooral de IgA-respons, was ook gerelateerd aan een snellere afname van klachten. We zagen meer klachten bij volwassenen dan bij kinderen. In hoofdstuk 4 vergeleken we de immuunrespons na infectie

vervolgens met de immuunrespons na een eerste vaccinatie. We lieten zien dat deze van elkaar verschilden, met een grotere IgA-respons na infectie, en meer IgG na vaccinatie. De functionaliteit van de antistoffen (hoe goed ze de ziekteverwekker konden blokkeren) was echter niet verschillend. Gezamenlijk tonen de resultaten uit hoofdstukken 2 tot en met 4 aan dat de lokale antistofrespons na een SARS-CoV-2 infectie, vooral de IgA-respons, geassocieerd is met een snellere afname van klachten en een lagere virale belasting. De immuunrespons na vaccinatie verschilt hiervan, met meer IgG-antistoffen en een minder uitgesproken IgA-respons. Dit is nuttige informatie voor het ontwikkelen van eventuele nieuwe COVID-19-vaccins.

Na de hoofdstukken over SARS-CoV-2 veranderen we van ziekteverwekker en onderzoeken we de antistofrespons na *B. pertussis*-vaccinatie en -infectie. In hoofdstuk 5 vergeleken we vier verschillende kinkhoestvaccinatie-combinaties met elkaar: de moeder kreeg wel of niet een kinkhoestvaccinatie tijdens de zwangerschap, en de baby kreeg óf het vaccin dat bestond uit de hele kinkhoestbacterie (wP), óf het vaccin dat bestond uit een aantal eiwitten van de kinkhoestbacterie (aP). In dit hoofdstuk toonden we aan dat de kinkhoestvaccinatie tijdens de zwangerschap zorgde voor veel antistoffen in de neus van de baby, als deze twee maanden oud was. Dit betekende dat we antistoffen in de neus van de baby konden meten, die tijdens de zwangerschap door de moeder waren aangemaakt en via de placenta aan de baby waren doorgegeven. Dit gold echter alleen voor IgG; IgA werd niet overgedragen van moeder naar kind. We lieten ook zien dat het wP-vaccin zorgde voor hogere IgG-antistof niveaus in de neus van de baby dan het aP-vaccin, vooral bij baby's waarvan de moeder een kinkhoestvaccinatie had gekregen tijdens de zwangerschap. IgA nam niet toe na aP- of wP-vaccinatie.

In hoofdstuk 6 onderzochten we de antistofrespons na een aP-vaccinatie in vrijwilligers die als kind ook gevaccineerd waren met het aP-vaccin. We maakten hierbij gebruik van een nieuwe methode, waarbij we de antistoffen die aangemaakt werden na vaccinatie konden onderscheiden van de antistoffen die aangemaakt werden na het doormaken van een infectie. We zagen dat oudere kinderen meer infectie-antistoffen hadden, voordat ze het aP-vaccin kregen. Door te kijken naar de hoeveelheid kinkhoestmeldingen in de twee leeftijdsgroepen, konden we de hypothese opstellen dat de hogere antistof niveaus kwamen door blootstelling aan de kinkhoestbacterie, die een infectie zonder klachten had veroorzaakt. Er waren namelijk meer kinkhoestmeldingen geweest in de oudere leeftijdsgroep, dus de vrijwilligers in deze groep hadden een grotere kans blootgesteld te zijn geweest aan *B. pertussis*.

Met deze informatie over IgA en blootstelling in ons achterhoofd, onderzochten we in hoofdstuk 7 de hoeveelheid kinkhoestinfecties in Nederland, door te kijken naar de IgA-antistofniveaus. In de voorgaande hoofdstukken hadden we al aangetoond dat IgA niet toenam na vaccinatie met het aP-vaccin. In dit hoofdstuk lieten we zien dat dit het geval was in vier verschillende vaccinatiecohorten uit verschillende landen en met verschillende vaccinatieschema's. Ook beschreven we dat een infectie met kinkhoest wel zorgde voor veel IgA-antistoffen, ook als er geen klachten waren. We beschreven dat IgA een goede parameter is om infectie van vaccinatie te onderscheiden. Hierna bestudeerden we de IgA-antistof niveaus van een groot Nederlands cohort van aP-gevaccineerde kinderen. Hierin zagen we dat de antistof niveaus al op jonge leeftijd hoog waren, en een leeftijdsafhankelijke toename lieten zien. Dit suggereert dat de hoeveelheid kinkhoestinfecties die niet gediagnosticeerd wordt in Nederland groot is, wat zou kunnen bijdragen aan de verspreiding van de bacterie.

Samenvattend blijkt uit dit proefschrift dat kinkhoestvaccinatie tijdens de zwangerschap de overdracht van IgG-antistoffen naar de neus van de baby bevordert, maar dat dit ook effecten kan hebben op de vaccinatierespons van de baby. Daarnaast werd aangetoond dat infecties met *Bordetella pertussis*, zelfs in de afwezigheid klachten, leiden tot een verhoging van de IgA-antistoffen, terwijl vaccinaties hier niet voor zorgen. Tot slot liet dit proefschrift zien dat er waarschijnlijk een grote hoeveelheid kinkhoestinfecties plaatsvindt die niet worden opgemerkt.

Gezamenlijk dragen de bevindingen uit dit proefschrift bij aan het beter begrijpen van de immuunrespons in de bovenste luchtwegen. Dit proefschrift benadrukt het belang van lokale antistofproductie in de slijmvliezen, en biedt belangrijke aanknopingspunten voor het verbeteren van vaccins tegen luchtweginfecties, zodat deze, naast bescherming tegen ernstige ziekte, hopelijk in de toekomst ook meer bescherming bieden tegen infectie zonder klachten.

English Summary

Respiratory infections are the most common infectious diseases worldwide, causing millions of deaths annually, especially among children and vulnerable groups. These infections can be caused by viruses such as SARS-CoV-2 (COVID-19) and influenza (flu), or bacteria such as *Bordetella pertussis* (whooping cough). Not every exposure to a pathogen leads to illness. For example, if you are exposed for a short period, the likelihood of getting sick is lower than if you are exposed for a longer period. The type of pathogen is also important; some "bugs" make you sick faster than others. Finally, your own immune system plays a crucial role: it can eliminate pathogens before symptoms appear or ensure that the pathogen is quickly cleared, keeping symptoms mild. If the immune system is not functioning well, severe symptoms can develop more quickly, or you may get sick more often than others. My dissertation focuses on the protective role of the immune system, with a special focus on the immune system of the upper respiratory tract; the nasal and throat cavities.

The respiratory tract is often the first place where a pathogen encounters the body: you continuously breathe in air that may contain pathogens. The immune system of the respiratory tract combats pathogens in three different, complementary ways:

- The physical barrier: Nasal mucus traps pathogens from inhaled air, and cilia remove them from the airways.
- The innate immune system: If the pathogen enters the body, the innate immune system prevents further infection through rapid, non-specific responses. The production of antibacterial substances can kill the pathogen. These substances also attract other immune cells specialized in clearing pathogens.
- The adaptive immune system: If the innate immune system is not enough to clear the pathogen, the body initiates a targeted immune response. Antigen-presenting cells, T cells, and B cells work together to attack the pathogen. Antigen-presenting cells present parts of the pathogen to the T cell, activating it. The T cell can then kill the bacterium or activate a B cell. B cells produce antibodies. The adaptive immune system also provides immunological memory, allowing the body to respond more effectively when it encounters the same pathogen again.

My dissertation mainly focuses on one part of the adaptive immune system, namely antibodies, also known as immunoglobulins. Antibodies are microscopic particles produced by activated B cells. They play a key role in combating respiratory

infections. There are five types of antibodies: IgG, IgA, IgM, IgE, and IgD, each with a different function. In this dissertation, I focus on IgG and IgA. IgG is the most common antibody in the blood and helps clear pathogens during an infection: it can bind to the pathogen, allowing other immune cells, such as macrophages, to clear the pathogen. IgA is known as the antibody of the mucosa (the mucous membrane): IgA is present in large quantities in the mucous layer of the respiratory tract and provides local defense against viruses and bacteria. IgA is particularly good at blocking pathogens, preventing them from causing damage. Infections often elicit both a local immune response in the mucosa and a systemic immune response in the blood, with an increase in IgG and IgA.

To prevent illness and death from infections, vaccinations are crucial. Vaccinations have saved millions of lives worldwide. Vaccinations, like natural infections, activate the adaptive immune system and create immunological memory. Most vaccines are administered via injections (intramuscularly) and primarily stimulate immunity in the blood, mainly IgG antibodies. While this is effective against severe illness, vaccinations sometimes provide insufficient protection against mild symptoms and the spread of pathogens through the mucous membranes. This is seen with pathogens like SARS-CoV-2 and B. pertussis. Individuals vaccinated against these pathogens can still become infected and spread the infection. In this dissertation, I focus on the antibody response after infection and vaccination against these two pathogens.

SARS-CoV-2 is a virus that causes COVID-19. The virus was first identified in China at the end of 2019. It belongs to the coronaviruses and spreads mainly through airborne droplets. COVID-19 can cause a wide range of symptoms, from asymptomatic infection (no symptoms) or mild symptoms like fever and cough, to severe pneumonia and respiratory problems. Although effective vaccines are now available, they were not available when I started my research. Most people had not yet been exposed to SARS-CoV-2 at that time. This gave me the unique opportunity to study the antibody response in the upper respiratory tract after a first infection and compare it to the response after a first vaccination.

Bordetella pertussis is the bacterium that causes whooping cough, a respiratory infection known for its prolonged, severe coughing fits ("the 100-day cough"). The disease is particularly dangerous for young babies who are not yet fully vaccinated and can lead to hospitalizations and death. Despite extensive vaccination programs and high vaccination coverage, whooping cough remains a global problem. This has several causes. Firstly, whooping cough is a highly contagious disease: an infected

individual can infect 10 to 15 other people. Additionally, an increase in whooping cough cases has been observed since the type of vaccine changed. The current whooping cough vaccine is very effective in preventing illness but less effective in clearing the bacterium: vaccinated individuals will be less sick but can still carry and spread the whooping cough bacterium. In my dissertation, I investigated the immune response after whooping cough vaccination and infection.

Over the past five years, I have been busy conducting research with other scientists, the results of which are presented in this dissertation. The main goal of this dissertation was to investigate how infection and vaccination affect the antibody response, with a focus on the antibody response in the upper respiratory tract. By comparing the antibody response to infection and vaccination, we can better understand how to improve protection against respiratory infections, not only against severe illness but also against spread. This is important for the development of better vaccines.

The first part of this dissertation (Chapters 2, 3, and 4) discusses the local immune response after SARS-CoV-2 infection and vaccination. Chapter 2 summarizes all information about the role of the local immune system in a SARS-CoV-2 infection. In Chapter 3, we investigated changes in the immune system of the nose after a first infection with SARS-CoV-2. We showed a clear increase in antibodies in the nose when someone was infected with SARS-CoV-2. We also demonstrated a correlation between a rapid IgA response and a lower amount of virus particles in the nose. The local antibody response in the nose, particularly the IgA response, was also related to a faster reduction in symptoms. We observed more symptoms in adults than in children. In Chapter 4, we compared the immune response after infection with the immune response after a first vaccination. We showed that they differed, with a greater IgA response after infection and more IgG after vaccination. However, the functionality of the antibodies (how well they could block the pathogen) was not different. Together, the results from Chapters 2 to 4 show that the local antibody response after a SARS-CoV-2 infection, particularly the IgA response, is associated with a faster reduction in symptoms and a lower viral load. The immune response after vaccination differs, with more IgG antibodies and a less pronounced IgA response. This is useful information for developing potential new COVID-19 vaccines.

After the chapters on SARS-CoV-2, we switch pathogens and investigate the antibody response after B. pertussis vaccination and infection. In Chapter 5, we compared four different whooping cough vaccination combinations: the mother

received or did not receive a whooping cough vaccination during pregnancy, and the baby received either the whole-cell whooping cough vaccine (wP) or the acellular whooping cough vaccine (aP). In this chapter, we showed that whooping cough vaccination during pregnancy resulted in many antibodies in the baby's nose at two months old. This meant that we could measure antibodies in the baby's nose that were produced by the mother during pregnancy and passed to the baby via the placenta. However, this was only true for IgG; IgA was not transferred from mother to child. We also showed that the wP vaccine resulted in higher IgG antibody levels in the baby's nose than the aP vaccine, especially in babies whose mothers received a whooping cough vaccination during pregnancy. IgA did not increase after aP or wP vaccination.

In Chapter 6, we investigated the antibody response after an aP vaccination in volunteers who were also vaccinated with the aP vaccine as children. We used a new method to distinguish antibodies produced after vaccination from antibodies produced after infection. We found that older children had more infection antibodies before receiving the aP vaccine. By looking at the number of whooping cough reports in the two age groups, we hypothesized that the higher antibody levels were due to exposure to the whooping cough bacterium, which caused an asymptomatic infection. There were more whooping cough reports in the older age group, so the volunteers in this group were more likely to have been exposed to *B. pertussis*.

With this information about IgA and exposure in mind, we investigated the number of whooping cough infections in the Netherlands by looking at IgA antibody levels in Chapter 7. In previous chapters, we had already shown that IgA did not increase after vaccination with the aP vaccine. In this chapter, we demonstrated that this was the case in four different vaccination cohorts from different countries with different vaccination schedules. We also described that an infection with whooping cough did result in high levels of IgA antibodies, even if there were no symptoms. We explained that IgA is a good parameter to distinguish infection from vaccination. We then studied the IgA antibody levels of a large Dutch cohort of aP-vaccinated children. We found that antibody levels were already high at a young age and showed an age-dependent increase. This suggests that the number of undiagnosed whooping cough infections in the Netherlands is high, which could contribute to the spread of the bacterium.

In summary, this dissertation shows that whooping cough vaccination during pregnancy promotes the transfer of IgG antibodies to the baby's nose, but this also

affects the baby's vaccination response. Additionally, it was shown that infections with Bordetella pertussis, even in the absence of symptoms, lead to an increase in IgA antibodies, while vaccinations do not. Finally, this dissertation revealed that there is likely a large number of undetected whooping cough infections.

Together, the findings from this dissertation contribute to a better understanding of the immune response in the upper respiratory tract. This dissertation emphasizes the importance of local antibody production in the mucous membranes and provides important insights for improving vaccines against respiratory infections, so that they may offer better protection against asymptomatic infections in addition to severe disease in the future.

Acknowledgements

Well well, look who we have here.. Have you read my whole thesis already, or will you decide on whether to do this after checking what/if I wrote about you?

Jokes aside, after almost five years thinking about this final chapter of my thesis, the time has finally come to put the words on paper and thank everyone who has contributed to my wonderful time at the Laboratory of Medical Immunology, and this book in front of you. I started my PhD in the middle of the COVID-19 pandemic, when life was weird and uncertain. Although life is still weird and uncertain, we luckily haven't been in a social lockdown forever, and I have gotten the opportunity to meet and collaborate with some amazing people. Without all of you, this thesis would not exist.

First and foremost, I would like to thank my team of supervisors; "De Mannen". **Dimitri**, as my daily supervisor you have provided me with essential support during this entire journey. Your critical thinking, enthusiasm and strive for perfection have tremendously improved our work, and it has been a real pleasure collaborating with and learning from you. At home, we jokingly call you "Dimeetring", as you are definitely on top of the list when it comes to the people I have meetings with. This is one of the things that I have valued most about you as my supervisor: There is always time for a meeting or a brainstorm session. However, next to the serious, work related discussions, there is always room for a joke or a side-story, which I have really appreciated. We have travelled to congresses in many places together in the past years, and (although you do really like to talk about work) it has been lovely to get to know more about your family and your personal life. I even got you to go on a run with me in Barcelona, which was a real highlight! Thank you, and keep being the inspiring researcher you currently are. I hope we keep collaborating for many more years.

Martijn, thank you for attending all the Monday morning meetings, and for somehow being able to come up with useful feedback. I has been a real treat to have you in my supervisors team, as you always provided a different perspective, and enabled us to stay pragmatic and focus on the end goal when we were hyper fixating on the details. Your dry humour has been a great addition to our Monday meetings, and although I apologize for not sharing your passion of wine, I would love to join you on a birdwatching trip one day.

Marien, as my promotor you have been the one to keep the overview from the sidelines, and guide me to the end product which lies in front of you now. You keep surprising me with your incredible memory, and immense database of knowledge/articles. Thank you for somehow always being able to come up with new suggestions, or interesting publications to get the work to another level.

Secondly, thank you to my manuscript committee prof. dr. Aura Timen, prof. dr. Patricia Bruijning-Verhagen and dr. Nathalie Mielcarek for accepting to be a part of my committee, and for reading and assessing my thesis. I am looking forward to hear your thoughts and questions. Additional opponents, prof. dr. Teun Bousema, prof dr. Peter Sebo, Dr. Nicoline van der Maas and dr. Gerco den Hartog, thank you for agreeing to join my thesis defence. You've all played a role in my career some in some way, whether this has been during my masters, my year as an epidemiologist at the RIVM, or during my PhD, and I am looking forward to our discussions.

Next to the official supervisors and the thesis committee, there have been many other colleagues that have helped me create this thesis. Two very important teams of people are the technicians and senior researchers of the LMI. Most of you will know that my strengths do not lie within the laboratory, nor in hardcore immunology, so without these people this book would not have become a reality. Thanks **Bram**, **Esther, Annemijn, Manon, Esther,** for all the practical help along the way, whether it was discussing laboratory set-up, Luminex data analysis, or R-coding. You're always there to give advice or have a coffee, thanks for that. **Hans, Ruben, Lilly, Hans, Xuehui, Renate, Jeroen, Evelien, Sajida,** thanks for all the interesting questions and discussions we've had throughout this PhD trajectory. I have learned a lot about immunology research from all of you, whether it was more about bacteria, cellular immunity or data analysis. **Saskia** and **Arnold**, thanks for giving me the opportunity to be involved in some teaching activities. I have enjoyed it a lot, and hope to continue to teach in one way or another.

Elles, thank you for being my great teacher in the lab, especially during the COVID-19 period. You were one of the few colleagues that was allowed to work in the lab when I started, and you have made my re-introduction into labwork a very enjoyable one. I will never forget the day we were called into work to "pretend to measure COVID-19 MLF samples" while Omroep Gelderland was interviewing Dimitri. **Christa**, thank you for always trying to provide an answer to my never ending stupid questions, and for not getting mad at me when I (again) forgot to put samples into Labguru. You have shown me that lab work can be really fun, as long as there are fun people around during the waiting steps (and you have enough

coffee breaks)! **Marc**, thank you for all the work you've put into the deposition assay. Without you the assay would never have been as advanced as it is today, and none of the Bp papers would have been there without your steady hands behind the scenes. It has been a pleasure to know that I can trust you with the samples. It is always fun to be in the lab with you and joke around, or talk about biking. **Fred**, thank you for all the mucosal lining fluid elutions you've performed. It was one of the most tedious parts about my research, but you've become a real expert in handling the forceps and getting those strips removed! I also always enjoy our puppy talks, and it was lovely to visit your place to see you amazing garden/horses (and have Luna traumatize the chickens…).

During this PhD, I've got the chance to work amongst a great group of fellow PhD candidates, of whom some have already finished their project. **Lucille**, you were still finalizing your PhD when I began, but I am so glad that you are still around! You're the Hermione Granger of the LMI; you seem to know everything and when in doubt, we ask you. Thanks for all the advice over the years, I'm sure there's more to come. **Josh**, thanks for collaborating with me on the COVID-19 paper; you made me believe everyone was doing data analysis with R, and learning this skill has been a real gift. **Evi**, our collaboration on the BERT vaccination paper was really enjoyable. It was a pleasure working with you and I hope you're enjoying your new adventure as a mom!

For the majority of my PhD, I shared an office with 12 PhD candidates! We have gone on several "PhD retreats" together, which was always a lot of fun. Thanks to all for making our time at the office feel so much like a second home, and for accepting Luna as our PhD mascot during our weekend trips!

Anna, you are always the first person I see when I walk into the office (no matter how early I arrive..), which is always a nice start of the day. Thanks for all the coffee breaks and great restaurant advice! **Geert**, I know you always joke that I am never in the office, but luckily I can depend on you always being around. Thanks for all the jokes and discussions, whether work related or not. **Gui**, you have taught me a great collection of facts, from the history of Portugal to why it is always a good idea to wear underwear. I'm afraid I have forgotten most of the things you've taught me, but thanks for always being up for a chat and a laugh! **Rienke**, you're the only PhD from SKI that moved with me to LMI. Thanks for making sure we got a nice desk with a view, and for being the fun grandma of the group. I cannot wait to celebrate our PhD's together. **Judith**, you've been my next desk neighbour since you started your PhD, which has been a lot of fun. Thanks for sharing my Radboudumc demolition

obsession, I think we've both got 50+ photos of the building site on our phones! **Aysel**, thank you for being a great desk-island buddy. Although you've unplugged my PC screen multiple times to charge your phone, you've made up for it with interesting and fun discussions. I hope you will run your marathon sometime in the near future! **Laurien**, my Twente buddy in the office, thanks for all the coffee break games and chats, and for providing the down-to-earth advice. **Daan**, thanks for all the random discussions and fun facts, it is always nice when you come and visit us from the RIVM. **Esther**, the same can be said about you, it was always nice to catch up when you visited us, and I think it is so fun that we are going to defend our thesis only one day apart! Thanks for sharing your experiences in the final phase, it was good to know that I was not alone in the misery called wrapping up a PhD thesis. **Sam**, you are one of the latest people to join our office, and I think my PhD got a little less productive when you arrived. Thank you for all the hilarious discussions about "research projects" and general life, it has been a blessing to be distracted by you. I am looking forward to the day that you get a grant to fulfil your weird research proposals. **Suzanne**, thanks for being such a big support in my last phase of this PhD, and for being my newest work bestie. Not only do we share the same supervisor and project, we also share a passion for baking and growing plants which has been the topic of many fun tea chats. I hope (and think) that the KIM-study will be a big success, but if not, at least we had fun!

Vera and **Ezgi**, thank you for all the hours we've talked during work breaks. Your little corner near the heater was a lovely place to digest the day and share all the daily gossip. I am very happy to say that I am a part of the "Three Musketeers" and I hope we stay weird for many years to come. Having you both as my paranymphs feels like a full circle moment, as without you two, I probably would not have made it until the end. The advice you've given me has always been very helpful. Vera, thanks for the great collaboration on the ACE-2 paper. It was a pain in the ass but we got some great hours in the lab together, perfecting (or messing up) the assay. Ezgi, a special teşekkürler for you becoming a surrogate mother to little Luna. Thanks for all the hours of dog-sitting and all the love. The fact that you have a shirt with Luna's face on it and I have not says enough I think, but I think that we would still hang out if it weren't for Luna… Right?!

Also a special thanks to my 4-daagse PhD buddies, Rienke, Gui, Aysel, Laurien and Judith. Thanks for the great time during the Vierdaagse of 2023. It was so much fun experiencing this event as walkers for the first time together, and I think we all learned a lot from/about each other during these 4 days and got a lot closer by the end of it. Another great memory is running the Zevenheuvelennacht with

Laurien, Aysel, Ezgi, Suzanne and Vera. It has been really great seeing you guys pick up running and coaching you through your first race!

Diane, **Ria** and **Joyce**, thank you for making my first project so much fun. Starting during the COVID-19 pandemic could have been a very lonely time, but it turned out to be a great adventure with you three. You made sure the MuCo study was a big success while I still found my way into this role as a PhD researcher, of which I am very grateful.

Kim, **Justin**, **Reza**, thanks for doing your internship under my supervision! I really hope you enjoyed your time here, your work on the deposition assay has been an important part of this thesis, and has led to a nice chapter!

All the collaborators of the **PERISCOPE** consortium, thanks for all the fruitful and interesting discussions! I have been very lucky to become a part of this consortium when all the data-collection was almost finished, resulting in some nice publications and very nice consortium meetings. **Anja**, thank you for all the hard work you've put into the GaPs mucosal paper, and for the joy it has been to write this paper with you. Showing you around Nijmegen on a bike when you were pregnant with Klara is such a fond memory, and I cannot wait to meet little Roko on my next trip to London. I hope we get our paper published soon, and that we stay in touch for more collaborations afterwards. **Beate**, thanks for all the wise words and amazing stories you've shared with me during post-meeting drinks. Your career is a very inspiring one. **Cecile** and **Jolanda** thanks for the collaborations on the Immfact data and fun chats during the meetings. **Hans**, thank you for all the laughs and the collaboration on the Southampton human challenge model data. The (very hot and sticky) car ride from Prague back to Nijmegen together with Suzanne and Dimitri will be a memory I will cherish forever. Although I probably won't invite you to go on a morning run with me again, I'm looking forward to more collaborations in the future. The rest of the Southampton team, especially **Rob, Diane** and **Alison,** thanks for the great teamwork. It was tricky, but in the end we got all the data of the human challenge study analysed and written down. I hope it gets published soon! The team of the UKHSA, **Andy**, **Breeze**, **Elodie**, **Stephen**, it is always fun to discuss all matters pertussis with you all, thanks for the helpful meetings and close collaboration. The team in Oxford, **Dominic**, **Marta**, **Kia**, thanks for the collaboration on the AWARE study, and for the useful feedback on the GaPs data. I think the community of pertussis researchers is an incredibly kind and welcoming one, thank you for welcoming me into this group.

Luckily, life does not only consist of writing up a PhD thesis; next to lots of work-related fun, there have been many great moments among friends and family in the past years.

First of all, thanks to the girls that have been there since the very first biology class at Zuid in Enschede, **Robin**, **Layla**, **Loubna**, and **Simone**. Thank you for always feeling like home, whether it has been two hours, two weeks, or two months since we've last spoken. It has been amazing to see how we've all gone in different directions, but remained close friends.

Elleke, thanks for going through the rollercoaster of adult life together with me. How lucky were we that we got accepted at the Venusstraat together in that first year of university, and how lucky am I that I can still talk about everything with you. I am curious to see what the next 5 years will have in store for us!

To "The BMW Chicks", **Cynthia**, **Bronke**, and **Eva**, thank you for being the supportive friend group every girl needs. Eva, during the COVID-19 lockdown and my first year as a PhD candidate, we walked around Nijmegen every week, and after every walk I felt a bit lighter. Thanks for always listening. Bronke, thank you for all the hours you worked at my place and watched Luna so I could go to the office. Not only did this give me more freedom, it also provided me with the perfect epidemiologist-therapist-friend to talk through the day! Cynthia, thank you for the many phone calls and supporting text messages throughout the years. Even though we don't live as close anymore, it has been great to be able to talk about anything with you, whether PhD related or not.

Fiek, thanks for the many runs and lunch dates together! It has become a bit less frequent now that little Jonas is making your life even busier, but I always enjoy our talks and runs!

Arwin, **Thyra**, **papa** and **mama**, thank you for putting the work into perspective and showing me that there are more important things in the world than p-values or deadlines. Coming home to Enschede always feels like a holiday and I love receiving updates from home or polarsteps updates from faraway lands during the week. A special thanks to our newest member of the family, little **Fiep**, for reminding me how amazing it is to learn and play. Maybe one day you will become a researcher too, but until then I love seeing you grow.

Biemansjes, **Heidi**, **Ad**, **Ynte** and **Ids**, thanks for always welcoming me into your weekends and for distracting me from work with fun chats, activities, and good food. I hope this thesis will bring you some inspiration for the coming Sinterklaas poems!

Last but definitely not least, endless thanks to my own little family. **Lars**, when I started this PhD we were living together in our tiny apartment on the Platolaan. Now we have our own home, and our baby dog Luna is already 4 years old! Time has really flown by but I am very glad I got to spend all of it with you. Thank you for growing together with me and for going through this adventure together. You have been the stable factor amongst all the chaos, and I am forever grateful that I have you to come home to at the end of the day. Who knows what the future will bring, but I am certain that you will be a part of it. I am ready for our next adventures.

Curriculum Vitae

Janeri Fröberg was born in Utrecht in 1996, and grew up in Enschede before moving to Nijmegen to study Biomedical Research. During her master, she got the opportunity to do two internships on global infectious diseases, which sparked her interest in this field of research. The first internship was on submicroscopic malaria infections in The Gambia, under guidance of Prof. Dr. Teun Bousema, and the second was on the incidence of tuberculosis infections in an indigenous population in Paraguay, under guidance of Dr. Cecile Magis-Escurra. Janeri obtained her Master's degree in Biomedical Sciences from Radboud University in 2018 and subsequently worked for a year as a Junior Epidemiologist at the National Immunization Program (RIVM). Here she learned more about the infectious diseases epidemiology of the Netherlands, and was responsible for the surveillance of diphtheria, polio, tetanus and pertussis cases. During her year at the RIVM, Janeri helped set up the surveillance for the maternal pertussis vaccine, and she collaborated with international institutes of health to write a paper on the burden of vaccine preventable diseases. In 2020, she began her PhD research at the Laboratory of Medical Immunology at Radboud University Medical Center, under the guidance of Dr. Dimitri Diavatopoulos, Prof. Dr. Marien de Jonge and Prof. Dr. Martijn Huynen. As she began in the middle of the COVID-19 pandemic, her PhD started with a study investigating SARS-CoV-2 antibody responses after a first-time infection and first-time vaccination. After the pandemic, she switched back to *B. pertussis*, and got the opportunity to work within a large research consortium called PERISCOPE. She mainly investigated the antibody responses in the nose after infection and vaccination, trying to detangle differences that could be used to increase vaccine effectiveness. Currently, she is continuing her research as a postdoctoral researcher at the Laboratory of Medical Immunology.

Research Data Management

This thesis is based on the results of research involving human participants (or existing data from published papers), which were conducted in accordance with relevant national and international legislation and regulations, guidelines, codes of conduct and Radboudumc policy. The privacy of the participants in these studies was warranted by the use of pseudonymization. The pseudonymization key was stored on a secured network drive that was only accessible to members of the project who needed access to it because of their role within the project. The pseudonymization key was stored separately from the research data. Informed consent was obtained from participants to collect and process their data for this research project.

For the research data in chapter 3 we made use of human mucosal lining fluid, serum, and naso/oropharyngeal swab samples that were collected for the MuCo study in 2020 (trial registration number NCT04590352, CMO Regio Arnhem-Nijmegen number: NL73418.091.20). We measured the presence of SARS-CoV-2 in the naso- and oropharyngeal swabs, and antibodies in the mucosal lining fluid and serum samples, and used the participant information collected in questionnaires in our analysis. The results of the research performed in this thesis were added to the 'MuCo-study (MuCo)' collection of the Radboud Data Repository (https://doi.org/10.34973/4x4j-w517). Access to this data can be requested. Additionally, the aggregated data from chapter 3 in this thesis has been published open access in the journal Nature Communications (NatComms, doi: 10.1038/s41467-021-25949-x). All the data can be opened with generally available software.

In chapter 4, the data used in chapter 3 was combined with mucosal lining fluid that was collected from healthy controls participating in the ReCoVaC-Immune Response study, a Dutch multicenter clinical trial that was performed in 2021 (trial registration number NCT05030974). Approval of this study was obtained from the Dutch Central Committee on Research Involving Human Subjects (CCMO, NL76215.042.21) and the local ethics committees of the participating centers, and written informed consent was obtained from all adult participants. All results from this thesis have been published open access in the Journal of Infectious Disease (JID, doi:10.1093/infdis/jiad385). The data underlying the published chapter are available in the 'RECOVAC' collection of the Radboud Data Repository (https://doi.org/10.34973/4xbc-te77).

In chapter 5, mucosal lining fluid and participant information was used that were collected as part of the PERISCOPE consortium. The PERISCOPE consortium was established as a public–private partnership, funded by the European Union Innovative Medicines Initiative and the Bill & Melinda Gates Foundation. The data and metadata is stored in the DANS Data Station Life, Health and Medical Sciences (doi:10.17026/dans-zcw-8x6j), and can be accessed upon request.

In chapter 6, mucosal lining fluid and participant information was used from the BERT-NL study (trial registration number NCT03697798), which were also collected as part of the PERISCOPE consortium. The data is stored in the DANS Data Station Life, Health and Medical Sciences (doi:10.17026/dans-zcw-8x6j), and can be accessed upon request. The chapter has been published open-access in Nature Communications (NatComms, doi: 10.1038/s41467-022-35165-w.)

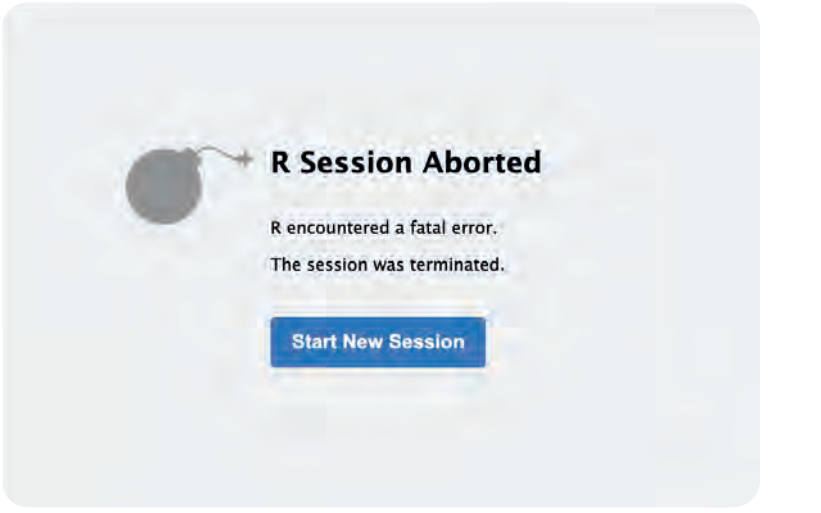
Chapter 7 combines data from the PERISCOPE consortium with data shared by the National Institute of Public Health and the Environment (RIVM). Bp-binding antibody levels from serum and participant information were used. Next to all data being stored on the PERISCOPE DANS network, all data published in chapter 7 of this thesis will be uploaded to the 'PERISCOPE_Exposure' Radboud Repository (https://doi.org/10.34973/269c-tm62).

Graduate School Portfolio

Department: **Laboratory Medical Immunology**
PhD period: **01/04/2020 – 31/12/2024**
PhD Supervisor(s): **Dr. D.A. Diavatopoulos**
PhD Co-supervisor(s): **Prof. dr. M.I. de Jonge, Prof. dr. M. Huynen**

Training activities	Hours
Courses	
• RIMLS - Introduction course "In the lead of my PhD" (2020)	15.00
• RU - Project management for PhD candidates (2021)	52.00
• RU - Analytic Storytelling (2021)	20.00
• Radboudumc - Scientific integrity (2021)	20.00
• RU - Education in a Nutshell (2022)	28.00
• Radboudumc - eBROK course (2022)	42.00
• RU - Design and Illustration (2022)	26.00
• RU - Analysing longitudinal and multilevel data using R (2022)	96.00
• RU - Effective Writing Strategies (2023)	75.00
• RU - Art of Finishing Up (2024)	10.00
Seminars	
• International Bordetella Lab Meetings (2020)	6.00
• Mini symposium modelling infectious diseases (2023) – oral presentation	12.00
Conferences	
• PhD retreat (2020) – poster presentation	4.00
• KNVM (2021) – poster presentation	24.00
• WEON (2021) – attendant	24.00
• Vaccine symposium (2021) – attendant	4.00
• PhD retreat (2021) – attendant	8.00
• PERISCOPE Gent meeting (2022) – oral presentation	32.00
• NVVI (2022) – poster presentation	16.00
• WEON pre-conference (2022) – online oral presentation	8.00
• 13th International Bordetella Symposium (2022) - poster presentation	40.00
• EuPertSTRAIN (2022) – oral presentation	24.00
• PERICSOPE meeting (2023) – oral presentation	24.00
• KNVM 2023 (2023) – attendant	16.00
• PhD retreat (2023) – poster presentation	8.00
• ESPID 2023 (2023) – poster presentation	48.00
• EUPertSTRAIN (2023) – oral presentation	16.00
• International Bordetella Symposium (2024) – two oral presentations	36.00
• HiCVac (2024) – helped with figures for oral presentation	14.00
Other	
• RIR theatre (2024)	1.50
• Career event for PhDs and Postdocs (2024) – organization committee	28.00

Teaching activities	
Lecturing	
• Dr House tour (2021)	3.00
• Dr House Tour (2022)	3.00
• MMC WG (2022)	3.00
• MGZ meets CSI WG (2022)	3.00
• Dr House Tour (2023)	3.00
• MGZ meets CSI WG (2023)	5.00
• R course to department (2023)	50.00
• WG BMW/GNK (2024)	4.00
• BMS88 Graphical abstracts WG (2024)	4.00
• WG Q1 rapportage (2024)	3.00
Supervision of internships / other	
• MIN29 coaching (2020)	20.00
• MIN29 coaching (2021)	20.00
• Intern supervision (2022)	70.00
• MIN29 coaching (2022)	20.00
• Intern supervision (2023)	53.00
Total	**1,041.50**


R Session Aborted
R encountered a fatal error.
The session was terminated.
Start New Session

9 789465 150659 >

Radboudumc university medical center

IN DEI NOMINE FELICITER

Radboud University