Antibody responses after influenza and SARS-CoV-2 vaccination and infection: Lessons across the ages

Nina Urke Ertesvåg

Thesis for the degree of Philosophiae Doctor (PhD)
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“Nothing in life is to be feared,
it is only to be understood.
Now is the time to understand more,
so that we may fear less”

– Marie Curie

List of abbreviations

ACE2	Angiotensin converting enzyme 2
ADCC	Antibody-dependent cellular cytotoxicity
ADCP	Antibody-dependent cellular phagocytosis
ANOVA	Analysis of variance
APCs	Antigen presenting cells
ARDS	Acute respiratory distress syndrome
BCR	B cell receptor
BSA	Bovine serum albumin
BSL	Biosafety level
CHMP	Committee for Medicinal Products for human use
CI	Confidence interval
COVID-19	Coronavirus Disease 2019
CRF	Case report form
DAMP	Damage-associated molecular pattern
DC	Dendritic cells
E	Envelope protein
EC	Emergency clinic
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
Fab	Antibody-binding fragments
Fc	Fragment crystallisable
FcR	Fragment crystallisable receptor
FFU	Fluorescent focus units
GC	Germinal centre
GISRS	Global Influenza Surveillance and Response System
GMT	Geometric mean titre
GP	General practice
H_2O_2	Hydrogen peroxide

HCW	Health care worker
HA	Hemagglutinin
HAT	Hemagglutination test
HAU	Hemagglutinating units
HCoVs	Human coronaviruses
HI	Hemagglutinin inhibition
HIV	Human immunodeficiency virus
HPR	Horseradish peroxidase
HR	Hazard ratio
IC_{50}/IC_{80}	Inhibitory concentration 50% or 80%
ICU	Intensive care unit
IFN	Interferon
Ig	Immunoglobulin
IIV	Inactivated influenza vaccine
IL	Interleukin
IRR	International Reagent Resources
JAK1/2	Janus kinase 1 or 2
LAIV	Live-attenuated influenza vaccine
M1/M2	Matrix protein 1 or 2
MBC	Memory B cell
MDCK	Madin-Darby canine kidney cells
MERS-CoV	Middle East respiratory syndrome-related coronavirus
MIS-C	Multisystem Inflammatory Syndrome in Children
MN	Microneutralisation
mRNA	Messenger ribonucleic acid
N	Nucleocapsid
NA	Neuraminidase
NAbs	Neutralising antibodies
NIBSC	National Institute of Biological Standards and Controls
NIPH	Norwegian Institute of Public Health
NP	Nucleoprotein

NS1/2	Non-structural protein 1 or 2
NTD	N terminal domain
OAS	Original antigenic sin
OR	Odds ratios
PA	Polymerase acid
PAMP	Pathogen-associated molecular pattern
PB1/2	Polymerase basic protein 1 or 2
PBS	Phosphate Buffered Saline
PBS-T	Phosphate Buffered Saline-Tween
PHE	Public Health England
PN	Pseudotype neutralisation
PRR	Pattern recognition receptor
RBC	Red blood cells
RBD	Receptor binding domain
RBS	Receptor binding site
RDE	Receptor destroying enzyme
RIG-I	Retinoic acid-inducible gene-I
RdRp	RNA-dependent RNA polymerase
RLA	Relative luciferase activity
RNA	Ribonucleic Acid
RR	Risk ratio
RSV	Respiratory syncytial virus
RT-PCR	Reverse transcription polymerase chain reaction
S	Spike
SA	Sialic acid
SARS-CoV-1	Severe acute respiratory syndrome coronavirus 1
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
SRH	Single radial haemolysis
ssRNA	Single stranded
TCR	T cell receptor
$TCID_{50}$	50% tissue culture infectious dose

TDN	Test negative design
Tfh	T-follicular helper cell
Th	T helper cell
TLRs	Toll-like receptors
TNF	Tumour necrosis factor
TMPRSS2	Transmembrane serine protease 2
Treg	T regulatory cell
VE	Vaccine effectiveness
V_HH	Heavy chain heavy antibody
VOC	Variant of concern
VN	Virus neutralisation
WHO	World Health Organisation

Preface

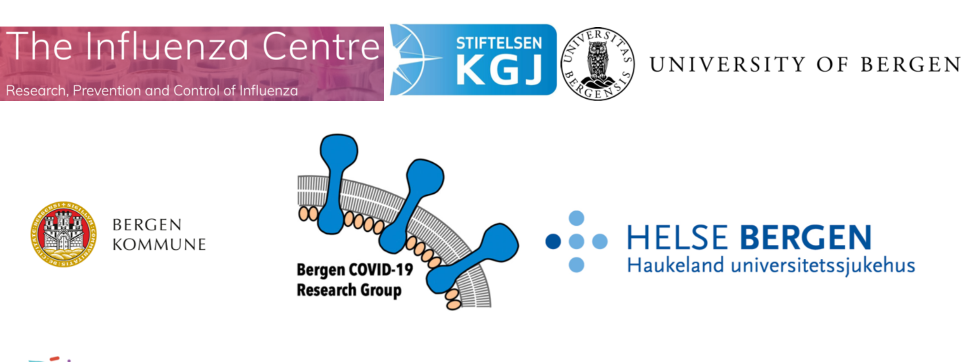
This thesis started with an aim to investigate immune responses to influenza viruses. The topic of my master thesis was obstacles to influenza vaccination in health care workers, and now I wished to continue studying the effect of vaccination on an immunological level. A cohort of health care workers recruited during the 2009 A/H1N1 pandemic were followed with blood samples for several influenza season, with various vaccine choices in this period. The serological samples provided a valuable opportunity to study antibody responses to the other influenza A virus, A/H3N2, circulating through the ages since 1968. I had an immense respect for the time and effort dedicated to follow these participants for four consecutive years, as I had some recent experience recruiting patients for an influenza study during my medical residency in the emergency department. These were the first steps on the journey I was about to undertake. Right around the corner awaited yet another pandemic, although not caused by an influenza A virus this time.

After decades of studying human immunity to influenza viruses, the Influenza Centre shifted focus to immunological and clinical research of the novel SARS-CoV-2 virus, forming Bergen COVID-19 research group. When the novel pandemic vaccines were rolled out, knowledge about the vaccine immunogenicity was highly sought after. The sense of doing something important was heavily present, both among study participants and us researchers. Contributing to the race for answers, motivated us to continue work on the five simultaneously ongoing clinical studies. Although challenging, the first pandemic years resulted in huge scientific development, and became essential for this doctoral work. This thesis includes immunological studies across age groups on both influenza and SARS-CoV-2, comprising infection and vaccination antibody responses and the long-term consequences of COVID-19.

Scientific environment

This PhD project was carried out at the Influenza Centre, Department of Clinical Science, University of Bergen, as part of the PhD program at the Faculty of Medicine, University of Bergen. Professor Rebecca Jane Cox was the main supervisor. Assistant Professor Kristin Greve-Isdahl Mohn and postdoctoral fellow Mai-Chi Trieu were co-supervisors.

The project was conducted over a four year-period (2018-2023), and was funded by the Influenza Centre, the K.G. Jebsen Center for Influenza Vaccine Research and the University of Bergen. Additional funding was received from the National Graduate School in Infection Biology and Antimicrobials (IBA) and Gades Legat. Patient studies from the Bergen COVID-19 Research Group were performed in collaboration with Haukeland University Hospital, Eidsvåg Family Practice and Bergen Muncipality Emergency Clinic, Bergen, Norway.



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I would also like to express my deepest appreciation to my co-supervisors *Associate Professor Kristin G-I Mohn* and *Doctor Mai-Chi Trieu. Kristin,* you are a force of nature, making an unmistakable and long-lasting impression on everyone you meet. Your enthusiasm for scientific dissemination is truly inspiring, setting a high standard for those under your guidance. You have safely mentored me through all the rough patches during the last years, sharing wise strategies. As you always reminded me, your phone line literally *has* been open 24/7. I could always trust your advice regarding the scientific requirements at the doctoral level. I thank you for these life-long lessons. *Mai-Chi* is truly one of the most brilliant and creative researchers I have met. You know it all, from lab to statistics. You have patiently taught me how to titrate, treat and organize serum samples, interpret, and illustrate data. I am fascinated by how your mind works, noticing every detail and yet with the bigger picture in mind. Thank you for all the contributions that improved the quality of my PhD.

Professor Nina Langeland came into my PhD later, but none the less made a substantial impact on my growth as a researcher. You never dwell on problems and lead with a steady professionalism, and for that I highly respect and admire you. Your door has always been open, with a loud laughter filling the corridor. I could always trust you for

a Mac charging cord, coffee, and nuts for all our late hours working on manuscripts, going through data, and submitting articles. I have appreciated your honest and straight-forward advice, reminding me that only I can take care of my own interests.

Thank you, *Associate Professor Bjørn Blomberg*, my trusted colleague, and the living image of a yes-man. You have helped me interpret data, choose the right statistical tests, and formulate manuscripts. On top of always being positive, funny and enthusiastic, you have a very creative mindset, with an appreciation of my scientific illustrations or "graphic designs" as you would call it.

To *Elisabeth* and *Lena*: I could not have completed this thesis without your company, support and positive energy. I have greatly appreciated your advice and input along the way, most recently on this thesis. My heartfelt gratitude also goes to *Sonja*, who spent countless hours with me in the lab, titrating plates. Thank you, *Fan*, for conducting all the neutralisation assays, and for sharing all your wisdom. I deeply appreciate the lab collaboration with *Sarah,* working with the LAIV samples, and *Stefan* for your steadfast control of all serum samples and willingness to conduct our last-minute assays. I owe *Karl* my sincerest thanks for teaching me immunology and providing perspective and technical help during this writing process. *Türküler* has been immensely patient and helpful with the statistics. Thank you, *Therese*, for all our honest conversations over a cup of coffee. *Sunniva* for being my PhD role model and in life in general. *Marianne, Hanne, Helene, Kanika, Kristin R* and *Kristin H*, thank you for making a fantastic team during the countless blood collections. A special thanks goes to the sporty relay team of Bergen City Marathon 2023, including *Jenny, Lukas, Karolina, Henriette* and *Dhana*. I also thank my other colleagues at the Influenza Centre and Bergen COVID-19 research group *Amit, Geir, Anders, Håkon, Abira, Juha, Linn, Richard* and *Shahinul,* and the other friendly faces on the 5th floor, especially *Kjerstin, Marianne* and *Tove.*

I appreciate my colleagues at the infectious disease department, led by *Associate Professor Trond Bruun*, and *Unni* at the medical outpatient clinic for always lending

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My parents *Nils* and *Randi* have always told me I could do anything and assured the best conditions for me to grow and thrive. This thesis could not be possible without their unwavering support. My sister *Anne-Stine*, brother-in-law *Robin* and my goddaughter *Tiril* have always cheered me on with love and encouragements. My parents-in-law *Elin* and *Morten* are true heroes and selfless beings with their generous efforts and devotion during this writing process. Thank you for the countless hours you have taken care of our daughter, I will be forever grateful.

Lamin, my love, my rock, and my number one team player. Thank you for running the household and being my go-to person in all crises. Your effectiveness and steadfast control in all practical matters has made this thesis possible. I love you. Finally, my daughter *Haddy* is the light and love of my life and the true inspiration behind this work. I hope you can use this work as an inspiration to stay curious, challenge established truths, and claim your place wherever you wish.

Nina Urke Ertesvåg,
Bergen 29.06.23

Summary

Influenza A and SARS-CoV-2 are respiratory RNA viruses which cause pandemics, and rapidly mutate ensuring their continuous circulation. There is a complex interplay between the host immune responses and the virus, influenced by prior memory from the initial infection, vaccination, and age. This thesis characterises antibody responses to influenza A/H3N2 and SARS-CoV-2 in different age groups after infection and vaccination.

The priming influenza infection, as well as the summary of life-time exposures, is known to affect subsequent immune responses. We found cross-reactive antibody responses in adults and children against A/H3N2 viruses back to their year of birth. Although antibodies to the most recent viruses dominated the antibody landscapes, antibodies also cross-reacted against future epidemic viruses.

Studies of SARS-CoV-2 immunity can be simplified by the use of a rapid, laboratory-based hemagglutination test (HAT) to measure surrogate neutralising antibodies. mRNA vaccines were less immunogenic in the elderly with lower cross-reactivity to new variants, unless they had been previously infected. We found that the elderly required two vaccine doses, to produce HAT antibodies comparable to after one vaccination in younger adults or previously infected subjects.

Long-term SARS-CoV-2 symptoms, known as post COVID-19 condition, are understudied in children and adolescents, especially after infection with the delta and omicron variants. In 10-20 year olds, we identified acute symptoms, older age, higher spike-specific antibody titres and female sex as factors associated with persisting symptoms. In the same cohort, we found higher antibodies and fewer omicron BA.1/2 reinfections in COVID-19 vaccinees than unvaccinated. However, vaccine effectiveness had a short duration of 22 days, despite hybrid immunity.

Our findings highlight the utility of rapid and simple assays for evaluation of infection and vaccination responses. There is a need for improved vaccine effectiveness to reduce the burden of COVID-19 and long-term symptoms in young people. Overall, cross-reactive antibodies can be favourable in the face of emerging respiratory viruses.

Samandrag

Influensa A og SARS-CoV-2 er RNA-baserte luftvegsvirus som forårsakar pandemiar og muterer raskt for å oppretthalde ein kontinuerleg sirkulasjon. Eit komplekst samspel mellom immunresponsar hjå verten og viruset er forma gjennom etablerte minne frå den første infeksjonen, tidlegare vaksinasjonar og alder. Dette doktorgradsarbeidet karakteriserer antistoff-responsar mot influensa A/H3N2 og SARS-CoV-2 i ulike aldersgrupper etter infeksjon og vaksinasjon.

Den første influensainfeksjonen, i tillegg til summen av eksponeringar gjennom livet, påverkar framtidige immunresponsar. Vi fann kryssreaktive antistoff-responsar hjå vaksne og barn mot A/H3N2-virus tilbake til fødselsåret deira. Sjølv om antistoff mot dei nyaste virusa dominerte landskapet, kunne antistoff også kryssreagere mot framtidige, epidemiske virus.

Ein laboratoriebasert og hurtig hemagglutinasjonstest (HAT) kan forenkle studiar om immunitet mot SARS-CoV-2 og brukast til å måle surrogat-nøytraliserande antistoff. mRNA-vaksiner var mindre immunogene hjå eldre, basert på lågare kryss-reaktivitet mot nye virusvariantar, med mindre dei hadde vore infiserte tidlegare. Vi fann at eldre trengte to vaksinedosar for å produsere tilsvarande HAT-antistoff mengder samanlikna med yngre vaksne med éin vaksinasjon eller tidlegare infiserte personar.

Det er få studiar på vedvarande SARS-CoV-2-symptom, også kjent som post COVID-19-tilstand, blant barn og ungdom, spesielt etter delta- og omikron-infeksjon. Faktorar assosiert med vedvarande symptom i aldersgruppa 10-20 åringar var akutte symptom, høgare alder, høgare antistoff-titer mot piggeproteinet og kvinneleg kjønn. I same kohort fann vi høgare antistoff og færre omikron BA.1/2-reinfeksjonar hjå COVID-19-vaksinerte samanlikna med uvaksinerte. Vaksineeffekten var derimot kortvarig, 22 dagar, trass i hybridimmunitet.

Desse funna understrekar nytten av raske og enkle analysar for å evaluere infeksjon- og vaksinasjonsresponsar. Det er behov for forbetra vaksineeffekt for å redusere byrden av COVID-19 og vedvarande symptom hjå unge menneske. Samla sett kan kryssreaktive antistoff vere gunstige i møte med nye luftvegsvirus.

List of Publications

I. **Ertesvåg NU**, Cox RJ, Lartey SL, Mohn KG, Brokstad KA, Trieu MC. Seasonal influenza vaccination expands hemagglutinin-specific antibody breadth to older and future A/H3N2 viruses. *NPJ Vaccines*. 2022;7(1):67.

II. **Ertesvåg NU,** Xiao J, Zhou F, Ljostveit S, Sandnes H, Lartey S, et al. A rapid antibody screening haemagglutination test for predicting immunity to SARS-CoV-2 variants of concern. *Commun Med.* 2022;2:36.

III. **Ertesvåg, NU,** Iversen A, Blomberg B, Özgümüş T, Pramila Rijal, Fjelltveit EB, et al. Post COVID-19 condition after delta infection and omicron reinfection in children and adolescents, *eBioMedicine* 2023;

IV. **Ertesvåg, NU,** Pramila Rijal, Nina Langeland, Rebecca J. Cox. COVID-vaccine protection against Omicron break-through infection in children and adolescents, submitted manuscript to *NPJ Vaccines* (2023).

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Paper IV is presented as a submitted manuscript.

Papers not included in this thesis

I. **Ertesvåg NU**, Sakkestad ST, Zhou F, Hoff I, Kristiansen T, Jonassen TM, et al. Persistent Fever and Positive PCR 90 Days Post-SARS-CoV-2 Infection in a Rituximab-Treated Patient: A Case of Late Antiviral Treatment. Viruses. 2022;14(8).

II. Hansen L, Brokstad KA, Bansal A, Zhou F, Bredholt G, Onyango TB, Sandnes HH, Elyanow R, Madsen A, Trieu MC, Sævik M, Søyland H, Olofsson JS, Vahokoski J, **Ertesvåg NU**, Fjelltveit EB, Shafiani S, Tøndel C, Chapman H, Kaplan I, . Mohn KGI, Langeland N, and Cox RJ. Durable immune responses after BNT162b2 vaccination in home-dwelling old adults. *Vaccine X*. 2023;13:100262.

Papers related to post COVID-19 condition

III. Fjelltveit EB, Blomberg B, Kuwelker K, Zhou F, Onyango TB, Brokstad KA, et al. on the behalf of the Bergen COVID-19 research group (**Ertesvåg NU** as a member). Symptom burden and immune dynamics 6 to 18 months following mild SARS-CoV-2 infection -a case-control study. *Clin Infect Dis*. 2022.

IV. Blomberg B, Mohn KG, Brokstad KA, Zhou F, Linchausen DW, Hansen BA, et al. on the behalf of the Bergen COVID-19 research group (**Ertesvåg NU** as a member). Long COVID in a prospective cohort of home-isolated patients. *Nat Med.* 2021;27(9):1607-13.

Papers related to SARS-CoV-2 immune responses

V. Trieu MC, Bansal A, Madsen A, Zhou F, Sævik M, Vahokoski J, et al. et al. on the behalf of the Bergen COVID-19 research group (**Ertesvåg N** as a member). SARS-CoV-2-Specific Neutralizing Antibody Responses in Norwegian Health Care Workers After the First Wave of COVID-19 Pandemic: A Prospective Cohort Study. *J Infect Dis*. 2021;223(4):589-99.

VI. Mohn KG, Bredholt G, Zhou F, Madsen A, Onyango TB, Fjelltveit EB, et al. on the behalf of the Bergen COVID-19 research group (**Ertesvåg NU** as a member). Durable T-cellular and humoral responses in SARS-CoV-2 hospitalized and community patients. *PLoS One*. 2022;17(2):e0261979.

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1. Introduction

1.1 A repeating history of pandemics and epidemics

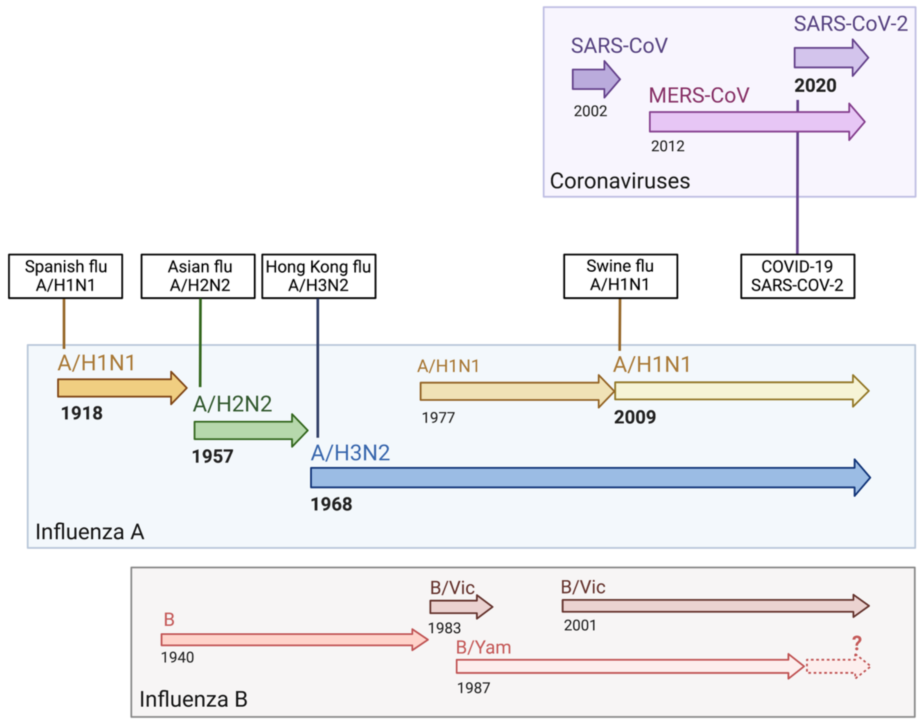
The work in this thesis covers both clinical aspects and immune responses to epidemic influenza A/H3N2 viruses and pandemic SARS-CoV-2. An *epidemic* is a disease outbreak occurring in a specific region during a limited time period, while a *pandemic* is an infectious disease spreading worldwide, affecting the global population (1). Throughout history, roughly 16 recorded pandemics caused by viruses or bacteria have claimed hundreds of million lives (2). Before the common era (BE), pandemics were often referred to as a plague, representing a sudden outbreak of disastrous affliction or "evil". With the lack of effective antimicrobial treatment against *Yersinia pestis* during The Black Death pandemic in 1347-1351, the practice of quarantine was introduced in 1377. A period of 30-40 days isolation was an effective infectious control measure to prevent the spread of disease (3).

1.1.1 Influenza pandemics and epidemics

The first well-documented, and most famous, global pandemic the "Spanish flu" was caused by the influenza A/H1N1 virus in 1918 (**Figure 1)**. It is considered one of the deadliest pandemics with its estimated 50 million deaths worldwide (4). Almost 30 years prior (in 1889), the Russian flu swept through Europe (5). As the name suggests, the Russian flu was believed to originate from an influenza virus, but no actual virological proof has been obtained. A contemporary theory is that the seasonal coronavirus HCoV-OC43 was the cause of this pandemic (6). However, the source of the Spanish flu is well-established as the influenza A/H1N1 virus. Although there was no knowledge of its nature at the time, the 1918 pandemic was the catalyst for modern influenza research. After causing three successive infection waves, the A/H1N1 virus continued to circulate in the population, with annual epidemics in both hemispheres. The A/H1N1 virus was isolated in 1933, a discovery that facilitated the development of the first influenza vaccine in 1936 (7). With knowledge of influenza and its symptomology, researchers discovered that not all influenza patients developed

antibodies towards the A/H1N1 strain. This finding led to the identification of another influenza type in 1940, influenza B, which was later included in the influenza vaccine.

Figure 1: Schematic overview over circulating influenza and sarbeco coronaviruses in man



The illustration was created with BioRender.

Since the 1918 pandemic, three successive influenza pandemics in 1957 (A/H2N2), 1968 (A/H3N2) and 2009 (A/H1N1) have followed (**Figure 1**). A pandemic vaccine was first developed for the global use during the 2009 swine flu pandemic, contributing to reducing the pandemic burden. All these pandemic influenza A viruses, in addition to influenza B viruses, have circulated in the population following their emergence. Although pandemics have a large societal and economic impact, the global burden from a severe influenza season may be comparable. Circulating influenza viruses represent a constant threat to human health, potentially causing the death of 290 000 to 650 000 people annually (8). The highest disease burden is in the youngest and elderly (U-shaped mortality). However, both A/H1N1 pandemics in the last century had unusual

mortality pattern, with high disease severity specifically in 20-40 year olds (9). In 2009, older adults had measurable pre-existing antibodies, induced by viral descendants of the 1918 pandemic H1N1 strain, whereas young adults were immunologically naïve (10). This likely contributed to the lower range estimates of mortality during this pandemic, comparable to an epidemic influenza A/H3N2 season (11). The influenza A/H3N2 dominated seasons are associated with the highest morbidity and mortality, attributed to a combination of viral and host factors (12). The A/H3N2 viruses disproportionally infect older adults and have the most rapid antigenic drift of all currently circulating human influenza viruses. Interestingly, even in seasonal influenza outbreaks, reduced viral infections and increased influenza vaccine effectiveness is found in certain birth cohorts, rooted in pre-existing immunity (13, 14).

1.1.2 The COVID-19 pandemic

Prior to the recent Coronavirus disease 2019 (COVID-19) pandemic, global pandemics over the last century were exclusively caused by influenza A viruses. However, two zoonotic coronaviruses, Severe Acute Respiratory Syndrome Coronavirus 1 (SARS-CoV-1) in 2002 and Middle Eastern Respiratory Syndrome Coronavirus (MERS-CoV) in 2012, caused epidemics in Southeast Asia and Saudi Arabia, respectively (15) (**Figure 1**). SARS-CoV-1 was eradicated through diligent infection control measures, while sporadic MERS-CoV cases are continuously reported in Saudi Arabia. Although these outbreaks never caused world-spanning disease, they should be regarded as early indicators of the pandemic potential of coronaviruses.

Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) emerged in late 2019, spreading quickly in an immunologically naïve population. Since its appearance, the virus has mutated continuously, giving rise to multiple variants of concern (VOC) (**Figure 2**). COVID-19 vaccines were rapidly developed and deployed and were initially highly effective in preventing infection and transmission. Viral evolution has now enabled immune evasion with frequent reinfections despite vaccination, although protection against severe disease and deaths is maintained.

Figure 2: SARS-CoV-2 variants of concern (VOC)

The illustration was created with BioRender.

Reduced transmission and infections could improve upon variant updated mRNA vaccines and the utilisation of next generation mucosal vaccines. SARS-CoV-2 is moving towards an endemic phase and might fluctuate in seasonal waves, much like influenza. If so, lessons can be drawn from the decades of influenza virus research. Conversely, the success of novel SARS-CoV-2 vaccine platforms could be readily implemented for influenza vaccine production, reducing the time between vaccine strain selection and vaccine deployment.

1.1.3 Viruses with pandemic potential

Viruses with pandemic potential have a set of distinct properties enabling their introduction to the human population (16, 17). Firstly, an essential precondition is an viral animal reservoir, serving as a constant origin of potential novel human viruses. Unique to all pandemic pathogens, is that they enter the human species through close interaction with animals (18). Many pathogens are able to transmit from animals to human, without being contagious between humans. If the pathogen further develops sustained human-to-human transmission, the groundwork for a future pandemic is built. There are numerous examples of pathogens that crossed the species from animals to man, such as smallpox, Ebola, and Human Immunodeficiency Virus (HIV). The natural hosts of both influenza and coronaviruses include multiple animal species. The influenza A/H1N1 pandemic virus of 2009 derived genetic segments from avian, swine and human influenza viruses. SARS-CoV-2 is thought to have originated from bats via an intermediate host (19), with a 96.3% shared genetic composition with a bat

coronavirus (CoV RaTG13), and only 82% similarity with SARS-CoV-1 which caused the SARS epidemic in 2002 (20).

A second requirement for the virus to adapt to a new host, is the ability to change or mutate. Both influenza and SARS-CoV-2 have RNA genomes, although of different polarities. Although the biggest distinction between their RNA genomes is the segmented influenza RNA, allowing an effective exchange of genetic material. Influenza, like most RNA viruses, does not have any proof reading mechanism during RNA replication and is therefore prone to high mutation rates. Thus, influenza respond readily to a changing environment and selective pressure. Although human coronaviruses have acquired a unique proof reading complex (21), the fidelity of the SARS-CoV-2 genome synthesis remains low, allowing emergence of new viral variants. Thirdly, the mode of transmission is fundamental for the spread of an infectious disease. The three most common infectious routes are through contact (direct or indirect), droplets or the airborne route. Respiratory viruses can spread by all these routes in the right circumstances. Measles, the most contagious disease, is a respiratory virus with prolonged aerosol formation and droplet transmission (22). Fortunately, lifelong immunity is accomplished through vaccination, due to low mutation rate and humans being the only viral host species.

Other viral factors that determine the successful transmission of a potential pandemic virus are the incubation period and viral pathogenicity. The incubation period is defined as the time from viral exposure to symptom development. If an infected individual is contagious during an extended incubation period, the disease will easily transmit and spread. Moreover, if the host is asymptomatic or has mild disease, they are less likely to refrain from social activities, providing transmission possibilities. However, these factors are relative. For example, the highly contagious SARS-CoV-2 omicron BA.1 variant has a reduced incubation period compared to the ancestral Wuhan Hu-1 strain (23). While several respiratory viruses have pandemic potential, only influenza A and SARS-CoV-2 have met the necessary preconditions defining a pandemic. The repeating history of epidemics and pandemics has affirmed the inevitable cycles of

novel viruses' emergence, spread and occasional establishment in humans. The next pandemic could be caused by one of the viruses already identified with pandemic potential, or by a completely unexpected, novel virus (disease X) (24).

1.2 Influenza and COVID-19

1.2.1 Common clinical features

Influenza usually has an abrupt symptom onset. Classical symptoms include systemic manifestations with fever or chills, arthralgia, myalgia, headache, and fatigue (25) (**Figure 3**). Unlike other respiratory viruses, common cold symptoms such as, pharyngitis and rhinorrhoea do not precede influenza, and cough develops later. Children in particular may experience gastro-intestinal symptoms, such as vomiting and diarrhoea. COVID-19 is characterised by many of the same symptoms as influenza (26). An exception is the loss of taste and smell (dysgeusia, anosmia), typically related to SARS-CoV-2 infection with Wuhan-Hu-1 through delta variants (**Figure 3**).

Symptoms have varied between SARS-CoV-2 variants, as opposed to the similar clinical presentation with different influenza types and subtypes (12). With the emergence of the gamma variant, and especially omicron variants, taste/smell disturbances were no longer dominating. Due to a shift in tropism to the upper airways, less lung affection is associated with the omicron subvariants (27). Delta has been linked to increased mortality compared to previous VOC, with a general increased risk of viral pneumonia, intensive care unit (ICU) admission, and need of ventilation support (28, 29), although severe disease could be attributed to lack of vaccination (30). Higher viral loads and prolonged viral shedding after delta infection have also been observed (31). The current omicron variants are mostly causing mostly mild upper respiratory symptoms such as rhinorrhoea and pharyngitis, which may reflect viral attenuation, but multiple vaccination and infections during the last three years may mask omicrons' true pathogenicity.

Figure 3: Common acute influenza and SARS-CoV-2 symptoms.

Loss of taste/smell is only associated with SARS-CoV-2 variants Wuhan, alpha, beta and delta. The illustration was created with BioRender.

A country such as China, with mostly naïve individuals due to extended lockdowns and limited vaccination coverage, may provide a clearer picture of the true severity of the omicron variants. The "zero COVID-19" restrictions in China were lifted, followed by a massive omicron wave in December 2022 and January 2023, infecting 80% of the population (32). However, reported death rates are uncertain since the Chinese definition of COVID-19 related death excludes people with comorbidities and those who die at home (33).

1.2.2 Severe disease and high-risk groups

In most cases, COVID-19 and influenza illness are self-limiting, although both COVID-19 and influenza are associated with significant morbidity and mortality. People with underlying comorbidities are at highest risk of severe disease, particularly immunocompromised individuals (34, 35) (**Table 1**). These include many older adults,

with age-related weakened immune responses (immunosenescence), and mortality is therefore highest in the elderly (36).

Table 1: Risk groups for severe influenza and COVID-19.

Risk groups	Influenza	COVID-19
	Influenza virus	SARS-CoV-2
Pregnant women	✓	✓
Adults >65 years old	✓	✓
Children <5 years old	✓	✗
People with chronic medical conditions*	✓	✓
Immunosuppressive conditions§	✓	✓

*Chronic cardiac, pulmonary, renal, metabolic (including obesity), neurodevelopmental, liver or hematologic diseases
§HIV/AIDS, receiving chemotherapy or steroids, malignancy

Risk groups for severe COVID-19 and influenza according to Norwegian Institute of Public Health (37, 38). The illustration was created with BioRender.

In addition to age and comorbidities, unvaccinated individuals develop more severe COVID-19 compared to vaccinated subjects (39). Progression to severe COVID-19 usually occurs one week after symptom onset (40). Hypoxemia, causing dyspnoea, can further develop into fulminant respiratory failure. Lung inflammation with vascular leakage which causes severe hypoxemia and bilateral radiographic infiltrations fulfil the criteria of acute respiratory distress syndrome (ARDS). Extrapulmonary disease, with systemic inflammation affecting many organs and immunothrombosis, is associated with severe COVID-19. ARDS is also a complication of severe influenza, in addition to bronchitis, viral or bacterial pneumonia. Excess inflammatory reactions and cytokine production can lead to a cytokine storm, ARDS and ultimately multi-organ failure (41). Influenza case fatality rates range from 60% in documented cases with zoonotic avian flu (influenza A/H5N1) (42), to approximately 0.05-0.1 % for epidemic influenza. Children have higher influenza attack rates compared to adults

(43), due to their immature immune systems and lack of immunity developed after previous infections or vaccinations, termed pre-existing immunity. In contrast to influenza, young children generally develop mild COVID-19, with the exception of the Multisystem Inflammatory Syndrome in Children (MIS-C) (44, 45). This is a rare, but severe delayed inflammatory response to SARS-CoV-2 with persistent fever, abdominal symptoms, skin lesions and ultimately hypotension and shock. High infection rates in younger people have occurred during the omicron wave, possibly due to increased socialisation and lower vaccination coverage in this age group. A priming infection early in life will likely protect children against severe acute outcomes of future SARS-CoV-2 variants, comparable to seasonal coronaviruses (46). However, the long-term consequences of repeated SARS-CoV-2 infections, regardless of pre-existing immunity, are unknown.

1.3 Diagnosis and treatment

1.3.1 Diagnosis

The gold standard for diagnosing acute influenza or SARS-CoV-2 infection is by reverse transcription (RT)-PCR of respiratory swabs. A swab is collected from the nasopharyngeal and/or the oropharyngeal cavity and tested for the presence of specific viral RNA sequences. With the COVID-19 pandemic, SARS-CoV-2 lateral flow-tests or rapid antigen tests became widely available and were implemented as a diagnostic tool. These over-the-counter tests spared and even replaced the more limited RT-PCR resources and improved rapid self-isolation. Dual rapid tests for both influenza and SARS-CoV-2 are now commercially available. Lateral flow-tests detect viral proteins and generally have a high specificity, the ability to rule out disease, both in symptomatic and asymptomatic individuals (47). Sensitivity, the ability to detect the virus, is higher in symptomatic than asymptomatic individuals, and in the first week after symptom onset. There is a chance of false negative results if tested early, although a recent SARS-CoV-2 challenge study found low viral emission before the lateral flow tests were positive (48).

Proof of previous infection is established by measuring antibodies, both for diagnostic and research purposes. Beyond the acute phase, the sensitivity for detecting an infection based on RT-PCR and lateral flow tests decreases, while the sensitivity for serological tests increases. Antibodies may be detected days to weeks after the infection and are directed against the viral proteins, most commonly the immunodominant spike or hemagglutination proteins of SARS-CoV-2 and influenza, respectively.

1.3.2 Treatment

Upon infection, specific antiviral treatment can be initiated to combat disease progression. Outside the hospital setting, treatment of influenza and SARS-CoV-2 is primarily limited to high-risk patients. For influenza, there are six licenced antiviral drugs, but two of them, amantadine and rimantadine, have high levels of resistance (>99%) and are not in use (49). The neuraminidase inhibitors (oseltamivir phosphate (oral), zanamir (inhalation) and peramivir (intravenous)) and the endonuclease inhibitor Baloxavir marboxil, are most frequently used and are effective against both influenza A and B. To provide the greatest clinical benefit antiviral treatment should be initiated as soon as possible, irrespective of lab-confirmation, preferably within 48 hours of symptoms onset. However, treatment is beneficial in hospitalised patients with evidence of continued viral replication up to 4-5 days after symptom onset (50).

SARS-CoV-2 antiviral therapies such as nirmatelvir/ritonavir, molnupiravir and remdesivir, have been shown to reduce hospitalisation and death in non-hospitalised patients if administered within the first week of symptoms (51). Currently licenced monoclonal antibodies have reduced or no effect against the dominating omicron variants (52), but may be considered for patients with immunodeficiencies without vaccine-induced immune responses (53). Hospitalised patients with dyspnoea and hypoxia are given oxygen through different modes of ventilatory support, anticoagulation prophylaxis/therapy and glucocorticoids such as dexamethasone (51). The need for immunomodulatory therapy with IL-1/IL-6 inhibitors and Janus kinase (JAK)1/2 inhibitors is evaluated in severely ill patients with rapid respiratory failure.

Monoclonal antibodies and antiviral drugs may also be given later (>7 days after symptom debut) to hospitalised patients with evidence of continued viral replication.

Additionally, non-pharmaceutical interventions such as mask wearing, social distancing and travel restrictions, to mention a few, have successfully been applied against SARS-CoV-2. Countries like Australia, New Zealand and China eliminated community transmission by closing international borders, widespread testing, and contact tracing, as well as short lockdowns (54, 55).

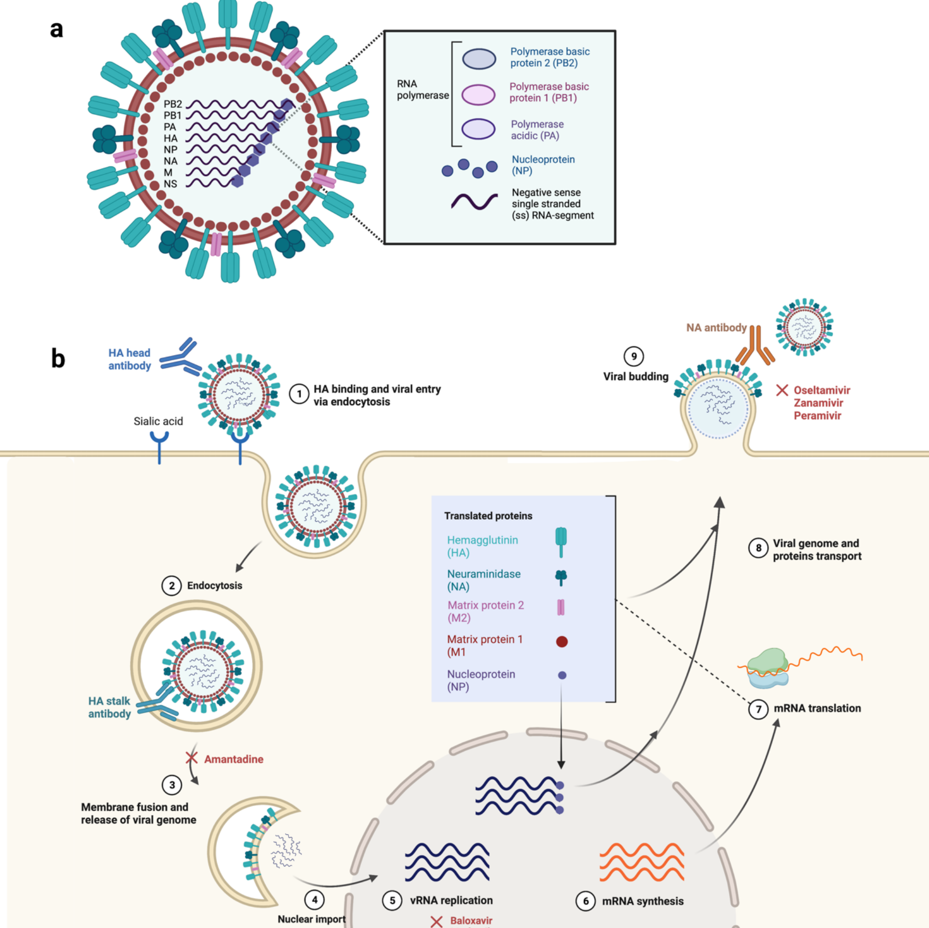
1.4 Viruses: Influenza and SARS-CoV-2

Both influenza and SARS-CoV-2 are enveloped, single stranded RNA viruses. The envelope contains virus-specific proteins important for viral attachment to the host cell. The two major surface proteins of influenza viruses, hemagglutinin (HA) and neuraminidase (NA), are pivotal for host cell entry and exit, respectively. SARS-CoV-2 uses the spike (S) protein for attachment to and fusion with host cells, and the envelope (E) protein is thought to play a role in viral assembly and release.

1.4.1 Viral structures and life cycles

Influenza belongs to the family *Orthomyxoviridae*. Influenza viruses can be divided into four types, A, B, C and D (56). Influenza A and B cause annual epidemics in humans, and type A has pandemic potential. **Figure 4a** shows the structure of an influenza virus A.

Figure 4: Influenza viral structure and replication cycle.



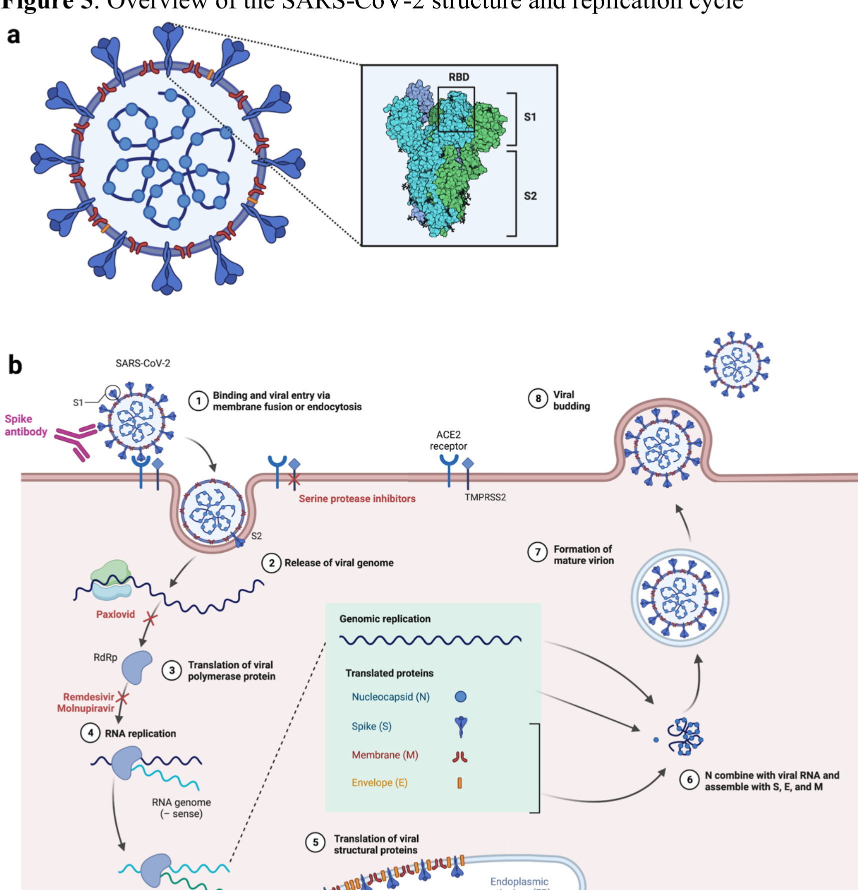
The genetic material of the influenza virus is 8 single stranded RNA segments (**a**). The RNA segments encode at least ten viral proteins, including HA, NA, matrix protein 1 (M1) and 2 (M2), polymerase basic protein 1 (PB1) and 2 (PB2), polymerase acidic (PA), nucleoprotein (NP), and non-structural proteins 1 (NS1) and 2 (NS2) (57, 58). Several other non-structural proteins are expressed in infected cells, but not in the mature virion. The internal proteins of the virus are highly conserved across influenza A subtypes. Each RNA segment is surrounded by multiple NPs and associated with the RNA-dependent RNA polymerase (RdRp) complex. This trimeric RdRp consists of PB1, PB2 and PA. Together with the RNA-bond NP, the RdRp complex form viral ribonucleoprotein complexes (vRNPs). Structural proteins are M1, forming the protein capsid, and the surface proteins embedded in the viral envelope: M2, HA and NA. The M2 protein is an ion channel that equilibrate the viral pH. The influenza replication cycle is initiated by HA binding to sialic acids (SA) on epithelial cells in the respiratory tract (**1**) (**b**). The virus is internalised by endocytosis (**2**) and the acidic pH in the endosome activates the HA transformation and the matrix 2 (M2) channel facilitates further acidification that mediates the membrane fusion and release of the viral genome (**3**). The genome is transported to the nucleus via nuclear transport (**4**) and viral RNA (vRNA) is replicated (**5**) and transcribed to mRNA (**6**). The mRNA is translated into the viral proteins (**7**) and assembled in the virion together with the viral genome (**8**) and transported to the cell membrane. The newly synthesised virus buds from the membrane (**9**) by NA cleavage of SA.
Antibodies against HA head can sterically interfere with receptor binding, while the HA-stem antibodies do not necessarily block cell entry but inhibit the fusion of the host and viral membranes by hindrance of the HA proteolytic cleavage. NA antibodies inhibit the release of progeny viruses from the host cell during budding. The illustration was created in BioRender.

The most abundant surface protein of the influenza virus is HA, followed by NA (ratio 300HA:40NA) (59). HA is a trimeric protein with a globular head domain (HA1) and a stem domain (HA2). The receptor binding site (RBS) is situated in the HA1 domain and is responsible for initiating the replication cycle by binding to sialic acids (SA) (**Figure 4b**). The NA is a tetrameric protein with enzyme activity, central for cleaving terminal SAs to facilitate viral budding from infected cells and movement through respiratory mucus. Antibodies may prevent infection by blocking the HA-SA binding or interfere with the proteolytic cleavage of HA. NA-specific antibodies inhibit viral budding and subsequent viral spread.

Influenza A is divided into subtypes based on the major surface glycoproteins HA and NA. There are 18 different HA subtypes (H1-H18) and 11 NA subtypes (NA1-11), with genetic and antigenic versatility. The 18 HA subtypes are categorised into two phylogenetic groups based on the conserved HA stem domain, group 1 (e.g., H1, H2, H5) and group 2 (e.g., H3, H7). The two influenza A subtypes currently circulating in humans, A/H1N1 and A/H3N2, are further divided into clades and subclades based on the HA gene sequence. Influenza B viruses are divided into two distinct lineages B/Victoria and B/Yamagata, although B/Yamagata has not circulated since 2020 (60).

The family of coronaviruses (*Coronaviridae*) belong to the order *Nidovirales.* Coronaviruses are large, enveloped viruses with a positive sense single-stranded RNA genome (+ssRNA) encapsidated by nucleocapsid (N) and three surface proteins: spike (S), envelope (E) and membrane (M) (**Figure 5a**) (61). The S protein radiates from the surface, giving the virus its characteristic crown (*corona*). SARS-CoV-2 forms a lineage that originates from the genus *betacoronavirus* and subgenus *sarbecovirus* or B lineage. This is the same subgenus that SARS-CoV-1 belong to. Other known betacoronaviruses are MERS-CoV virus (C lineage), and two viruses that cause common colds, HCoV-OC43 and HCoV-HKU-1 from the A lineage. The other genera of coronaviruses are *alphacoronavirus*, *deltacoronavirus* and *gammacoronavirus*. The seasonal coronaviruses HCoV-229 and HCoV-NL63 are alphacoronviruses. The delta- and gammacoronviruses infect avian species and not humans.

Figure 5: Overview of the SARS-CoV-2 structure and replication cycle



The structural proteins spike (S), membrane (M) and envelope (E) are embedded in the viral SARS-CoV-2 membrane (**a**). The S protein is a trimeric protein that can be divided into two functionally distinct regions: S1 and S2. The S1 domain contains the receptor binding site (RBD), while S2 is a transmembrane domain that mediate the fusion of viral and cellular membranes. The M protein is essential for viral assembly, together with the E protein, and is the most abundant protein in the membrane. The E protein is also believed to regulate the viral pH and facilitate viral release. The RNA genome is encapsidated by nucleocapsid protein (N) and encodes several non-structural and the structural proteins. The betacoronaviruses from the B-lineage bind to the ACE2 receptor via the receptor binding domain (RBD) in the S1 region of the spike protein (**b**) (**1**). The S2 transmembrane domain mediate the fusion of viral and cellular membranes, after the host transmembrane serine protease 2 (TMPRSS2) cleave the fusion site (**2**). The virus can also enter cells via the endosomal cysteine proteases cathepsin B and L. The RNA-dependent RNA polymerase (RdRp) is translated via host ribosomes in the cytoplasm directly upon entry (**3**). Synthesis of the (-sense) viral RNA is mediated by the RdRp and its integrity maintained by an RNA proofreading complex (**4**). However, mutations by natural selection still occur. The negative sense RNA serves as template for the viral genome and subgenomic RNA that encode structural (S, M, E, N) and non-structural proteins (NPS1-16). Proteins are transported to the endoplasmic reticulum (ER) (**5**), and then assembled with the encapsidated viral genome through a complex cooperation with the E and M structural proteins (**6**). The final steps are the formation of the mature virion (**7**) and exocytosis by viral budding (**8**). The illustration was created with BioRender.

1.4.2 Viral tropism

Viral tropism is the summary of factors dictating which cells (cellular tropism), tissues (tissue tropism) or hosts (host tropism) a virus is able to infect (62). For example, human influenza A usually infects respiratory cells by binding to sialic acid receptors (SA) in airway tissue and are endemic in humans and in several animal species, including pigs, wild aquatic birds, domestic poultry, horses, dogs and bats (hosts) (63). Human influenza viruses prefer α2.6-linked SA present in the upper airways, while avian and equine influenza viruses preferentially attach to α2.3-linked SA. In human airways, most respiratory non-alveolar cells express α2.6-linked SA, but bronchiole and alveoli cells in the lower airways express α2.3-linked SA. The tropism of avian viruses explains their high lethality, causing severe pneumonia in humans. In contrast to human influenza binding to sialic receptors, SARS-CoV-2 binds to angiotensin receptor 2 (ACE2). ACE2 is widely distributed in human tissues, including the respiratory, cardiovascular, gastrointestinal, urogenital and nervous system (64). Coronaviruses circulate in multiple species, e.g., mammals, birds, bats, livestock (cows, pigs) and pets (dogs and cats). After ACE2-binding, membrane serine protease 2 (TMPRSS2) facilitates cleavage of the S-protein that initiates membrane fusion (65) (**Figure 5b**). If this protease is not present, or there is insufficient TMPRSS2 expression, the virus may enter the cell via endocytosis. Omicron BA.1 and BA.2 had attenuated ACE2/TMRPSS2 entry, but the BA.5 sublineage reverted back to utilising this pathway similarly to pre-omicron variants (66). Some COVID-19 patients have experienced neurological symptoms similar to encephalitis, and post-mortem brain tissue of COVID-19 patients have revealed viral RNA (67). However, whether the virus enters the brain by primary neuron infection or if entry is secondary by other routes (such as blood or the olfactory bulb) remains unclear.

1.4.3 Influenza viral evolution by antigenic drift and shift

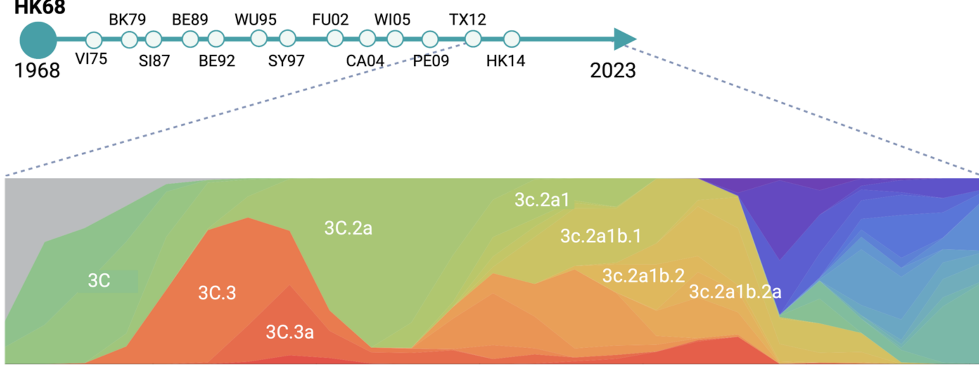
Seasonal influenza epidemics occur annually in the temperate climates, while influenza continuously circulates in the tropical zones, although peaks can be observed during the rainy season. The influenza season in the Northern hemisphere is during the winter

months, from October to April (8). Recurrence of influenza epidemics due to high genetic diversity and rapid viral evolution of influenza viruses, leaving population immunity one step behind. Due to the lack of proof-reading during replication, the virus can accumulate point mutations in a process known as antigenic drift. Selective pressure from influenza specific antibodies in the community directs viral drift, allowing virus to escape pre-existing immunity. Occasionally, novel influenza A viruses can appear through antigenic shift. The mechanism behind their origin is mixing of genetic segments from two or more influenza viruses, facilitated by co-infection in a host. The reassortant influenza virus with novel HA and NA may be capable of causing a pandemic by respiratory spread between humans in an immunologically naïve population.

The pandemic A/H3N2 virus that emerged in 1968, was derived from an avian A/H3 species re-assorted with the human A/H2N2 virus (68). Initially, the A/H3N2 virus maintained some of the avian receptor recognition. With evolution, the virus gradually lost affinity for avian receptors, adapting to the human species. The adaptation and immune evasion by the A/H3N2 viruses can be explained by different modification mechanisms of the surface viral proteins, HA and NA. Antigenic sites of the globular HA head of influenza A/H3N2 are designated sites A-E (69). Amino acids near the receptor binding sites of the HA may be replaced, or sugar molecules can be added to existing structures, known as glycosylation, reducing antibody binding to antigenic sites (70). The original A/H3N2 pandemic virus had 2 glycosylation sites, compared to up to 7 in the globular head of the HA on current viruses. By comparison, the A/H1N1pdm viruses are not prone to this shielding process, with minimal glycosylation. Parallel to the increasing glycosylation, the net charge of the virus has increased, which may have cancelled out the negative effects of glycosylation and helped increase the avidity to its SA receptor. However, most A/H3N2 vaccine mismatches have been linked to mutations in the antigenic site B of the HA, not yielding any new glycosylation sites (71).

Consequences of the accumulating point mutations are that novel A/H3N2 viruses have appeared sporadically every 2-5 years, measured as a leap in antigenicity (72). Since the 2014/15 influenza season, clades and subclades of A/H3N2 viruses have been identified (**Figure 6**). Previously circulating viruses had belonged to the 3c.1 clade (exemplified by A/Texas/50/2012) but were replaced by the 3c.2a clade representative virus A/HongKong/4801/2014 with 10-12 amino acid substitutions (68, 73). Subclades from 3c.2a1b.1 (newly designated clade **1**) and 3c.2a1b.2 (**2**) have circulated until a dramatic drop in all circulating influenza viruses followed the emergence of the SARS-CoV-2 pandemic due to infection control measures (74). An increased influenza activity has, however, been observed during the 2022/23 season due to reopening and lifting of social restrictions and removal of other infection control measures for COVID-19.

Figure 6: Influenza A/H3N2 clades and subclade circulation, 2011-2023.



The illustration shows the different clades and subclades of influenza A/H3N2 that have circulated from 2011 to 2023. After the emergence of the COVID-19 pandemic in 2020, there was an abrupt decrease in H3-viruses, and currently subclades of 3C.2a1b.1 (**1**) and 3C.2a1b.2 (**2**): **1a.1**, **2a.1b**, **2a.3a**, **2a.3b** and **2b** circulating (blue and ocean green). The global viral HA frequencies (y axis) based on 1477 genomes were generated from nextstrain.org and modified with BioRender.

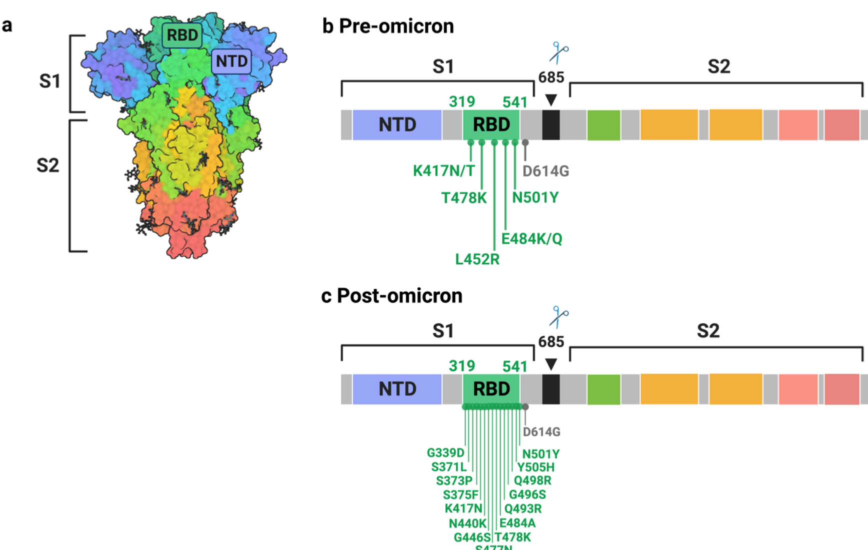
The reduced influenza transmission rates during the COVID-19 pandemic were not only observed for influenza viruses, as other contagious diseases, such as respiratory syncytial virus (RSV), also plummeted due to social distancing. Interestingly, the B/Yamagata lineage has not been conclusively detected since March 2020 (60). Moreover, the other influenza viruses (A/H1N1, A/H3N2 and B/Victoria viruses) have circulated with less diversity, which is unlike any previous observations. For instance,

three subclades of A/H3N2 have not been detected since April 2020. The influenza season 2021/22 was characterised as mild, with mainly A/H3N2 viruses circulating in two delayed epidemic waves. During the 2022/23 season, influenza circulated in October 2022. Influenza A (of both subtypes) dominated during the first wave, followed by a spring wave of influenza B/Victoria in Europe (75). The estimates for the season are only preliminary, but an overall higher influenza activity was observed compared to the previous season (76).

1.4.4 SARS-CoV-2 viral evolution and variants of concern

Following the spread of SARS-CoV-2 in the community, it was believed that SARS-CoV-2 would have low mutation rate due to its proof-reading mechanism. However, millions of infected people allowed for a vast selection pressure. A spike mutation from D614 to G614 gave rise to the D614G variant in March 2020, which quickly dominating worldwide (77). The D614G mutation was advantageous and resulted in increased viral replication and transmissibility (78) (**Figure 7**). In the following months, several novel VOC emerged independently across the globe, defined as viral variant which impacts epidemiology by increased transmission and reduced population immunity, eradicating previous variants. The alpha variant (B.1.1.7 lineage) was detected in the UK in September 2020 (79), beta (B.1.351) in South Africa October 2020 (80), gamma (P.1) in Brazil in November 2020 (81) and delta (B1.516.2) in India in January 2021 (82), all containing mutations in the spike protein that increased infectivity by different mechanisms. The receptor binding domain (RBD) of the spike comprises residues 319-541 (**Figure 7**). The N501Y mutation was introduced with the alpha variant, which enhanced binding affinity to ACE2 (83). In addition to the N501Y mutation, the beta and gamma VOC had the common K417N/T and E484K/Q RBD mutations with reduced or diminished antibody binding. The delta variant had the novel L452R and T478K mutations which both significantly enhanced affinity with the ACE2 receptor. All these variants have currently been descaled by the World Health Organisation (WHO) because they are no longer detected or detected at very low levels (84).

Figure 7: Virus spike protein structure and RBD domain with significant mutations



Cryo-electron microscopy structure of the spike protein of SARS-CoV-2. The spike protein is composed of the two subunits S1 and S2. The N terminal domain and receptor binding domain (RBD) is located in the S1 subunit (a). The RBD constitutes spike residues 319-541. Important RBD mutations found in variants of concern (VOC) pre-omicron (b) and post-omicron (c) are highlighted. The post-omicron mutations are those found in the first omicron sublineage BA.1. Part of illustration made by Diana Sofia Mollocana Yanez. Created in BioRender.

The omicron variant (B.1.1.529) emerged in South Africa, November 2021, and quickly spread worldwide (85). Unique to this variant is the immense number of mutations, with 32 spike mutations compared to the ancestral Wuhan-Hu-1 (86). The 15 RBD mutations (**Figure 7**) facilitate immune evasion by enhanced ACE2 affinity (G339D, N501Y), higher antibody resistance (S371L, N440K, G446S, Q493K) and decreased protein stability and increased infection risk (S373P, S375F, K417N, S477N, T478K, E484A, G496S, Q498R, Y505H). Furthermore, this variant has developed into multiple widely circulating subvariants, initially BA.1 and BA.2, followed by BA.5, BA.212.1, BA.2.75, BQ.1, XBB (87), and most recently XBB.1.5 (88). Early reports have found that XBB.1.5 has increased ACE2 binding and antibody evasion, explaining its rapid rise (89).

Immune pressure from an increasingly infected and vaccinated population is likely the main facilitator of viral evolution. There is currently a large proportion of hybrid

immunity from both infection and vaccination in the community. Interestingly, SARS-CoV-2 variants alpha through delta emerged prior to the wide distribution of COVID-19 vaccines. Only 25% of the population was immunised when omicron appeared in South Africa, but the country had three prior infection waves with high number of community infections (78). Omicron was detected after yet another surge in infections, many of which were reinfections.

1.5 Immunity to influenza and SARS-CoV-2

The host immune responses are essential in the clinical outcome of influenza and SARS-CoV-2 infections. Although many lessons have been learned from influenza immunology, the acute B cell responses has been more extensively characterised to SARS-CoV-2 than no other pathogen (90). The immune responses against the two respiratory viruses have similarities and differences, although the general immune mechanisms are similar. Both develop local immunity in the respiratory tract through infection or mucosal vaccines, and systemic immunity is conferred by both vaccines and infection.

1.5.1 Innate immunity

The main function of the innate immune system is to prevent and eliminate infection, promote tissue repair, and stimulate the adaptive immune response. The airways are routinely exposed to large numbers of potential pathogens and have developed several non-specific mechanisms as a first line of defence. These are physical barriers, such as mucus and ciliated cells, preventing virus from interacting with the underlying cells and transporting virus away from the airways (91). Furthermore, innate immune cells, such as macrophages and dendritic cells express pattern recognition receptors, PRRs, that can detect and act upon foreign molecular structures (pathogen-associated molecular patterns, PAMPs or damage-associated molecular patterns DAMPs). Activation of PRRs leads to production of inflammatory cytokines and chemokines and induction of cell death in infected cells (92).

Several types of PRRs are important in response to influenza and SARS-CoV-2, particularly Toll-like receptors (TLRs) and retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs). Attenuated SARS-CoV-2 inflammation and reduced cytokine production have been confirmed in TLR2-deficient macrophages. Similarly, mutations in the TLR3 gene are associated with influenza encephalopathy in humans. Increased viral loads of both SARS-CoV-2 and influenza virus have been detected in TLR3-deficient mice. Single stranded RNA (ssRNA) can be detected by RLRs, in addition to intracellular TLRs. Activation through these signalling pathways generally induce pro-inflammatory cytokines such as tumour necrosis factor (TNF), interleukin (IL)-6, IL-1, and type I and III interferons (IFN). The role of type I IFN in SARS-CoV-2 pathogenesis has been demonstrated in several studies and is likely a major immune evasion mechanism for the virus (93). A rapid and robust IFN response likely contributes to eliminating the disease, while a prolonged stimulation and increased levels over time is associated with mortality. Effector cells of the innate immune system, like neutrophils, natural killer cells, dendritic cells and macrophages are attracted to the infection site by chemokines and contribute to elimination and activation of the adaptive immune system, essential for clearing infection and induction of memory responses.

1.5.2 Adaptive immunity

The adaptive immune system is characterised by its ability to create immunological memory and will adjust and improve upon repeated exposure to an antigen. Upon the first encounter with an infectious agent, the naïve lymphocytes of the adaptive immune system will be activated within hours, whereas memory cells are activated within minutes in a secondary encounter. In both cases the lymphocytes' proliferation process requires days before it is possible to detect and measure (94). Dendritic cells (DC) are the connection between the innate and adaptive immune responses. DCs migrate from the site of infection to draining lymph nodes (LN) and present the viral antigens to B and T lymphocytes or B and T cells. B and T cells use two different molecules to specifically recognise and respond to antigens, namely the B cell receptor (BCR) and T cell receptor (TCR). Antibodies are produced by B cells together referred to as

humoral immunity. Cellular immunity is mediated by two main types of T cells, CD4+ and CD8+ T cells.

Cellular immune responses

Cellular immune responses by CD4+ and CD8+ T cells are often directed against internal virus peptides and are less prone to antigenic variation. T cell targeted proteins are NP, M and the PA for influenza, and N, M, NSP and E for SARS-CoV-2 (95). Therefore, cellular responses can provide broader immunity by protection against several SARS-CoV-2 variants or by *heterosubtypic* protection (protection against another influenza A subtype with different HA or NA) (96-98). There are epidemiological data to support that pre-existing T cell memory responses induced by earlier HCoV infections contribute to clearance of SARS-CoV-2 infections (98).

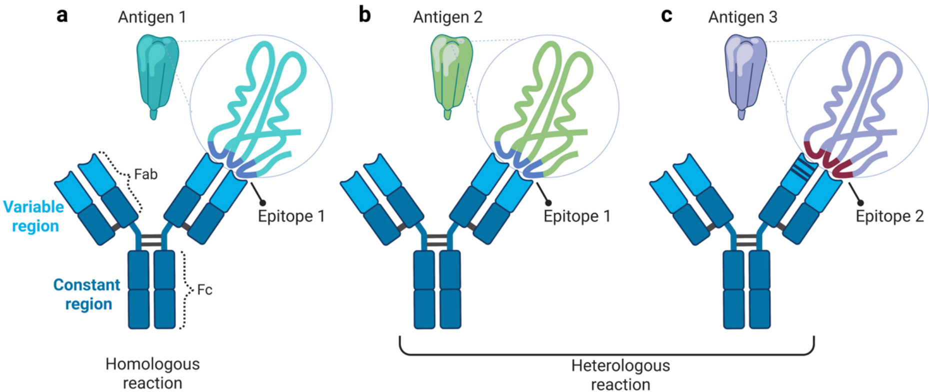
CD8+ T cells or cytotoxic T cells kill infected cells, as well as produce cytokines like IFN-γ and TNF-α inhibiting viral replication. CD4+ T cells have several different functions and are divided into subsets (T helper (Th)1, Th2, Th17, T follicular helper cells (Tfh) and regulatory T cells (Treg)), defined by the cytokines they produce (99). Tfh cells produce cytokines that help support B cells to differentiate into memory B cells (MBCs) and plasma cells secreting class-switched and high-affinity antibodies. Furthermore, CD4+ T cells recruit CD8+ T cells to lymph nodes and the infection site and secrete IFN-γ important for generating memory CD8+ T cells. CD4+T cells can additionally provide cytotoxic effects, activate antigen presenting cells (APCs) and other components of the innate immune system (100). As intracellular pathogens, both influenza and SARS-CoV-2 infections are biased towards Th1 responses. After the infection is cleared, immunological memory is established by memory T cells (long-lived central memory T cells, effector memory T cells and tissue resident memory T cells).

Humoral immune responses

Antibodies are produced by terminally differentiated B cells called plasma cells and may also be referred to as immunoglobulins (Ig). Antibodies exist in two forms, either membrane bound to the B cell (as B cell receptors, BCR) or in secreted form. The

secreted antibodies are important for neutralising virus and preventing viral attachment to host cells and thereby infection. All antibodies have the same basic structure, with two identical light and heavy chains, each chain comprising variable and constant domains. The Ig can further be defined by a fragment crystallisable (Fc) and a variable region that contains two identical antigen-binding fragments (Fab) (**Figure 8a**). The Fc region define the effector functions and the Ig *isotype* of the antibody. IgG, IgA and IgM are the main antibody mediators of influenza and SARS-CoV-2 immune responses. IgA is present in mucosal secretions, where it can neutralise and prevent viral entry, and also in serum. IgM is expressed on the surface of B cells and mediates the primary antibody defence by activating the complement system (101).

Figure 8: Antibody structure and recognition by homologous and heterologous reactions



An antibody can be divided into the constant region (dark blue) and variable region (light blue), or by the antigen-binding fragment (Fab) and the fragment crystallisable (Fc). An antibody recognises an antigen 1 with an epitope 1 in a homologous reaction (**a**). Upon antigenic drift, the antigen will change (antigen 2), yet the epitope remains unchanged (epitope 1), and the antibody induced by a previous exposure can recognise antigen 2 by a heterologous reaction (**b**). Antigenic drift may also change the epitope (epitope 2) on an antigen 3 (**c**). The antibody is able to recognise epitope 2 due to affinity maturation in the B cell pool (mutations in the B cell receptor in one of the antigen-binding fragments of the variable domain is illustrated by stripes), facilitating antibodies with improved binding to a non-identical, yet similar sequenced epitope 2. The illustration was created with BioRender.

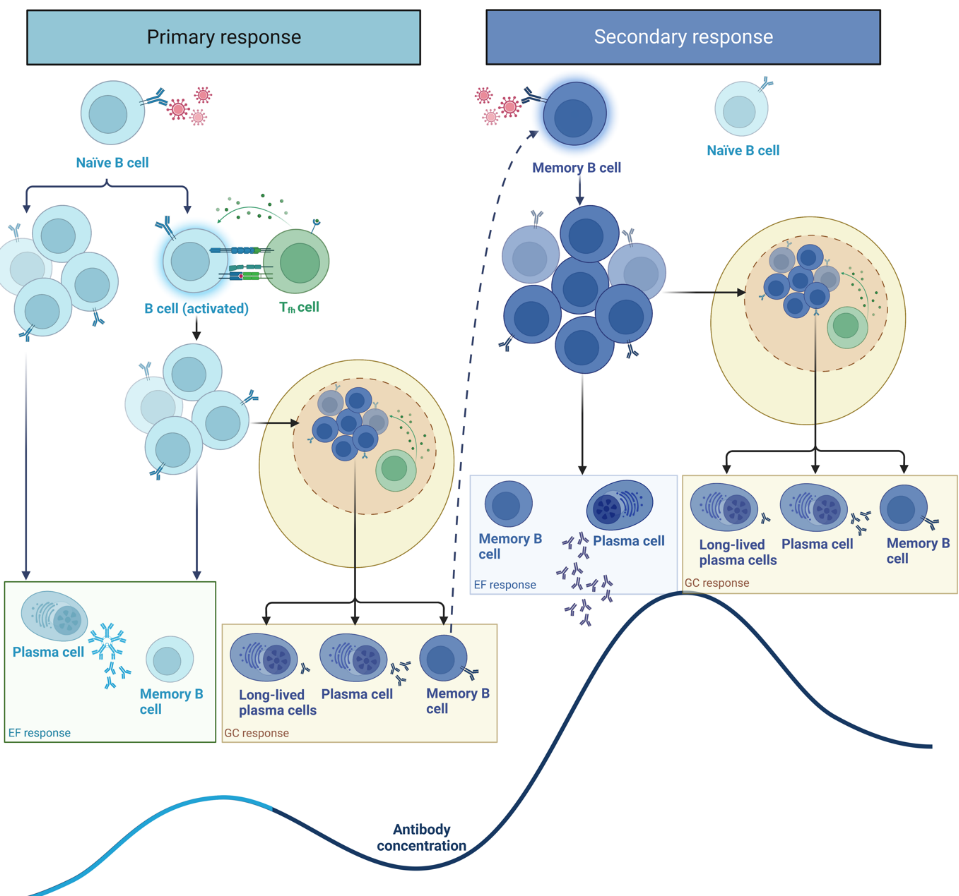
IgG constitutes the main systemic isotype detected in plasma or serum and has several important effector functions. IgG antibodies neutralise virus, activate the complement pathway, target cells for lysis by the innate immune cells (antibody-dependent cell-mediated cytotoxicity, ACDC), coat (opsonize) antigens to promote phagocytosis (antibody-dependent cellular phagocytosis, ADCP), and provide feedback inhibition to

activated B cells. Activation of complement, ACDC and ADCP mechanisms are viewed as a connection between adaptive and innate immunity and are often termed non-neutralising attributes of antibodies or effector functions of the Fc-domain or Fc-receptor (FcR). Non-neutralising antibodies to influenza NP and M2 have been found to reduce disease in animal models (102), and specifically ADCC and ACDP mechanisms contribute to clearance of influenza infected cells in humans (103). Animal studies have found enhanced Fc effector functions (supplementary to neutralising activity) in animals with complete SARS-CoV-2 infection protection. Unlike influenza, the ADCC activity may be of secondary importance due differences in the replication cycles of the two viruses.

Humoral immune responses are initiated by the binding of a viral antigen to an IgM (or IgD) B cell receptor on a naïve B cell. The next phase of the response may either be *T cell dependent* or *T cell independent* (**Figure 9**). B cells have been found to respond to influenza vaccination without T cell help, but these responses are often transient and dominated by low-affinity IgM. However, most B cells acquire help from CD4+ T cells to differentiate, switch isotype and undergo *affinity maturation*. B cells can differentiate into antibody-producing plasma cells in the respiratory tract within 3 days after influenza infection. The systemic antibody response can be detected approximately 3 days later (day 6-7) (102). B cells are able to increase BCR through somatic hypermutation in the germinal centres (GC) on lymph nodes. Here, T cells may select high-affinity B cells for differentiation to long-lived plasma cells that reside in the bone marrow or MBCs eventually localised in tissues. Proof of GC formation after influenza vaccination has recently been demonstrated in humans (104). MBCs are generated both after SARS-CoV-2 infection and vaccination. With their extensive BCR repertoire, MBCs are an important B cell population for combatting diverse influenza and SARS-CoV-2 viruses (90).

Neutralising antibodies (nAbs) induced by infection or vaccination can prevent viral attachment to host cells and thereby prevent infection and are an important early defence mechanism. These antibodies target the immunodominant viral surface proteins such as HA on influenza or the S protein of SARS-CoV-2.

Figure 9: Primary and secondary B cell responses



The primary B cell response is initiated by the naïve B cell binding an antigen. The B cell can proliferate independently of T cell help in the extrafollicular compartment, ensuring the rapid plasmablast production of IgM, IgG and IgA and later generation of memory B cells (MBCs). The B cell may alternatively acquire T cell help, further proliferate and differentiate to plasma cells or MBCs. Some of the clonally expanded B cells enter the germinal centre (GC) for affinity maturation. Long-lived plasma cells and MBCs are generated in the later phases of the immune response. Upon the secondary B cell response, MBCs may quickly recognise the antigen, proliferate to plasmablasts producing rapid and robust IgG responses. Some of these MBCs may re-enter the GC for further affinity maturation. The naïve B cell may also be stimulated in secondary immune responses but constitutes a smaller part of the overall response.

Vaccine-induced antibodies that match the viral antigen are often referred to as *homologous* antibodies. However, due to the frequent mutations in HA and S, the binding sites for nAbs can be lost, in worst case rendering viral escape. Yet, MBCs established from an earlier exposure can be restimulated by vaccination or infection to produced *heterologous* or *cross-reactive* antibodies (**Figure 8**) with maintained

protection (97). Generally, cross-reactive antibodies are associated with non-neutralising antibodies directed against more conserved epitopes, such as the HA-stem, NA or internal proteins (105, 106). These epitopes remain unchanged, providing a simple route for cross-reactivity (**Figure 8b**). However, cross-reactivity has also been demonstrated by several studies measured in the HI assay (107, 108), illustrating the plasticity of the B cell pool to develop affinity matured antibodies able to recognise conserved epitopes on mutated viral antigens (109) (**Figure 8c**).

1.6 Correlates of protection

A correlate of protection is defined as the immune measurement corresponding to either protection against infection, known as sterilising immunity, or protection from disease (110). Although immune responses are multifaceted, and the immune system have developed multiple layers of defence mechanisms, a single arm of the response can correlate with protection (111). This is very useful during evaluation and approval of new vaccines. In most cases, correlates of protection are based on antibodies measured by serological assays because they are easier to measure and standardise than cellular responses.

1.6.1 Influenza correlate of protection

The principle of hemagglutination as a diagnostic tool was, first described for influenza viruses in 1941, when erythrocytes would agglutinate if a blood vessel was penetrated during the harvest of influenza virus grown in hen's egg (112). Since then, the technically simple and inexpensive hemagglutination inhibition (HI) assay has been utilised to evaluate influenza vaccine responses and has gained reference status for evaluating influenza vaccines and seroconversions. The basis for this correlate is a human influenza challenge conducted in 1972 by Hobson *et al.*, a serum HI antibody titre between 18-36 was shown to provide 50% protection from influenza A or B challenge (113). Later studies have confirmed that a HI titre of ≥40 is a good measurement of the 50% vaccine protective titre, and is generally a good measurement of protection in healthy young adults (114). The HI assay utilises the principle that

influenza virus can bind to sialic acids on the surface of certain avian and mammal erythrocytes, causing them to agglutinate. If virus-specific nAbs are present, they will bind the influenza virus and release the red blood cells (RBCs), inhibiting the agglutination. In this manner, “nAbs” can be measured and quantified by serial titration. Drawbacks of the assay are differences in laboratory practices, as well as the cut-off being considered too low for children, and lower sensitivity to influenza B antibody responses. Furthermore, the evolution and adaptation of the A/H3N2 viruses to the human species has resulted in reduced affinity to avian receptors, and a reduced ability to agglutinate turkey RBCs (115). To characterise the modern A/H3N2 viruses, the use of mammalian guinea pig RBCs has been applied. Furthermore, some of the newer A/H3N2 strains have acquired a mechanism to agglutinate RBCs both by the HA and NA, which may be solved by addition of oseltamivir. The NA agglutination is associated with viral passage in cell cultures and is not a property of clinical isolates (116).

There are several other immune measurements that correlate with influenza virus protection, but without any agreed threshold. The microneutralisation (MN) assay is a more time-consuming alternative to the HI assay, measuring serum antibody titres able to prevent virus infection of mammalian cells. The MN assay is generally more sensitive than the HI assay, although the two often strongly correlated (117). IgG antibodies may also be measured by single radial haemolysis (SRH) or enzyme-linked immunosorbent assay (ELISA). ELISA can additionally quantify local and systemic IgA titres, which have been linked to reduction in duration of viral shedding (118). NA inhibiting antibodies correlate with protection, particularly associated with reduction of symptoms and viral shedding (119). Furthermore, non-neutralising antibodies confer protection through Fc-mediated functions, exemplified by HA stem antibodies which were found to protect against challenge with pandemic influenza A/H1N1 (120). T cell mediated responses, CD4+ and CD8+ T cells in adults (121, 122), and INFγ in children, have shown potential as independent correlates of protection (123).

1.6.2 SARS-CoV-2 correlates of protection

As most viruses with immunodominant glycoproteins, protection from SARS-CoV-2 infection show a clear correlation to nAbs which prevent viral attachment to host cells (124). Yet, binding antibodies against the S1 domain are even more highly correlated with protection (125). The evidence for a neutralising correlate was established by studies of reinfection with the Wuhan-strain. One study found 50% protective neutralisation level was equivalent to an *in vitro* neutralisation titre between 1:10 to 1:30 (126), whereas another study showed that individuals with neutralising titres above 20 were protected from reinfection (127). Lower titres were required to prevent severe infection. However, the relationship between nAbs and protection against infection has become complicated by the emergence of new variants, especially omicron. While studies have found a linear relationship between higher antibody levels and reduced risk of delta infection, a recent study found no difference in rates of omicron BA.1/2 reinfections compared to binding spike Wuhan IgG titres (128). However, omicron-specific antibodies were not measured in the present study, which might still correlate with protection. Despite frequent SARS-CoV-2 reinfections by the omicron variants, the incidence of severe disease has not increased. There might therefore exist separate correlates for protection against mucosal infection, and another correlate against severe or systemic disease (129).

Similarly to influenza, SARS-CoV-2 specific nAbs can be measured using pseudotype or live viruses. Although pseudotype viruses merely require biosafety level 2 (BSL-2), the method is costly, time-consuming, and not readily available globally. Furthermore, working with live SARS-CoV-2 requires BSL-3 facilities. We utilised an hemagglutination-based test that was shown to highly correlated with nAbs against Wuhan and other VOC. By serum titration, the antibody levels may be quantified without the requirement of expensive or specialised lab equipment and can be used in evaluating the need for booster doses essentially everywhere in the world (130, 131).

Besides the neutralising properties of antibodies, other immune mechanisms have been found to correlate with protection from COVID-19. Convalescent serum therapy has

highlighted the additional benefit of non-neutralising antibodies (125). FcR-effector functions such as opsonophagocytosis may mediate the protection from severe COVID-19 by rapid viral clearance (132). Mucosal immune responses are less extensively studied compared to serum immunity, although higher nasal fluid spike-specific IgA are correlate with milder disease and reduced risk of break-through infection (133-135). Both local and circulating memory T and B cells established from earlier infection confer protection from SARS-CoV-2. Multiple studies have found that early and potent T cells responses are associated with better clinical outcomes (98). Tfh cells providing help to B cells, correlate with antibody responses. Local tissue resident T cells generally contribute to protection from reinfections, and have been detected both in the upper and lower respiratory tract up to 10 months after infection (136, 137). An animal model found protection from SARS-CoV-2 infection by tissue-resident cells, in the absence of nAbs (138).

Finally, lab assays produce varying results based on the source of red blood cells or other laboratory reagents, protocols, and defined endpoints. The lack of standardisation of neutralising assays during the SARS-CoV-2 pandemic has challenged comparison between studies and poor reproducibility. Systematic protocols and use of international standard reagents are needed to reduce inter-laboratory variability. A SARS-CoV-2 correlate of protection would largely benefit future vaccine development and guide further immunisation recommendations.

1.7 Seasonal influenza vaccines

1.7.1 Historic vaccine development

Following the isolation of the influenza A virus in 1933, development of influenza vaccines started (139). Cultivation of influenza in embryonated eggs yielded high virus concentrations and facilitated a method for viral propagation. Although cultivation in cell culture was developed in the 1930s, egg propagation is to this day the most widely used method for producing seasonal influenza vaccines, constituting approximately 90% of all vaccines (140). The first influenza vaccine was a live-attenuated influenza

vaccine (LAIV) given intranasally, developed in Russia in 1936, but had several weaknesses, including side reactions and low effectiveness. A few years later, in 1942, the first bivalent inactivated influenza vaccine (IIV) was successfully tested in a clinical study and provided to the general public in 1945 (141). Until 1960, the IIV was composed of whole virion particles, before methods of splitting virus particles were implemented in the 1960s, reducing side reactions particularly in children. In the same decade, LAIV were cold-adapted, yielding temperature sensitive viruses that only replicated in the nasal cavity, with enhanced safety. Subunit HA and NA based influenza vaccines were developed in the 1970s, and recombinant influenza vaccines containing synthetically produced HAs was introduced in the 2000s (141). The European Committee for Medicinal Products for Human Use (CHMP) licensure criteria for new seasonal influenza vaccines are based on the HI correlate and require (I) a seroconversion rate 40%, or (II) seroprotection rate of 70%, or (III) a mean fold change >2.5 in adults (142).

The constant antigenic drift of influenza viruses led to the establishment of a global monitoring network for circulating influenza viruses, the World Health Organisation's (WHO) Global Influenza Surveillance and Response System (GISRS) in 1952 (143). By characterising viruses antigenically and genetically, as well as their spread and evolution, the network informs WHO biannually on candidate influenza viruses for seasonal influenza vaccines (February and September, for the northern and southern hemisphere, respectively). Initially, vaccines were trivalent, containing two influenza A subtypes (A/H1N1 and A/H3N2) and one B lineage. After the cocirculation of both influenza B lineages since 2002, quadrivalent vaccines were recommended from the 2012/13 influenza season. However, with the absence of B/Yamagata circulation, vaccines may again return to trivalent.

1.7.2 Current vaccines

Influenza vaccines primarily induce strain specific immunity, and the constant antigenic drift requires biannual vaccines updates. The rapid drift has compromised the

vaccine effectiveness (VE), especially for influenza A/H3N2 virus, often referred to as the "headache" virus. The A/H3N2 vaccine component has been updated twice as often as the A/H1N1 virus (29 updates compared to 15, respectively), to match circulating strains (144). Despite the frequent updates, VE ranges from 10-60%, with low effectiveness against A/H3N2 viruses in recent years (145). The low VE represents an urgent area for vaccine improvement, with attempts underway to develop a broadly protective influenza vaccine – termed universal influenza vaccine.

Immune responses induced by current IIVs are mainly antibody mediated and are measured in serum, predominately as IgG. The humoral response will primarily be directed against HA (146). Antibodies can be detected 2-6 days post-vaccination, peaking at 14-21 days, followed by gradual waning and a 50% reduction by 6 months (147). With the exception of children <4 years, induction of T cell responses after IIV are limited, and mainly attributed to follicular CD4+ T cells (148). Parental vaccination is not able to induce tissue resident B or T memory cells, but low transient amounts of mucosal secretory IgA may be detected in previously infected individuals. LAIV induces a more balanced humoral and cellular response with stimulation of both subsets of CD4+ and CD8+ T cells, similarly to an influenza infection, by replicating in cells of the nasal cavity (149). Immune responses are detected both locally and systemically, preserving memory cells in the respiratory tract able to rapidly respond upon pathogen exposure. Consequently, LAIV has a high VE (up to 80%) in children <6 years old. To improve the immunogenicity of IIVs, adjuvants can be added to enhance reactivity by stimulating the innate immune system. Adjuvants are particularly convenient for use in IIVs for elderly to overcome immunosenescence or during pandemics for dose-sparing and enhancing immunity against a novel pathogen.

Most licenced influenza vaccines are egg-based, except one cell-culture subunit vaccine and the recombinant HA vaccine (150). In addition to the prolonged manufacturing time and demand/supply issues, egg manufacturing may introduce egg-adapted changes in the vaccine virus HAs (151). Immune responses can be directed towards these egg-adaptations, leading to reduced vaccine effectiveness. To what

extent the immune response is focused against egg-adapted epitopes varies with prior immunity, and thus differs across age groups (152). However, during the influenza seasons in 2016/17 with an antigenically matched A/H3N2 vaccine virus, the reduced VE was linked to egg-adaptations in the vaccine virus compared to the circulating strain (71). There was a significant advantage of cell-based vaccines in the 2017-18 season, although age was the primary driver of varied VE in the 2018-19 season (153). This suggest that VE is complicated by currently unmeasured factors such as previous influenza exposure.

Risk for severe disease listed in **Table 1** are recommended annual influenza vaccines in addition to health care workers, household contacts of immunocompromised and pig farmers. Vaccination policies for children vary by different countries. Healthy children are not recommended influenza vaccination in Norway, despite children having prolonged viral shedding and representing the main transmitters of influenza due to the lack of pre-existing immunity. The split and subunit inactivated influenza vaccines are approved for people ≥6 months, recombinant vaccines for adults 18-49 years and LAIV for children and adolescents 2-17 years in Europe (2-49 years in the US) (154). Generally, healthy adults respond best to influenza vaccines, while the youngest and oldest have poorer responses, thus reduced VE and the highest influenza-associated morbidity. Children ≤9 years old who have not been previously vaccinated are offered two doses due to their immature immune system. Older adults ≥65 years are offered vaccines with a higher antigen content, or influenza vaccines with adjuvants such as MF59 to increase immunogenicity.

1.7.3 Next-generation influenza vaccines

There is an urgent need for improved influenza VE, especially for A/H3N2 viruses. The focus has been to move away from the rapidly mutating and immunodominant globular head of the HA to more conserved regions such as the stem HA (chimeric or headless HAs, HA nanoparticles), and other viral proteins (NA, M2 ectodomain, M1 and NP) (155). Requirements for universal vaccines are high, generally stating that

they should induce (**I**) cross-protection against all influenza A subtypes, (**II**) at least 70% VE (**III**) for all age groups and (**IV**) durable protection across multiple influenza seasons (156).

The implementation of several new influenza vaccine platforms, including nanoparticles, viral-vectors and nucleic acids, will be a major step towards reducing the vaccine manufacturing time and egg-adaptive mutations. Universal influenza vaccine candidates were already formulated in a mRNA-lipid nanoparticle format prior to the COVID-19 pandemic, with promising results in mice (157). Several influenza mRNA vaccine candidates are currently being tested in phase 3 trials (158). The main strategy is broader coverage by including multiple (or all 20) HA antigens, or both HA and NA antigens, or combination vaccine candidates containing multiple respiratory viruses such as influenza, SARS-CoV-2, and RSV. Recently, Arevalo *et al.* published results on mice immunised with a 20-valent HA-based influenza mRNA nanoparticle vaccine (159). One dose of the vaccine successfully induced strain-specific (homologous) antibodies against all 20 HA proteins, but two doses were needed to fully protect against a heterologous challenge. Moderna recently reported results from the phase 3 safety and immunogenicity trial of a quadrivalent influenza "mRNA-1010" vaccine in adults (160). While the influenza A subtypes met the criteria of seroconversions and geometric mean titre ratios, these requirements were not fulfilled for the influenza B lineages.

1.7.4 Repeated vaccination and vaccine effectiveness

Since the first article reporting reduced VE after repeated vaccination in 1979 (161), an increasing focus on whether annual influenza vaccination can negatively impact the immune responses has resulted in numerous studies (reviewed in (145, 162, 163)). Although epidemiological studies demonstrate that annual influenza vaccination is associated with better health outcome, results from serological studies do not always support this (164, 165). The underlying immune mechanisms associated with repeated influenza exposure have been troublesome to determine and have varied by prior

vaccination status, age and circulating influenza virus, suggesting a combination of influencing factors determined by exposure history. Moreover, the way in which we measure VE is flawed by not including these factors (166).

The easiest way to measure VE is by the retrospective cohort design named *test-negative design* (TND) (167). Prospective cohorts or randomised placebo-controlled trials (the latter measuring vaccine *efficacy*) are comparably very expensive and challenging to conduct. Briefly, TND recruit patients seeking medical care based on influenza-like illness and are tested for laboratory confirmation of infection. The prevalence of vaccination in the two groups (positive or negative test outcome) are compared. VE is defined as the reduction in hazard ratio (HR) or odds ratio (OR) of influenza infection:

$$VE = 1 - HR \qquad \text{or} \qquad VE = 1 - OR$$

The TND usually adjusts for age, sex, risk-status, time in the influenza season, and in/outpatient. However, the design often falsely claims to exclude bias from health-seeking behaviour, although it may be reduced. Moreover, TND does not take into account an individual's susceptibility or exposure risk to influenza, which influence vaccination decisions. In recent years, the TND has been accused of causal inference, questioning results that associate vaccination with increased susceptibility to influenza (166). Perhaps the most fundamental flaw of the TND is that it does not consider pre-existing immunity.

Pre-existing immunity may interfere with VE, but does not necessarily arise from the original *infection* (original antigenic sin, OAS) but can be a result of the previous influenza seasonal *vaccination*. Moreover, memory responses are induced at the *expense* of responses to the new antigen. Perhaps the most accredited theory explaining the heterogeneity of repeated influenza vaccination is *negative interference* by antigenic distance (168). In this hypothesis, VE against the epidemic strain is based on both the antigenic distance between the current vaccine and the previous season vaccine strain, and between the epidemic virus. Here, pre-existing antibodies from a previous vaccination that recognise common epitopes on a new circulating strain will

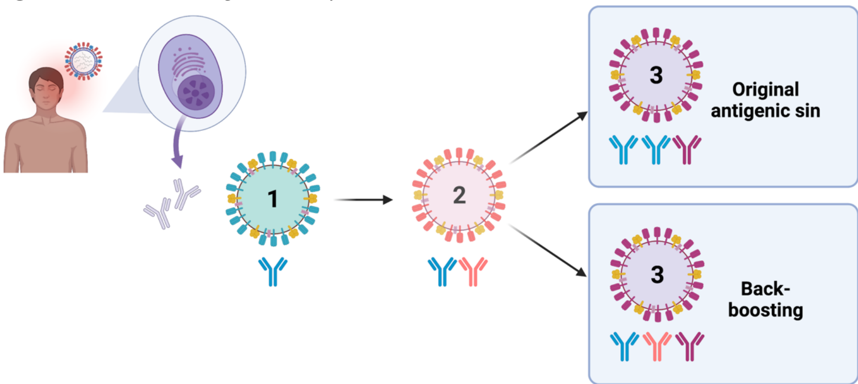
bind and mask these epitopes, thereby inhibiting antibody responses, *epitope masking* (169). Moreover, pre-existing antibodies will determine the amount of boosting, with a lower fold-increase when pre-existing titres are higher, the "ceiling effect".

To further complicate matters, certain birth cohorts have been linked to variable VE against A/H1N1 over time (170, 171). In order to estimate the most accurate VE, considering past exposure, both previous vaccinations and infections would be ideal. The effect of pre-existing immunity was recognised by the WHO in 2017, when human sera extended their vaccine antigenicity measurements beyond only ferret sera.

1.8 Pre-existing immunity - the original antigenic sin?

How pre-existing immunity shapes and directs the antibody response to a new influenza antigen has been a revisited subject since it was first introduced in the 1950s (172). Most people will experience their first influenza infection in childhood within their first 6 years of life, with highest attack rates around 3-4 years old (173, 174). Upon this initial influenza infection, influenza-specific antibodies are produced towards epitopes on antigens of the surface glycoprotein (**Figure 10**). Upon secondary exposure with the identical virus, memory immune responses will ensure a rapid and effective clearance, inhibiting reinfection. However, the constant antigenic drift of influenza ensures an everchanging antigen. Despite their elusive nature, antibody responses to influenza viruses are strongly driven by memory. "The doctrine of original antigenic sin" (OAS) state that secondary exposure to a drifted influenza A/H1N1 virus (either by natural infection or vaccination) will recall antibodies that preferably interact with common conserved epitopes (175) (**Figure 10**). The "sin" refers to the imprint from the initial infection that will dominate the antibody landscape of a birth cohort throughout life. Contrary to later interpretation, OAS did not dimmish responses to contemporary strains, but induced a strong anamnestic antigen-specific response to previously encountered influenza A/H1N1 that could be boosted with later monovalent A/H1N1 vaccinations (176). It was even suggested that the sin could be turned to a blessing by a priming vaccination (175).

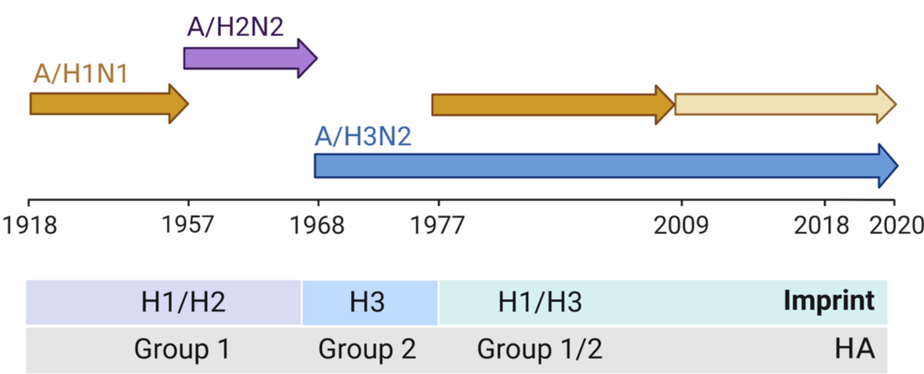
Figure 10: Pre-existing immunity to influenza



The immune system generates antibodies directed against the primary infecting influenza virus (1). Upon the second influenza encounter with a drifted virus (2), memory responses will ensure antibodies are directed both against virus 1 and virus 2. In the case of influenza exposure, there are several theories about the combination of *de novo* and memory responses, and the focus of cross-reactive antibody responses (3). The theory of original antigenic sin states that a current day infection will boost the antibodies mainly against the first or primary infecting virus. The back-boosting theory claims that antibodies can be back-boosted to all previously encountered influenza viruses of the same subtype. The illustration was created with BioRender.

The unique age-distribution of disease during the 2009 A/H1N1 pandemic reignited interest for OAS due to the protection from severe disease in A/H1N1 imprinted older individuals. The term "antigenic seniority" extended OAS to A/H3N2 viruses in 2012, suggesting that repeated influenza exposure would result in highest antibody titres against the more "senior" or earlier virus encountered in life in a form of hierarchy (177). The term "back-boosting" was introduced in 2014, demonstrating that a current vaccination or infection boosted antibodies against multiple previously circulating influenza A/H3N2 strains (107) (**Figure 10**). Vaccine-induced back-boosting beyond merely the original infecting strain was already documented in different birth cohorts by Davenport *et al.*, although the highest titres were observed to newer and contemporary viruses. Furthermore, a pre-emptive vaccine update with an advanced strain could back-boost antibodies in previously exposed subjects. Focusing on the clinical implications of an imprinting effect, Gostic and colleagues found protection from avian viruses belonging to the same HA group as the imprinting group (178) (**Figure 11**).

Figure 11: Influenza A priming patterns by birth year



Influenza A viruses have confirmed circulation since 1918, with the emergence of the Spanish flu (A/H1N1). In 1957, the A/H1N1 subtype was replaced by the new A/H2N2 virus, belonging to the same HA group 1. The Hong Kong pandemic in 1968 introduced the HA group 2 with the A/H3N2 subtype. The A/H1N1 subtype was reintroduced in 1977, and both HA groups have co-circulated ever since. Depending on birth year, and individual will identify with a specific birth cohort, with a unique priming pattern of either HA group 1 or 2. The illustration was created with BioRender.

Although the original formulation of OAS did not indicate any reduction in antibodies to contemporary viruses, some studies have suggested that the memory responses suppress antibody responses to new viral strains. However, pre-existing immunity is generally regarded as an advantage in lowering infection susceptibility, although in certain cases it might represent a cost. For example, in certain influenza seasons, some individuals have a disadvantageous priming pattern and higher infection rates. This was the case during the 2013-14 influenza season when middle aged individuals were highly susceptible to severe influenza disease due to a drifted circulating A/H1N1, which contained the mutated K166Q HA epitope (179). Although pre-existing immunity had protected them during the 2009-pandemic, it had cost them the opportunity to induce *de novo* responses to other A/H1N1 epitopes present in the drifted virus.

The immune mechanism explaining the intricate immunological imprint is the infection-induced memory responses that are elicited to common epitopes shared between previous and contemporary influenza viruses. Simultaneously, naïve B cells directed against new viral epitopes compete with the higher affinity B cells that possess a lower activation threshold. MBCs may undergo affinity maturation to increase their

affinity against new viral strains. Multiple exposures throughout life will lead to multiple opportunities for the B cells to evolve, gradually increasing the pool of cross-reactive antibodies (180).

1.9 SARS-CoV-2: the unexpected pandemic

1.9.1 The emergence of SARS-CoV-2 and the COVID-19 pandemic

Until 2020, influenza A was regarded as the greatest pandemic threat. No other pathogen had been as carefully monitored through global surveillance. At the top of the list of feared pandemic candidates was the highly pathogenic bird flu, that continues to pose a significant threat with widespread infection in mammals. In addition to Ebola, Zika and Nipah viruses among others, SARS-CoV and MERS-CoV were listed with potential to cause a public health emergency in 2018 (181). Moreover, in a report from 2019, coronaviruses were included in the list of viral groups with pandemic potential (182). Despite the awareness and warnings at the time, the world was unprepared for the emergence of SARS-CoV-2 in late 2019, reminiscent of previous pandemics.

The first reports of pneumonia of unknown aetiology originated in December 2019, all cases were connected to a seafood market in Wuhan, China. It was soon established that the disease showed human-to-human transmission, and that spread was possible in the asymptomatic or pre-symptomatic phase (183, 184). Thirty-two percent of the first described COVID-19 patients (n=41) needed intensive care unit treatment and 15% died, spreading fear around the globe (185). The genome sequence of the disease-causing agent; a new coronavirus was made publicly available on January 10th, 2020. By the end of January, the WHO declared an outbreak of public health emergency of international concern, with confirmed cases in 18 countries outside China (186). The first lock-down was initiated in Wuhan and the Hubei province from January 23rd, 2020, affecting millions of people for 76 days. Other countries around the world prepared their disease outbreak responses. The pandemic was officially declared on March 11th, and six days later all countries in Europe had confirmed COVID-19 cases.

The importance of a rapid pandemic response was catastrophically illustrated by the huge outbreak that affected the Lombardy region in Northern-Italy with over-filled hospitals, shortage of ventilators and high death rates (187). The strategy of "flattening the curve" was communicated in many countries to spare the health care systems and delay the viral spread to allow for vaccine and antiviral development. The vaccine race was already well established with the first vaccine phase I clinical trial initiated March 16th, 2020 (185). By the end of the first pandemic year, approved COVID-19 vaccines presented high vaccine efficacy, rendering new hope for the containment of the pandemic. With the approaching vaccine distribution, WHO called for vaccine equity with initiatives such as COVAX, aiming to guarantee vaccine access across the globe (188). However, vaccines were first and foremost deployed in high-income countries, despite the notion that "no one is safe, until everyone is safe".

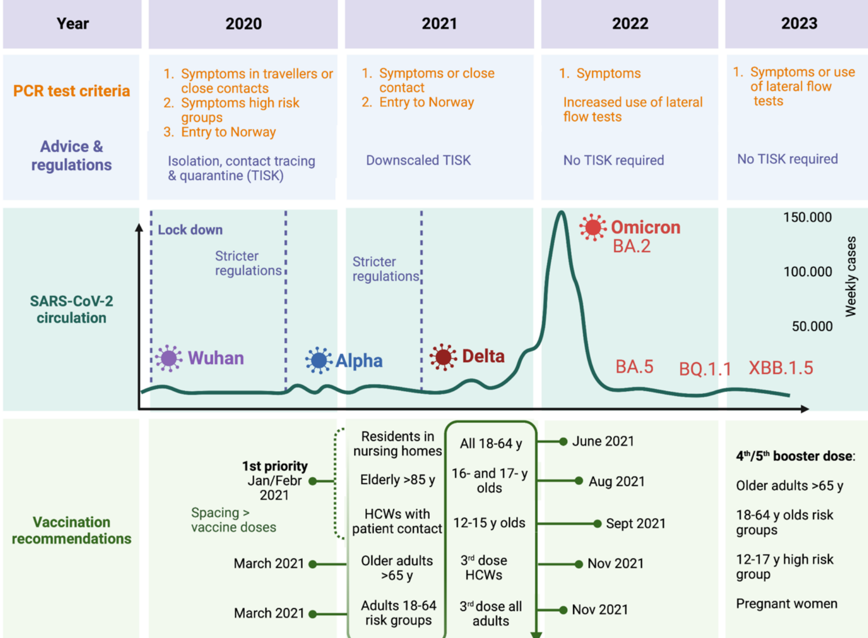
With the devastating impact the pandemic has had on society at large, with economic crises, drained health care systems, and millions affected by mental and physical disease, the numbers of infections and hospitalisations have eventually decreased. On May 5th, 2023, the WHO declared that COVID-19 no longer represents a public health emergency of international concern. However, globally many ten thousand continue to succumb to COVID-19 every day.

1.9.2 The COVID-19 pandemic in Norway

The first SARS-CoV-2 infected individual was registered in Norway February 26th, 2020, quickly passing 100 infections by March 6th. Norway introduced comprehensive infection control measures 12th March 2020, including closure of schools and national borders, right before the first pandemic wave (189) (**Figure 12**). About one month later, the Norwegian Institute of Public Health (NIPH) declared the outbreak under control, and a gradual reopening was initiated. The emergence of VOC reinforced infection control measures at regular intervals, with outbreaks concentrated in highly populated areas, primarily Oslo, but also Bergen. At the start of the pandemic, Norway had the greatest number of people tested per inhabitant in the world (190). Testing, isolation,

contact tracing and quarantine were conducted until September 2021, with increasing use of lateral flow tests, especially from January 2022. Overall, COVID-19 mortality has been low in Norway, without excess deaths the first 18 months of the pandemic (191).

Figure 12: COVID-19 pandemic in Norway



The PCR-criteria, advice and regulations, SARS-CoV-2 circulation, and vaccine recommendations in Norway during the COVID-19 pandemic, 2020-2023 (192).

1.9.3 COVID-19 vaccines

Unparalleled by any previous historic vaccine development, COVID-19 vaccines were developed and distributed at an unprecedented speed through scientific collaborations, largely helped by government funding. In a record-breaking two months after the viral sequence was available, the first vaccine phase I vaccine clinical trial was initiated by Moderna. While the vaccines have saved millions of lives, a few vaccinees have suffered rare, yet severe side effects from some vaccine candidates. The ChAdOx1-S vaccine was quickly suspended from the Norwegian Coronavirus Immunisation Programme due to the rare but severe haematological side effects (193). The increased

risk of myocarditis and pericarditis in young males after the mRNA-1237 vaccine consequently led to its contraindication in this age group (194). Overall, vaccine immunology has taken giant leaps with many lessons learned along the way. However, COVID-19 vaccines largely benefited from considerable previous research on other coronavirus vaccine candidates. The knowledge of the S-protein's immunodominance, and the introduction of two mutations that yielded a stabilised S-structure paved the way for the successful accelerated vaccine composition (195, 196).

Production of traditional vaccines is slow (>6 months), which is disadvantageous in a pandemic setting. However, rapid manufacturing platforms such as mRNA and adenoviral vector vaccines were widely utilised for the first time during the COVID-19 pandemic. The vaccines that were granted emergency use and distributed in early 2021 included the mRNA vaccines encoding the S-protein (mRNA-1273 and BNT162b2) and the adenoviral vector vaccines (197). There are multiple other vaccine-candidates, such as the Novavax recombinant protein and adjuvanted vaccine and inactivated virus vaccines (**Figure 13**). However, the mRNA vaccines, and especially the BNT162b2 vaccine, was the most widely distributed vaccine in Norway and the focus of this thesis will therefore be on this vaccine platform. mRNA vaccines are composed of a lipid nanoparticle carrying mRNA encoding the S protein (198). Upon administration, the lipid membrane fuse with the host cell and releases the mRNA to the cytoplasm for direct translation. Synthetic vaccine mRNA is recognised by host PAMPs and induces robust immune responses without requiring adjuvants. The mRNA is quickly degraded, but the translated viral antigen may persist for several weeks, ensuring prolonged stimuli.

High-risk groups, such as the elderly, residents of nursing homes, people with chronic diseases and front-line health care workers were prioritised for vaccination. The first COVID-19 vaccine recipient in Norway was a nursing home resident on the 27th of December 2020, followed by an extensive immunisation of >90% of the adult (>18 years) Norwegian population (199) (see **Table 2** for details).

Figure 13: Examples of different COVID-19 vaccine platforms.

Viral vector vaccines (ChAdOx1-S and Ad26.COV2.S), mRNA vaccines (BNT162b2 and mRNA-1273) and the subunit vaccine Nuvaxovid were the COVID-19 vaccines with initial marketing authorisations in Norway. However, the viral vector vaccines were suspended from the Coronavirus Immunisation Programme in Norway April 2021 due to the rare but severe risk of vaccine-induced thrombosis and thrombocytopenia. The illustration was inspired by Professor Jamie Triccas, The University of Sydney, and created in BioRender.

1.9.4 COVID-19 vaccine effectiveness

The initial licensing clinical trials for the mRNA vaccines showed very high efficacy in adults against symptomatic disease; 95% for BNT162b2 and 94% for mRNA-1273 (2 dose regimes), while the viral vector vaccines demonstrated efficacy around 70% (197). Real-world observations confirmed these data by demonstrating robust protection against infection, disease, hospitalisation and death as well as induction of high level of nAbs. As the mRNA vaccines became available for children and adolescents, a comparably high efficacy (>90%) was demonstrated in these age groups. However, older people >85 years old were not included in the initial COVID-19 vaccine trials (200). Furthermore, the age composition of clinical trial participants revealed barely 10% over the age of 65 and <2% over 75 years. Paradoxically, older adults are at highest risk for severe disease, with people over 60 years having at least five times higher risk of COVID-19 associated hospitalisation and death (201). Previous experience from influenza vaccines suggested that the COVID-19 vaccines might be less immunogenic in older individuals due to immunosenescence, or at least require multiple doses. In the months after vaccination, vaccine-induced antibodies and thereby vaccine effectiveness (VE) against infection waned. The VE was further compromised by spread of novel viral variants (202). While mRNA VE in adults

remained high against the alpha variant (90%), a reduced effectiveness was observed against the beta (79%), gamma (72%) and delta (83%) variants, and lowest against the omicron variants (56%) (203). Fewer studies have assessed the vaccine antibody responses and VE across variants in adolescents or elderly, but general findings imply the VE in younger age groups was comparable to adults, while older adults required multiple immunisations to achieve high antibody responses (204).

Fortunately, vaccine protection against severe disease and death has remained high, and monovalent mRNA booster doses have been able to improve cross-reactivity to omicron variants (205). Updated bivalent mRNA vaccines containing both the ancestral strain and different omicron subvariants (BA.1 or BA.4/5) are now available, although only a modest superior protection against omicron subvariants were achieved compared to the monovalent vaccines (206). Furthermore, the BA.1 and BA. 4/5 variants were quickly replaced by other omicron subvariants. The updated vaccine composition recommendation was monovalent and included the XBB.1.5 subvariant. Boosters were primarily advised for high risk groups, such as elderly and health care workers (HCWs).

1.9.5 SARS-CoV-2 immune responses

The following section will cover specific aspects regarding SARS-CoV-2 immune responses after vaccination, infection or the combination of the two, known as hybrid immunity, relevant to this thesis.

Antibody mediated protection

The SARS-CoV-2 specific B cell response after infection can be heterogenous, with interindividual variance in peak antibody titres. This contrasts with the robust and more homogenous mRNA vaccine-response observed in healthy adults (207). Vaccines primarily induce spike and RBD-specific IgG antibodies, most of which are nAbs. The antibodies peak at 3 weeks post-vaccination followed by a decline. Vaccine elicited antibodies wane more rapidly compared to those induced by infection, as a result of

fewer long-lived plasma cells maintaining a stable antibody titre (208). MBCs will conversely increase to a steady level in the months following infection or vaccination. MBCs are capable of being remarkably long-lived, and thus far SARS-CoV-2-specific MBCs are detected at least 15 months after infection, with increasing somatic hypermutations in the immunoglobulin genes up to 12 months (90). Similarly high levels of affinity matured MBCs are found after vaccination, although with reduced affinity maturation.

Higher serum antibody titres and B_{Mem} cells have been detected in subjects with severe disease, whereas lower antibody concentrations were measured in individuals with milder and asymptomatic infections (207). The high antibody levels are likely mediated by extrafollicular B cell responses, by evidence of dysfunctional GC responses with low levels of Tfh cells and B cell somatic hypermutations in individuals with severe outcome. Simultaneously, impaired, and delayed T cell responses are observed in connection to critical disease. Mild infections are characterised by both robust extrafollicular B cell responses and GC formation. The GC responses may be ongoing for several months by prolonged antigen presence in draining lymph nodes and intestine. As an example, cross-reactivity to VOC were increased in the months following infection and vaccination and improved after booster doses. An increased spacing between the primary and secondary mRNA doses was found advantageous, which improved GC formation (90). However, persistent antibody responses following COVID-19, perhaps due to preserved viral antigen, have also been connected to long COVID or post COVID-19 condition.

NAbs have been the main focus when measuring immunity against SARS-CoV-2. Most nAbs target the immunodominant spike RBD (~90%), although some are directed to the N terminal domain (NTD) (208). NAbs can be divided into different classes depending on their binding site (209). Antibodies targeting the RBD and consequently blocking ACE2 binding are grouped in classes 1 and 2. Class 3 includes antibodies binding the RBD without blocking the binding to ACE2, and nAbs that bind

outside the RBD belong to class 4. As previously covered, antibody Fc effector functions have an essential role in disease attenuation.

T cell mediated protection

T cell responses of both subsets are important contributions to both COVID-19 vaccination and infection responses, although CD4+ T cells are more readily detected compared to CD8+ T cells (98). While nAbs play a central role during the early phase of infection, T cells are able to effectively clear and kill infected cells once the infection is established. SARS-CoV-2 infection has a slow nature, readily escaping innate immune mechanisms with a prolonged incubation period and severe disease generally developing in the second week of infection. These factors further strengthen the role of T cells in resolving infections. Infection induced CD4+ T cells have Th1, Tfh and cytotoxic characteristics. Multiple studies have found early and potent CD4+ and CD8+ T cells responses associated with better clinical outcomes. The CD4+ Tfh subset improves the magnitude and quality of antibody responses, consistent with the correlation between the two. In contrast to CD4+ T cells and antibody responses, CD8+ T cells have not been further stimulated by booster vaccine doses. On the other hand, vaccine-induced T cell memory is maintained for at least 8 months, effectively recognising new viral variant which explain to the observed preservation of protection against severe disease (90). Similarly, T cell memory (especially CD4+ T cells) following infection show little waning, supported by evidence of persistent T cells up to 18 years after SARS-CoV-1 infection. Furthermore, pre-existing CD4+ T cells from HCoV-infections are linked to improved infection and vaccination responses. Similarly to persistent antibody responses, continued T cell responses have been associated with post COVID-19 condition.

Mucosal immunity

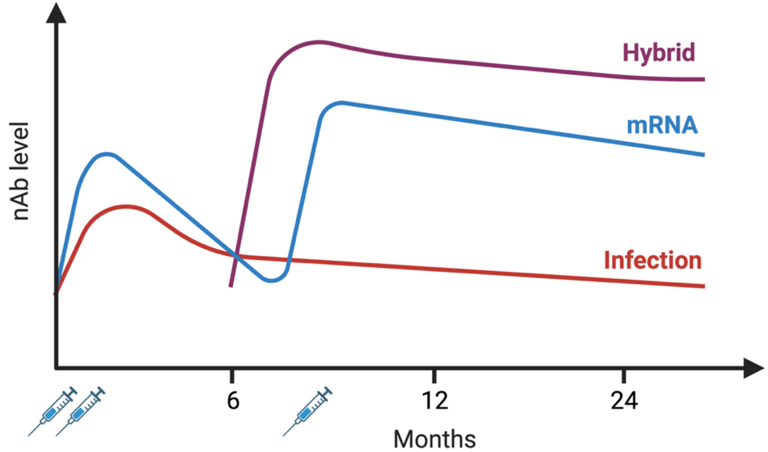
As expected, mucosal responses are superior after natural SARS-CoV-2 infection compared to intramuscular vaccination. Lung tissue resident T and B cells are induced following COVID-19 and higher nasal fluid spike-specific IgA have been found to correlate with milder disease (133). Although parental vaccines primarily stimulate

robust systemic responses, low level mucosal IgA and IgG have been detected after mRNA immunisation (134, 210). These IgA responses are associated with protection from infection, but it is unclear if the antibodies are locally produced or originate as a transudate from serum (135). To improve local mucosal immunity and preferably reduce infections and transmission, COVID-19 mucosal vaccine candidates are being developed (211). Four mucosal vaccines are approved for emergency use. Vaccine formats are predominantly based on viral vectors due to their natural mucosal tropism, although anti-vector immunity is a challenge for the viral vector vaccines with high seroprevalence in the community. Furthermore, a deeper understanding of the mucosal SARS-CoV-2 immune responses correlating with protection is needed for these vaccines to be successful.

Hybrid immunity

The extensive spread of SARS-CoV-2 in the community has resulted in nearly 80% of the worlds' population having previous SARS-CoV-2 infection or vaccination, or both. The combination of immunological memory by infection and vaccination is termed hybrid immunity. Vaccination followed by infection is sometimes referred to as break-through infection, but the order of the events is otherwise largely insignificant. However, it remains unclear whether vaccination is able to boost tissue resident T cells induced by infection, and if a primary vaccination followed by an infection will improve mucosal T cell responses. The rational for singling out this hybrid classification is the superiority in immune memory, especially in the magnitude and durability of antibody responses (90) (**Figure 14**). Also characteristic for this type of hybrid immunity is the breadth of nAbs, with some antibodies being able to neutralise different VOC including omicron variants, and even SARS-CoV-1. Recall MBCs and CD4+ T cells induced by either previous infection or vaccination are facilitating these antibody responses. Not just the systemic responses, but also the local IgG and IgA immune responses in the upper airways are higher and more durable (212).

Figure 14: SARS-CoV-2 neutralising antibody levels by infection, mRNA vaccination and hybrid immunity

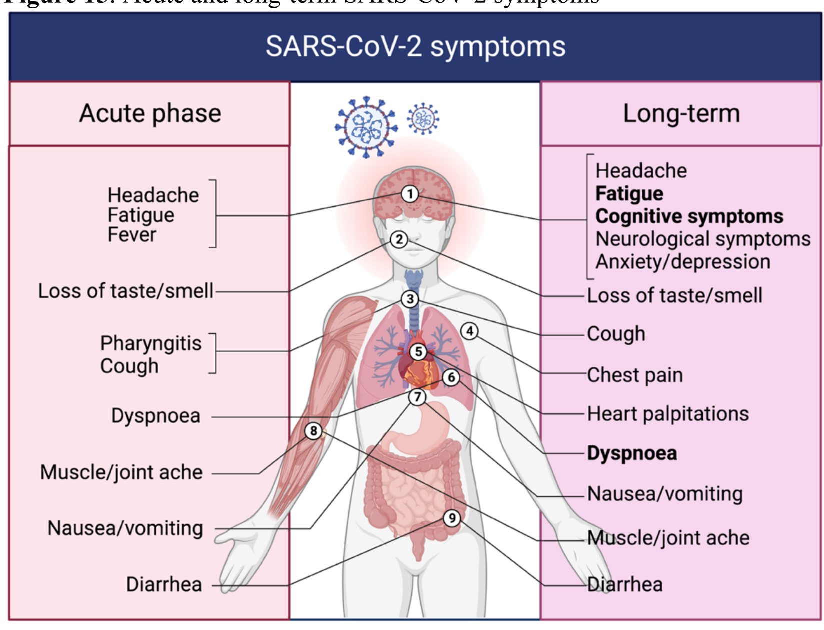


Different dynamics of SARS-CoV-2-specific neutralising antibodies (nAbs) after infection, 2 doses of mRNA vaccination followed by a booster dose and hybrid immunity (infection followed by vaccination). Figure inspired by Sette *et al.* (90).

1.9.6 Post COVID-19 condition

The first clinical reports on persisting symptoms after SARS-CoV-2 infection surfaced early spring of 2020. Patients complained to their doctors about persisting symptoms across many organ systems, such as continuous fatigue, “brain fog” that resulted in concentration and memory problems, headache, and dyspnoea several months post-infection (213) (**Figure 15**). While critically ill patients admitted to the ICU commonly experience post-intensive care syndrome, the majority of people with persisting COVID-19 symptoms experienced mild acute illness (214). The condition affects 10-20% of SARS-CoV-2 infected individuals of all ages, but predominately those between 36-50 years old. Certain risk factors have been identified, for example female sex, specific comorbidities, and lack of rest after acute disease. The ensemble of symptoms has been named long COVID or post COVID-19 condition and are recognised as a severe threat to public health with at least 65 million sufferers affected world-wide.
The three symptoms highlighted by the WHO are fatigue, dyspnoea, and cognitive dysfunction.

Figure 15: Acute and long-term SARS-CoV-2 symptoms



Loss of taste and smell is typically associated with SARS-CoV-2 variants pre-omicron.

The adult post COVID-19 definition requires a previous SARS-CoV-2 infection, the persistence, or newly developed symptoms at least 3 months after the diagnosis and a duration of at least 2 months, although symptoms may fluctuate. Differential diagnosis should be ruled out, and the symptoms' impact on daily life considered. The newly formulated definition for post COVID-19 condition in children contains the same criteria, although a wider range of symptoms may be considered (215).

Post-viral fatigue syndrome is not unique and equivalent symptoms have been observed after other pandemics and epidemics. Coinciding with the 1918 pandemic, a surge of what became known as encephalitis lethargica, with an acute and chronic phase describing influenza-like symptoms followed by an increased need for sleep, confusion and fatigue was described (216). In Norway, the 2009 influenza pandemic was associated with a 2-fold increase in chronic fatigue syndrome (217). Similar findings of prolonged fatigue up to 39 months after SARS-CoV or MERS-CoV infections were reported in a meta-analysis (218). Furthermore, other infections, such as Epstein Barr, *Coxiella burnetiid* (causing Q fever), West Nile virus and Giardia lamblia can cause long-term symptoms and have been associated with the onset of chronic fatigue

syndrome. These historic events demonstrate the ability of infectious pathogens to trigger prolonged symptoms beyond the acute phase of the illness.

The pathogenesis of post COVID-19 condition is controversial, with many differing causal and overlapping explanations. Some of the suggested underlying causes are dysregulations in (**I**) the immune system (including autoimmunity), (**II**) the microbiota, (**III**) endothelia and (**IV**) in neurological signalling (214). Although no specific treatment is currently available, acute COVID-19 antiviral therapy with Paxlovid resulted in a 25% reduction of post COVID-19 condition (219). COVID-19 vaccination has also been associated with reduction of long-term symptoms, although more research is needed. Furthermore, knowledge regarding the impact of novel variants, reinfections and prevalence in vulnerable and understudied groups like children and adolescents is largely lacking.

The last literature search was performed in June 2023.

2. Aim and objectives

The aim of this thesis was to characterise age-specific immune responses towards epidemic and pandemic respiratory viruses.

Primary objective:

The primary objective was to investigate antibody responses in different age groups after influenza or COVID-19 vaccination and/or infection.

Secondary objectives:

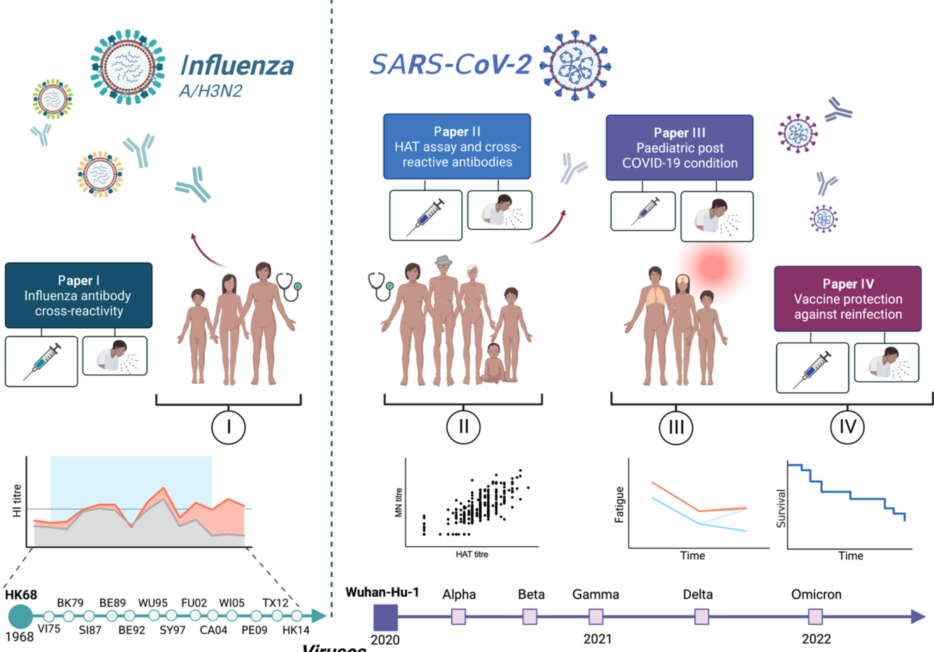
- Characterise the impact of imprinting and antibody breadth after live-attenuated or inactivated influenza A/H3N2 vaccination and infection in children and adults (**paper I**)
- Compare SARS-CoV-2 infection and mRNA vaccine elicited antibody responses to variants in younger and older adults using a novel surrogate neutralisation assay (HAT) (**paper II**)
- Evaluate clinical and immunological risk factors for post COVID-19 condition after delta infection in children and adolescents (**paper III**)
- Investigate vaccine protection against break-through omicron BA.1/2 infection, in previously delta infected children and adolescents (**paper IV**)

3. Methods

3.1 Overview of study populations and study design

This thesis includes four papers studying antibody responses to either influenza A/H3N2 (**paper I**) or SARS-CoV-2 (**papers II-IV**) (**Figure 16**). The studies were based on eight different cohorts across the ages 1-99 years. **Paper I** included children (3-17 years) immunised with the live-attenuated influenza vaccine (LAIV) and young adults (21-61 years) vaccinated with the inactivated influenza vaccine (IIV) or A/H3N2 infected. **Paper II** compared two cohorts of younger (23-77 years) and older COVID-19 vaccinees (80-99 years), and a cohort of SARS-CoV-2 Wuhan infected cases (1-89 years). The final cohort was delta variant SARS-CoV-2 infected children (10-15 years) and adolescents (16-20 years) (**papers III**, **IV**).

Figure 16: Overview of the four papers included in the thesis



Paper I investigated antibody influenza A/H3N2 responses in children and younger adults, who were health care workers (HCWs), symbolised by a stethoscope. Papers II-IV studied SARS-CoV-2 antibody responses of different age cohorts. All studies evaluate both infection and vaccination responses, with the larger symbols "vaccine" or "infected person" indicating the main focus in each study. A graph of the main findings from each paper and timelines with the viruses included in the studies are shown at the bottom.

All papers include study participants recruited in Bergen, Norway, either from Bergen Municipality (**papers II-IV**) or Haukeland University Hospital (**papers I**, **II**) (**Table 2**). To confirm the correlation of HAT and neutralisation with VOC in **paper II**, additional serum samples were provided by the UK co-authors from SARS-CoV-2 convalescents or vaccinees. Influenza infection was defined by seroconversion (**paper I**). Wuhan, delta and omicron BA.1/2 infections were detected by RT-PCR of nasopharyngeal oral swabs. Alternatively, Wuhan infections were confirmed by the presence of serum antibodies (anti-spike IgG) and clinical symptoms (**paper II**), and omicron BA.1/2 infections were confirmed by lateral flow tests (**paper III-IV**).

Table 2: Overview of cohorts in papers I-IV

Virus	Cohort	Age (years)	Paper(s)	No. (n)	Study period	Ethical number	Infection or vaccine	Site of recruitment
Influenza A/H3N2	Adults	21-61	I	42	2010-14	2009/1224 2012/1772	IIV[1] & infection	Haukeland[2]
	Children	3-17	I	42	2012-13	2012/1088	LAIV[3]	Haukeland
SARS-CoV-2	Adults	22-77	II	316	2021	218629	BNT162b2[4]	Haukeland
	Elderly	80-99	II	96	2021	218629	BNT162b2	Eidsvåg GP[5]
	Children to elderly	1-89	II	307	2020	118664	Infection	Bergen, EC[6]
	Adults[7]	16-65	II	420	2020	BSCR20047 BSCR20051	Infection	UK[8]
	Adults[9]	16-65	II	124	2020-21	GI Biobank 16/YH/0247	Infection & BNT162b2	UK
	Children & adolescents	10-20	II-IV	276	2021-22	118664	Infection & mRNA[10]	Bergen, EC

[1]Inactivated influenza vaccine (IIV), [2]Haukeland University Hospital, Bergen, Norway, [3]Children <9 years received two vaccine doses live-attenuated influenza vaccine (LAIV) at 28 days interval, [4]Vaccinees received two doses BNT162b2 at 28 days interval, [5]Eidsvåg General Practice, [6]Bergen Municipality Emergency Clinic, [7]Included in correlation analysis, [8]United Kingdom, [9]Included in correlation of variants and finger prick test [10]103 vaccinees, n=97 one dose BNT162b2 (except n=6 mRNA-1273), n=6 two doses

From a larger cohort of HCWs (147), appropriate subjects with a single IIV in 2010 or 2012, or both seasons, and infected subjects with A/H3N2 seroconversion were included (**paper I**). The children included had participated in a LAIV trial, in either

2012 or 2013, only a few non-responders were excluded (220). Influenza infected individuals were sampled once a year from 2010-2014, and SARS-CoV-2 infected were sampled at 3-10 weeks post Wuhan infection and at 3 and/or 8 months post delta infection. All recruited vaccinees provided blood samples on the day of each vaccination and 21/28 days post-vaccination. Influenza vaccinees provided additional serum samples 6 and 12 months post-vaccination, and five individuals provided long-term samples at 36 and 48 months.

3.1.1 Vaccines

The trivalent IIVs (2010-14) were either subunit (Influvac, Abbott Laboratories) or split-virion (Vaxigrip, Sanofi Pasteur) containing 15 μg HA per strain (**paper I**). Children received the trivalent LAIV (2012-13) (FLUENZ, AstraZeneca), containing 10^7 fluorescent focus units (FFU) of each strain. The A/H3N2 vaccine viruses changed from A/Perth/16/2009 in the 2010-11 influenza season to A/Victoria/361/2011 in seasons 2012-13 and 2013-14.

COVID-19 vaccinees received the BNT162b2 mRNA vaccine (Comirnaty, Pfizer), although six adolescents included in **papers III**, **IV** received the mRNA-1273 vaccine (Moderna Inc). The BNT162b2 mRNA and mRNA-1273 both contain purified single-stranded, 5′-capped mRNA, encoding the spike (S) protein from the Wuhan-Hu-1 strain, although with a lower mRNA content of 30 μg in the BNT162b2 compared to 100 μg in the mRNA-1273 vaccine.

3.2 Ethical considerations

The studies included in this thesis were performed according to the Declaration of Helsinki (2008) and the principles of Good Clinical Practice. All included study participants provided written or digital informed consent prior to inclusion. The studies conducted in Bergen, Norway, were approved by the Western Norway Ethics committee (see **table 2** for ethical numbers). No monetary compensation was provided to study subjects. The UK samples for **paper II** were approved by the National Blood

Supply Committee for Audit, Research Ethics of National Health Service Blood and Transplant Research and Audit Committee, and the research ethics committee at Yorkshire & The Humber-Sheffield (**Table 2**).

Influenza study participants were provided oral and written information about the study in person. Due to the extraordinary pandemic situation with home-isolation of SARS-CoV-2 infected subjects with mild disease, all participants were contacted by telephone with study information from the municipality testing station and provided written informed consent prior to blood collection a few weeks later or digitally. For children <16 years parents or legal guardians consented, although children were actively informed. In the LAIV study, both parents or legal guardians and children from 12 years provided written consent before inclusion in the study. For the questionnaires of acute and post COVID-19 symptomology in **paper III**, children <16 years were actively involved in providing the information. A total of four delta infected children withdrew consent for further participation at the 3 months (n=1) and 8 months follow-up (n=3). The older adults (>80 years) included in **paper II** were home-dwelling and healthy for their age. None were diagnosed with dementia. Some older individuals had substantial hearing loss; therefore, extra care was taken during follow-up visits by planning enough time to ensure that all information was correctly understood.

Demographical information of study subjects was recorded on paper case report forms (CRFs) (**paper I**). For **papers II-IV,** demographical and clinical information was recorded in an electronic CRFs (RedCap©, Vanderbuilt, US) (**papers)**. Designated study personnel had access to CRFs. Further data analysis was conducted on de-identified data.

3.3 Laboratory assays

3.3.1 Blood samples

Blood samples were collected as part of all studies and labelled with a unique identification number, as well as date of sampling. In **papers I-IV**, 10 ml serum

samples were collected from all subjects, except blood volume from LAIV immunised children was by children's weight and collected as heparinised plasma. Blood was clotted for serum separation for at least 60 minutes in room temperature or overnight at 4°C before centrifugation at 2000 rpm at 4°C. The separated sera or plasma were aliquoted and frozen at -80°C. Prior to use in serological assays, the sera were thawed at room temperature or at -4°C. The serum samples used for the HAT assay were heat inactivated for 30-60 minutes at 56°C (**paper II-IV**). One volume of serum and plasma samples analysed in the HI assay were treated with four volumes of receptor destroying enzyme (RDE) (Denka Seiken, Japan) and incubated at 37°C overnight before heat inactivation for 30 minutes at 56°C (**paper I**). Serum and plasma samples were then preadsorped with turkey or guinea RBCs for 1 hour at 4°C before use in the HI assay.

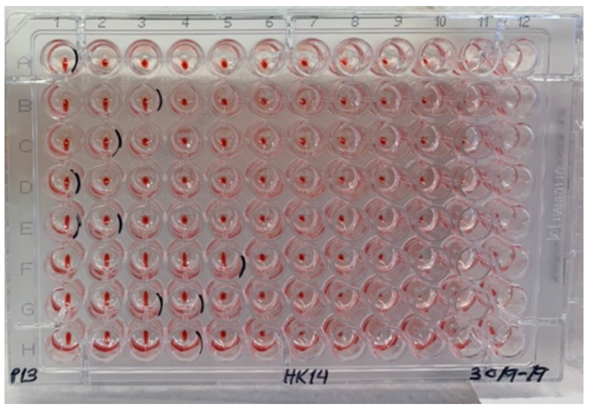
3.3.2 Hemagglutination inhibition assay (HI)

The hemagglutination inhibition assay (HI) was utilised to quantify influenza A/H3N2 specific antibodies and was performed as previously described (221). Starting from a 1:10 dilution, 25 µl RDE treated serum/plasma was added to 25 µl phosphate buffered saline (PBS), and two-fold serially diluted in a V-bottom 96-well microtiter plate (**Figure 18**). The plates were incubated with 25 µl of 4 hemagglutinating units (HAU) for one hour at room temperature, before 50 µl 0.5% (volume/volume) turkey or guinea pig RBCs in PBS was added for a 30 minute incubation. The HI titre was read as the reciprocal of the highest serum/plasma dilution inhibiting a complete hemagglutination (100%).

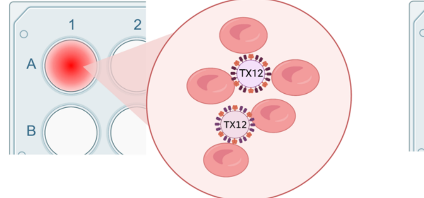
Thirteen inactivated influenza A/H3N2 virus antigens were obtained from the National Institute of Biological Standards and Controls (NIBSC), UK, and the International Reagent Recourses (IRR). The HK68 virus was egg-propagated in-house on a PR8 backbone (see table of viruses in **paper I**). Negative (human depleted Ig serum) and a panel of positive controls were included in every run, including H3 reference ferret/sheep sera, a human control, and a strain-specific control for some of the oldest viruses (BK79, BE92). Additionally, a serum control (serum incubated with only

RBCs) was run with every sample. All samples were tested in duplicate by independent replication, in which the same virus was titrated with two separate sources of RBCs. This was performed to ensure a more accurate comparison between different viruses. Negative HI titres (<10) were assigned a value of 5. A fourfold or higher HI titre was considered as seroconversion.

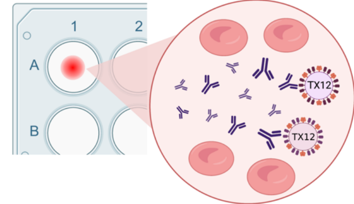
Figure 17: Hemagglutination inhibition assay readout.



Hemagglutination



Hemagglutination inhibition



The picture shows an example of an HI plate and the readout. The principle of hemagglutination and hemagglutination inhibition are shown below. When no influenza-specific antibodies are present, the virus will bind the red blood cells (RBCs) causing hemagglutination, visualised as dispersed blood cells. If the serum sample contains influenza HA-specific antibodies, the antibodies will bind the virus, inhibiting hemagglutination, and the RBCs will sink to the bottom of the well forming a button. The button will run when the plate is tilted.

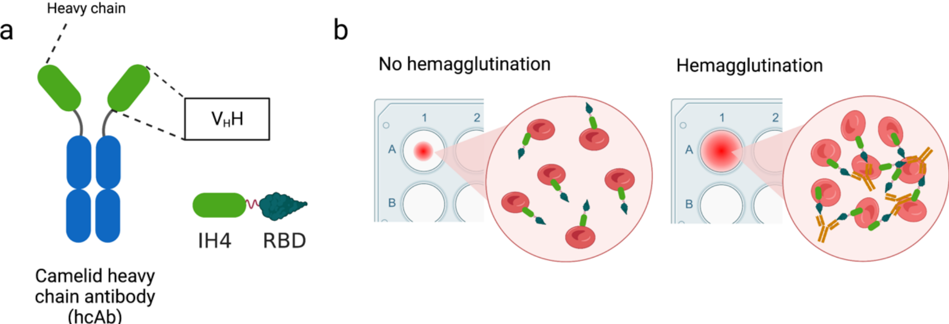
3.3.3 Hemagglutination test (HAT)

The hemagglutination test (HAT) was used to measure SARS-CoV-2 receptor binding domain (RBD)-specific antibodies (**papers II-IV**), as previously described (130). Since SARS-CoV-2 does not bind to sialic acids on red blood cells, a bi-specific fusion protein of two moieties was constructed. One moiety is made up of the viral antigen

(RBD) linked to a second moiety; an antibody specific for RBCs. Glycophorin A is highly expressed on the surface of RBCs, and heavy chain antibodies against this structure may be derived from camelids immunised by human blood transfusion. The variable domain of the heavy chain heavy antibody (V_HH) against glycophorin A, named IH4, is called a nanobody (222) (**Figure 18a**).

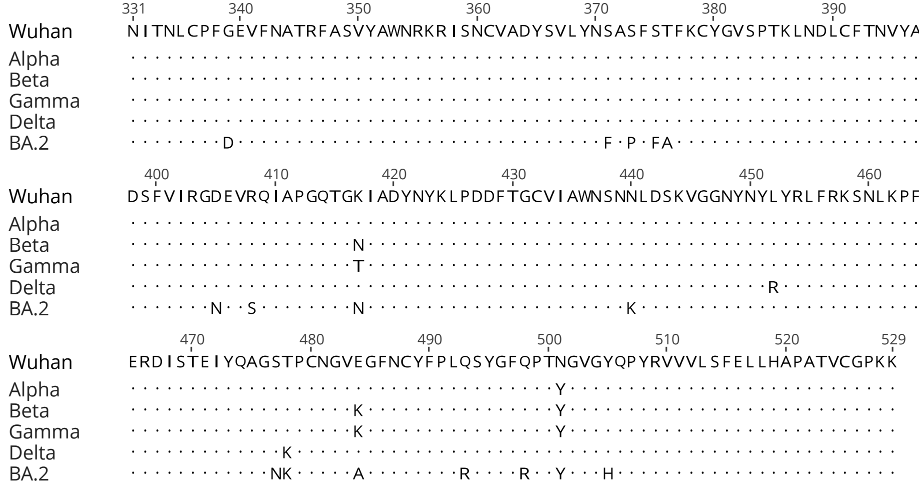
Reagents with the specific RBD amino acid sequences for the ancestral Wuhan virus, as well as the VOC alpha, beta, gamma, delta and omicron BA.2 were generated using codon-optimised IH4-RBD sequences (**Table 3**), expressed in Expi293F cells and purified by the c-terminal 6xHis tag by Ni-NTA chromatography. The RBD HAT reagents may cross-link RBCs in the presence of RBD-specific antibodies, as visualised by the hemagglutination reaction (**Figure 18b**). If no antibodies are present, the RBCs will not be able to cross-link, forming a button at the bottom of the well.

Figure 18: The principle of the hemagglutination test (HAT).



The camelid heavy chain antibody is composed of two heavy chains (**a**). The variable domain of the heavy chain antibody (V_HH) is the antigen binding domain. The V_HH domain IH4, specific for glycophorin A, is coupled to the receptor binding domain (RBD) of SARS-CoV-2 to make the HAT reagent (**b**). When this HAT reagent is mixed with O negative donor red blood cells and patient serum containing RBD-specific antibodies, the hemagglutination reaction can be visualised in a 96-well V-bottom plate The illustration was created with BioRender.

Table 3: Receptor binding domain (RBD) sequence alignment for the HAT reagents



HAT screening

RBCs from a O negative human donor in EDTA were washed in PBS, and a 1.0% RBC solution was prepared. Sera were pre-screened at a dilution of 1:40 in PBS with a 1.0% RBC solution in a 96-well microtiter and added 2.5 µg/ml IH4-RBD. Plates were incubated for 1 hour in room temperature before tilting to allow the RBCs in the control wells (RBCs without IH4-RBD) to reach the bottom (at least 20 seconds). No teardrop formation was defined as a positive screening, whereas a partial or complete teardrop were scored as negative and assigned a value of 5. All plates were run in duplicates.

HAT titration

Titration was performed on duplicate plates directly. Sera were double diluted from a starting dilution of 1:40. A 1.0% RBC solution containing 2.5 µg/ml IH4-RBD was prepared and added to the wells and incubated for 1 hour. Control wells had RBCs without IH4-RBD. The last well without teardrop formation is defined as the HAT titre. Negative controls (PBS) and a positive control (monoclonal antibody EY-6A (223)) were included in each run. All VOC RBD share the conserved epitope recognised by the positive control, and the IH4-RBD reagents for each VOC were standardised by agglutination of RBCs occurring at the same endpoint dilution (~16 ng/well).

HAT fingerprick

Both HAT screening and titration may be performed on autologous blood obtained from a finger-prick or venous blood sample. Whole blood was diluted in PBS (1:40) and mixed with the IH4-RBD reagent, followed by a one hour incubation. Hemagglutination was scored as a positive sample. Alternatively, diluted whole blood is centrifuged. The supernatant is titrated and the IH4-RBD reagent added with either autologous or O negative RBCs (washed and diluted in 1:40 PBS). Negative controls were whole blood dilution mixed with PBS.

3.3.4 Enzyme-linked immunosorbent assay (ELISA)

Enzyme-linked immunosorbent assay (ELISA) was used to detect binding spike SARS-CoV-2 antibodies (**papers II**, **III**), as described previously (224). Sera were pre-screened in duplicate in 96-well plates for IgG antibodies against the Wuhan RBD (100 ng/well) (**paper II**). Briefly, plates were coated overnight with RBD antigen and blocked with blocking solution (PBS with 5% milk, 0.1% Tween-20, 1% BSA) for one hour. Serum samples were diluted 1:100 in PBS with 1% milk and 0.1% Tween-20 and 100 µl/well was incubated for 2 hours at room temperature, followed by 6 washes of PBS containing 0.05% Tween-20 (PBS-T). A secondary horseradish peroxidase (HRP)-labelled anti-human IgG antibody was added, and plates were incubated for one hour. Following incubation, the plates were washed with PBS-T and the chromogenic substrate 3,3′,5,5′-tetramethylbenzidine was added. The colour development was stopped by adding hydrochloric acid and the absorbance was read immediately at 450/620 nm by spectrophotometer.

All sera (**paper III**) or sera positive by RBD screening (**paper II**) were analysed in duplicate by spike ELISA. The plates were coated with the Wuhan spike protein (2µg/ml, 50 µl/well). Then, sera starting at a 1:100 dilution followed by a five-fold serial dilution were added. The plates were incubated for 2 hours at room temperature, and bound IgG antibodies were detected and measured as described above in the RBD screening. The mean endpoint titre was calculated for each sample, and samples with no detectable antibodies were assigned a value of 50. Positive and negative controls

were included in every run, with serum from a COVID-19 patient infected with Wuhan-Hu-1 D614G virus and the monoclonal antibody CR3022 as positive controls (225), and pooled pre-pandemic sera (n = 128) as a negative control (226).

3.3.5 Neutralisation assays

The neutralisation assays were used to characterise nAbs in sera from convalescents and vaccinees in Bergen, Norway (**paper II)**. Neutralisation assays were also performed in the UK for subjects included in the correlation analysis. The SARS-CoV-2 isolates used in the microneutralisation (MN), virus neutralisation (VN), and pseudotype neutralisation (PN) assays are listed below (**Table 4**).

Table 4: Virus isolates utilised in neutralisation assays.

Virus	Location	Assay	Clinical isolate, accession ID
Wuhan-like	Bergen, NO	MN[1]VN[2]	SARS-CoV-2/Human/NOR/Bergen1/2020, GISAD EPI_ISL_541970
		PN[3]	pHR'CMV-Luc, pCMVRΔ8.2 and pCMV3 encoding Wuhan spike protein transfected in HEK293T cells
	PHE[4], UK	MN	England/02/2020, GISAD EPI_ISL_407073
	Oxford, UK	MN	Victoria/01/2020, GenBank MT007544.1, B hCoV-19_Australia_VIC01_2020_ EPI_ ISL_ 406844_ 2020-01-25
Alpha-like	Oxford, UK	MN	2/UK/VUI/1/2020, H204820430
Beta-like	Oxford, UK	MN	20I/501.V2.HV001
Delta-like	Oxford, UK	VN	Genbank ID OK622683.1
	Bergen, NO	PN	pHR'CMV-Luc, pCMVRΔ8.2 and pCMV3 encoding delta spike protein transfected in HEK293T cells

[1]Microneutralsation, [2]Virus neutralisation, [3]Pseudotype neutralisation, [4]Public Health England

Pseudotype neutralisation (PN) assay

The SARS-CoV-2 PN assay was performed in biosafety level (BSL) 2 laboratory, as previously described (226). Briefly, HEK293T cells were transfected with TMPRSS2 and human ACE2 encoding constructs to make target cells for the assay. Heat inactivated serum samples were analysed in duplicates, serial diluted (from 1:10) and mixed with Wuhan or delta pseudotype viruses corresponding to 20 000 - 200 000

relative luciferase activity (RLA) in 96-well plates and incubated at 37 °C for 1 hour. The serum-pseudovirus mixture was added to plates seeded with ACE2-TMPRSS2 co-transfected HEK293T cells and incubated for 72 hours. RLA was measured by a BrightGlo Luciferase assay and the PN-based neutralisation titres inhibiting concentration (IC_{50} and IC_{80}) were calculated as the reciprocal of the sera dilution giving 50% and 80% reduction of RLA, respectively. Negative titres (<10) were assigned a value of 5.

Microneutralisation (MN) assay

The MN assay was performed in a certified BSL-3 laboratory by a qualified scientist with clinical isolates of SARS-CoV-2 (**Table 4**), as previously described (224). Heat inactivated serum samples were analysed in duplicate of serial dilutions (from 1:20) and mixed with 100x 50% tissue culture infectious doses ($TCID_{50}$) in 96-well plates and incubated for 1 h at 37 °C. Serum-virus mixtures were transferred to 96-well plates seeded with Vero cells and incubated at 37 °C for 24 hours. Cells were fixed and permeabilized with methanol and 0.6% H_2O_2, and a secondary rabbit IgG antibody against SARS-CoV2 nucleocapsid was added. Following incubation, the plates were incubated with a biotinylated goat anti-rabbit IgG, followed by (HRP)-labelled streptavidin. The plates were developed with the substrate o-Phenylenediamine the absorbance was read at 490 nm by a spectrophotometer. The MN titre was determined as the reciprocal of the serum dilution giving 50% inhibition of virus infectivity. Negative titres (<20) were assigned a value of 5.

Virus neutralisation (VN) assay

The VN assay was performed with clinical isolates of SARS-CoV-2 (**Table 4**) in a certified BSL-3 laboratory by a trained operator, as previously described (226). Heat inactivated serum samples were serial diluted (from 1:20) and analysed in duplicates. Sera were mixed with 100x $TCID_{50}$ of Wuhan or delta viruses in 96-well plates followed by 1 h incubation at 37 °C. Mixtures were transferred to 96-well plates seeded with Vero cells and incubated at 37 °C for 4–5 days. All wells were microscopically

examined for cytopathic effect (CPE). The VN titre was the reciprocal of the highest serum dilution with no CPE. Negative titres (<20) were assigned a value of 5.

3.4 Methodological considerations

Three papers in this thesis (**papers II-IV)** were based on a prospective study design, while **paper I** had a retrospective approach. The prospective design was appropriate when following participants over time after vaccination and infection, ensuring high quality of the collected data and the ability to measure multiple outcomes. Limitations to this design are risk of selection bias (see details below) and the high cost and resource demand. Although there are many aspects to methodological considerations, the main considerations of each paper will be discussed in the following sections.

Paper I

Egg-propagation associated changes have reduced VE against the A/H3N2 component in influenza vaccines. The loss of an important glycosylation site present in the wild-type HK14 were detected in the vaccine strain in the 2016/17 influenza season, resulting in reduced VE (71, 227). However, most historical A/H3N2 viruses that circulated before 2014 were not glycosylated at antigenic site B and were antigenically similar to the egg-based vaccine viruses. By using egg-grown viruses and the traditional HI assay, we were able to assess antibody responses in our cohorts of vaccinated or infected individuals against a total of 14 A/H3N2-viruses spanning 1968 to 2018. It was not feasible to obtain older cell-grown viruses that circulated back to 1968, due to the long-standing practice of isolation of influenza viruses on eggs within the global WHO network. Moreover, the WHO recommendation of using cell-based viruses for vaccine production only started in the 2020/21 season for the Northern hemisphere and in the 2021/22 season for the Southern hemisphere. The first cell-based vaccine in the EU was approved in 2007 and produced in Madin-Darby Canine Kidney (MDCK) cells from egg-adapted influenza viral seeds, thus may also contain egg-adapted viruses. Therefore, it would not be possible to have carried out the study with

this factor completely eliminated. However, our results regarding the HK14 strain should be interpreted with this in mind.

Papers II-IV

Paper II aimed to show the correlation between HAT and nAbs. To fully investigate its utility, serum samples from large cohorts were collected from multiple sites and several types of neutralisation assays and ELISA were conducted. WHO anti-SARS-CoV-2 international standards were added for comparison to account for interlaboratory variability, as the neutralisation assays were conducted in a different laboratory. The correlations are shown primarily in Wuhan convalescent sera, although a small group of delta infected and vaccinees were included. The finger-prick HAT provides an opportunity to measure HAT antibodies directly using autologous blood, which simplifies and allows the method suitable for limited resource settings. The correlation to venous blood were shown as an alternative source of blood. The HAT assay is generally useful for seroprevalence and research studies.

HAT favours the detection of nAbs, which is reasonable considering the high frequency of nAbs targeting the RBD. After RT-PCR verification of infection, the sensitivity of HAT is around 90%, with 99% specificity (130). IgM antibodies are often better at cross-linking HAT reagents, therefore improved HAT sensitivity and specificity is expected shortly after infection or primary vaccination. HAT sensitivity can be improved by prolonged incubation or centrifugation of the plates, although at the expense of specificity (131). However, HAT is not restricted to only measuring nAbs, as it may also detect non-neutralising, binding antibodies which cross-link. HAT antibody responses to omicron BA.2 were measured in **papers III** and **IV**. The level of nAbs to the omicron variants are generally reduced compared to pre-omicron VOC. In hindsight, the correlation between omicron-specific HAT and nAbs would strengthen our findings, however, the omicron variants were not circulating at the time **paper II** was conducted. By only measuring HAT BA.2 antibodies, the total antibody levels targeting the omicron subvariants, particularly BA.1, could be underestimated.

Moreover, the stabilising mutations introduced in the omicron HAT reagents could have reduced the affinity for neutralising antibodies.

Paper III

The ideal study design for **paper III** would be a case control study. However, more than 80% of the Norwegian population has been exposed to SARS-CoV-2. An unexposed control group would be very challenging to identify, considering the late pandemic stage at which our study was conducted. Thus, our data cannot estimate the prevalence of long-term symptoms in the ages 10-20 year olds compared to the uninfected population. However, our research questions were primarily focused on comparison of long-term symptoms after delta infection in different age groups, children versus adolescents, and adolescents versus adults. Furthermore, we aimed to study how the reported higher viral loads after delta infection would impact persisting symptoms compared to our previous study regarding the Wuhan variant. Specifically, we wished to compare antibodies between the groups with and without persisting symptoms. To approach these research questions, we chose a prospective cohort study design.

Specific challenges occur when conducting research during a pandemic, due to the impossibility of predicting its trajectory. The emergence of omicron BA.1 and BA.2 which caused reinfections in our delta infected cohort, and evolving COVID vaccination recommendations complicated our initial research questions. The group in which we could investigate delta long-term symptoms was halved by the 8 month follow-up. Furthermore, the large vaccination coverage made it impossible to estimate the effect of vaccination on long-term symptoms without a control group of unvaccinated individuals. However, the omicron reinfections facilitated an opportunity to study the possible impact on delta long-term symptoms and the period of vaccine protection against reinfection.

3.4.1 Generalisability, bi as and confounding factors

In the unique setting of a pandemic, such as the COVID-19 pandemic, the motivation in the general public to participate in clinical studies may increase. There was no knowledge of the virus or the severity of disease when the novel SARS-CoV-2 first appeared, which could motivate people to contribute to scientific research. In Norway, there is generally a high trust in policy makers and official advice communicated from the government and public authorities. Vaccine hesitancy is limited, with few people choosing not to follow the official childhood vaccination programme. During recruitment, our experience was that people were highly motivated to be included, with curiosity and altruistic interest in contributing to filling the knowledge gaps. This is reflected in our high recruitment rate of home-isolated Wuhan-infected individuals with a large age range (1-89 years) during the first pandemic wave, representing 92% of SARS-CoV-2 positive individuals in Bergen, Norway, this specific period (**paper II**).

However, the risk of certain types of bias and confounders are present in our studies. The adult vaccinees in our studies are health care workers (HCWs) (**papers I, II**). While not representative of the general population, partly due to a higher occupational exposure to infectious diseases, they are easily accessible for inclusion and follow-up in vaccine studies. The children included in **paper I** were scheduled for an elective tonsillectomy, but were otherwise healthy. Moreover, the small sample size limited generalisability. In **paper II**, home-dwelling older adults aged 80-99 years old were included. As expected, the older study participants used more prescription drugs and had more comorbidities compared to younger adults, but nonetheless they represented a healthy older population.

In **paper III**, all patients (10-20 years old) who tested PCR-positive for SARS-CoV-2 during the initial delta wave in Bergen municipality (between August 1st and September 15th) were invited to participate in our study. The intention was to capture all cases, but approximately 40% of all eligible participants in our municipality consented to participate. The prospective design probably limited the selection bias that could be

introduced when participants are retrospectively recruited, since individuals with persisting symptoms might be more likely to participate. Additionally, our drop-out rate was low with a 74% responding at the 8-month follow-up. We could not control for pre-COVID symptoms, however, very few studies are able to adjust for pre-COVID factors that may influence the reporting of post-COVID symptoms. The indirect data collection by parents of participants <16 years could introduce confounders, although these children actively participated in the interviews during both follow-ups, which facilitated opportunities to ask follow-up questions if any question was unclear, and we could identify potential misinterpretations. Only a subset of participants provided blood samples in **paper III** and **IV**, which may lead to selection bias when describing the immune responses in this age group.

As the immune response is multifaceted and this thesis aimed to characterise antibody responses, there are limitations in transferability to the overall immune response. However, other studies by our group have investigated T cell responses to influenza and SARS-CoV-2 which were beyond the scope of thesis (228, 229).

3.4.2 Statistical methods

Continuous variables were assessed for normality by the Shapiro-Wilk test and Kolmogorov-Smirnov test. Association between categorical variables were evaluated by the Chi square test. For Gaussian distributed data, ANOVA was used to assess differences of geometric means between groups. For non-parametric variables, differences in two independent groups were compared by the Mann-Whitney U-test, and the Kruskal-Wallis test was used when analyses included more than two independent groups. Furthermore, non-parametric variables with repeated measures were assessed by Wilcoxon test (two groups) and the Friedman test (more than two groups). Corrections for multiple comparisons were performed when appropriate (Dunn's test or Holm-Šídák method). Additionally, the log-rank Mantel-Cox test was used to compare the survival distributions. The statistical significance level was set to p=0.05, except for **paper I** where some p values were reported as # for levels <0.1.

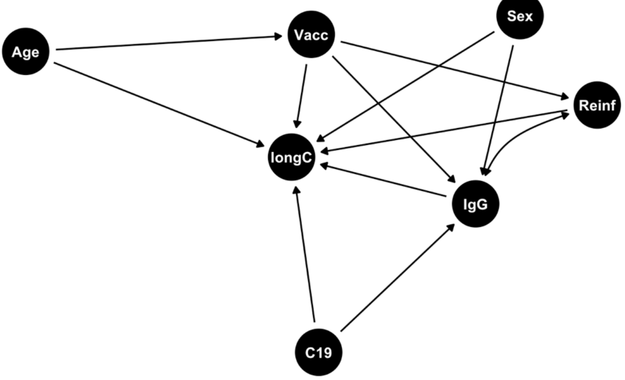
In **paper I**, a few missing values at the 6 month time point (n=6) were interpolated using linear regression for individuals with day-21 and 12-month data. Other missing data (from n=6) were interpolated by using the geometric mean titre (GMT) of the group. Pre- and post-infection or vaccination titres against n=14 viruses within the same group were compared using Friedman test with Dunn's multiple comparison or Wilcoxon matched-pairs signed-rank test with individual ranks computed for each comparison and Holm-Šídák method for multiple comparisons. The groups with missing samples were analysed using Wilcoxon matched-pairs signed-rank test. When calculating differences between groups with different priming patterns, we applied the two-way ANOVA and Holm-Šídák method for multiple comparisons. For the plots of differences in seroprevalence in different groups, the log-rank Mantel-Cox test was used.

In order to compare ranks in HAT titres between the older and adult vaccinees in **paper II**, the two-tailed Mann–Whitney U test with 95% confidence level was used. Correlations between nAb titres and HAT titres were calculated by the non-parametric two-tailed Spearman R correlation with 95% CI.

For **paper III**, we calculated both odds ratios (OR) and risk ratios (RR), since both are frequently used in epidemiological and immunological studies. In accordance with our study design, we chose to use ORs, as we were comparing different groups within an infected population, and not infected cases versus uninfected controls. Univariate and multivariate binomial logistic regression were used to identify associations between predefined predictors and the binary outcomes "any symptoms", "fatigue", "dyspnoea", "neurological symptoms", "cognitive symptoms". The predictor variables were chosen based on a priori hypothesis and suspicion of confounding (especially age and vaccination status) (**Figure 19**), and the linearity assumption was checked both for univariable and multivariable analysis by using Box-Tidwell test. Percentages of reported symptoms by age groups (> or ≤16 years) were calculated with 95% confidence intervals (CI), using the Wald interval. Differences between groups were

calculated as crude risks, as an excess percentage of symptoms in one group compared to another group.

Figure 19: Direct acyclic graph (DAG) used to define predictors.



Predictors of the outcome "long C" (long COVID or post COVID-19 condition). Vacc=vaccination, reinf=reinfection, C19=COVID-19, IgG=immunoglobulin G (spike-specific)

The survival plot in **paper IV**, was derived from the latest COVID-vaccination date and participants were followed until omicron BA.1/2 reinfection (the event of interest) or censoring events. Censoring events were the end of follow-up or loss to follow-up. There were no competing events. Spike-specific IgG and HAT antibody titres in papers **III** and **IV** were compared as untransformed continuous variables by the Mann-Whitney U test.

All data were analysed and visualised in GraphPad Prism and R (R Foundation for Statistical Computing, Vienna, Austria).

4. Summary of results

4.1 Paper I

“Seasonal influenza vaccination expands hemagglutinin-specific antibody breadth to older and future A/H3N2 viruses”

Influenza is an epidemic respiratory virus, usually infecting and priming the population at a young age. Pre-existing immunity, especially the priming infection, impacts immune responses later in life. The two main types of influenza vaccines are IIV and LAIV, the latter is only licensed in children in Europe. We investigated how vaccination and infection impacted the breadth of antibody responses against 14 antigenically distinct historical and future epidemic A/H3N2 viruses in adults (n=42, 22-61 years) and children (n=42, 3-17 years). Adults received either single or repeated IIV over a 3-year period, whereas children received LAIV. HI titres were evaluated pre- and up to 4 years post vaccination, as well as post-infection.

We found broader HI antibody responses in adults than children, which were more cross-reactive in previously vaccinated or A/H3N2 primed adults. Vaccination induced antibodies against viruses encountered in childhood, spanning to future viruses. Back-boosted responses were primarily directed against the newer strains, and less focused on historical A/H3N2-viruses, contrary to the theory of original antigen sin. Repeatedly vaccinated adults had broader and more durable antibodies compared to previously unvaccinated children and adults.

In conclusion, the vaccine-induced cross-reactive antibodies recognised multiple H3N2-strains, even viruses the individual had not encountered. We observed that an increased life-time exposure to influenza A/H3N2 viruses broadened the antibody responses. These cross-reactive antibodies may be favourable in the face of new epidemic influenza viruses.

4.2 Paper II

"A rapid antibody screening haemagglutination test for predicting immunity to SARS-CoV-2 variants of concern"

Measuring SARS-CoV-2 neutralising antibodies (nAbs) responses to live infectious virus is laborious and costly, requiring laboratory biosafety level 3. There was a need for a cheap and simple rapid-test to study and compare SARS-CoV-2 antibodies in different age groups after the implementation of COVID-19 vaccines. Especially since older adults >80 years had not been included in the vaccine licencing trials and were the first vaccine recipients. We aimed to show the utility of a novel, rapid hemagglutination test (HAT) by demonstrating its correlation to the gold standard neutralisation assay. Furthermore, we compared mRNA vaccination (BNT162b2) induced HAT antibodies in younger and older adults >80 years and to a cohort of naturally infected individuals.

We found a high correlation (R=0.74-0.88) between HAT and nAbs in previously Wuhan-infected subjects (n=798), which was maintained after delta infection (R=0.72-0.82). Older, naïve adults (n=89) had significantly lower Wuhan-specific antibodies (32% seropositivity) after the first dose compared to younger adults (n=309) and convalescents (74% and 94% seropositive, respectively). The second dose particularly boosted Wuhan-specific antibodies in older adults (78% seropositivity). Younger adults and infected individuals generally had higher specific and cross-reactive antibodies to VOC than older adults. Interestingly only after previous infection did older adults achieve comparable antibody responses to younger adults after the first vaccination, with limited boosting after the second dose.

In summary, HAT was found to be a simple, inexpensive surrogate measurement for nAbs against emerging VOC. Older adults were more dependent upon a priming stimulus, either through vaccination or infection, to elicit SARS-CoV-2 antibody responses comparable to younger adults or previously infected individuals.

4.3 Paper III

"Post COVID-19 condition after delta infection and omicron reinfection in children and adolescents"

Children are an understudied group regarding long COVID, or post COVID-19 condition, and there is with limited knowledge concerning risk factors. Long-term symptoms after SARS-CoV-2 infection include fatigue, dyspnoea, and cognitive impairment, which affect daily function. In a cohort of delta infected children and adolescents 10-20 years old (n=276), we addressed the knowledge gap of persisting and long-term COVID-19 symptoms and their association to antibody responses elicited after infection.

We identified persisting (3 months) and long-term symptoms (8 months) after infection in children and adolescents, and their impact on daily life. Serum spike IgG and HAT antibody titres at 3 months post-infection were higher in young people with persisting symptoms. Experiencing acute symptoms, such as dyspnoea and fatigue, was associated with persisting and long-term symptoms. Children 10-15 years had significantly less long-term fatigue, dyspnoea and cognitive symptoms compared to adolescents 16-20 years. Adolescents reported 71% of any long-term symptoms 8 months post-infection, compared to 28% of children, despite their higher vaccination coverage. Absenteeism from work and/or extracurricular activities was more prevalent in the symptomatic group (40% versus 16%), especially in those reporting fatigue (OR 3.1, 95% CI 1.6–5.9). Females were at higher risk for long-term dyspnoea compared to males.

To summarise, adolescents compared to children experienced more frequently post COVID-19 condition, with symptoms similar to adults. Young people with persistent symptoms had higher antibody titres, and long term symptoms were associated with acute phase symptoms and absence from work and extra-curricular activities.

4.4 Paper IV

"COVID-vaccine protection against Omicron break-through infection in children and adolescents"

COVID-19 vaccines have been highly successful in preventing infection with the ancestral SARS-CoV-2 and severe illness and disease from all VOC. However, vaccine protection waned quickly, and was especially compromised by the omicron variants' nAb escape. A combination of previous vaccination and infection, known as hybrid immunity, is superior in protecting against break-through infection by all SARS-CoV-2 variants. In the same cohort of children and adolescents aged 10-20 years from paper III, a large proportion had hybrid immunity by previous delta infection and recent COVID-19 vaccination (n=88). We assessed cross-reactive antibody responses and the duration of vaccine protection against break-through infection with the widely circulating omicron BA.1/2 variants.

We found a high overall omicron BA.1/2 reinfection rate of 55% in our cohort. Three months after delta infection, vaccinated individuals had significantly higher omicron BA.2 antibodies than unvaccinated subjects, which reduced the risk of omicron BA.1/2 break-through infection. However, hybrid protection from omicron BA.1/2 was short-lived, and lasted only 22 days after monovalent vaccination, corresponding to the peak of the vaccine-induced antibodies. In summary, our results demonstrate that higher omicron-specific antibodies resulted in fewer reinfections, although not always protective from reinfection. This was confirmed by the high infection rates in both unvaccinated and vaccinated individuals, with a short duration of vaccine protection.

5. Discussion

5.1 Impact of age on antibody responses and long-term symptoms

The work in this thesis has focused on antibody responses after infection and vaccination with the two major respiratory viruses; influenza and SARS-CoV-2, across different age groups. The work has covered the breadth of antibody responses elicited after administration of different vaccines, such as inactivated and live-attenuated influenza vaccines and COVID-19 mRNA vaccines. Furthermore, vaccine responses were compared to infection and between children and adults, as well as between younger and older adults. Finally, the duration of COVID-19 vaccine protection and long-term consequences after SARS-CoV-2 infection were evaluated in children and adolescents.

As a prototype drifted respiratory virus, influenza has taught us several important lessons about immunity. Firstly, the primary influenza A infection leaves a lasting immunological imprint which may direct subsequent immune responses (172, 174, 175, 230). Influenza A/H3N2 has undergone continuous antigenic drift and circulated in man since its pandemic emergence in 1968. Children are exposed to influenza viruses early in life, most often the A/H3N2 subtype, supported by the finding of influenza-specific antibodies in half of all 2 year old children (173). The children (aged 3-17 years) included in **paper I** had pre-existing antibody titres to A/H3N2 strains that circulated in the years after their birth, especially the older children (10-17 years). Thus, almost every influenza exposure throughout life will be secondary. In our influenza study, the birth cohort born 1967-1976 (H3-primed) had high pre-existing antibodies to the oldest A/H3N2 strains (HK68-BK79), indicating childhood infections with these viruses. However, the oldest cohort (born 1948-1966) had higher pre-existing titres to HK68 than the H3-primed cohort. This may reflect an infection in early adult life with this pandemic virus, inducing a robust MBCs response. This leads us to the second lesson; decades of exposure both by infections and vaccinations to a range of drifted influenza A viruses, broadens the antibody repertoire (231-233). This is in agreement with our findings of broader antibody responses in adults, especially those with

previous vaccinations, compared to children. A/H3N2-specific B cells will have many opportunities to increase their breadth of response through affinity maturation over the years. Moreover, the sequence of exposures may also influence the antibody repertoire (180, 234), as illustrated by the diversity of individual antibody landscapes. In addition to the imprinting infection, the sum and succession of lifetime infections and vaccinations against drifted virus is relevant to contemporary antibody responses.

Upon encounter with a novel antigen such as SARS-CoV-2, all age groups are immunologically naïve. In such a scenario, age groups with the best health outcome are immunocompetent younger adults. In comparison, the older adults are at high risk for severe disease. Consequently, older age groups were prioritised for vaccinations with the newly developed COVID-19 vaccines. Experience from influenza immunisation in the elderly has illustrated the attenuated vaccine responses in this age group due to immunoscencence. Two mRNA immunisations were necessary to develop high antibody responses in the older adults compared to younger adults, who responded well after the primary dose (**paper II**). Furthermore, the younger adults in our study had better cross-reactivity to VOC. Immunoscencence involves both the innate and adaptive immune responses. However, the adaptive responses are most relevant in vaccine responses, as development of immunological memory is the key concept. While the pool of naïve T cells decreases with age due to thymic atrophy, the number of peripheral memory T cells expands (235, 236). This phenomenon is advantageous for regularly encountered antigens, such as influenza, but unfavourable in the face of novel viruses. The smaller pool of B and T cells reduce the initial antibody vaccine response and durability of antibodies. Indeed, we observed faster antibody waning was observed in older compared to younger adults (228).

As older age groups were protected by the primary vaccination series, booster doses and risk reducing activities, infection rates with the highly infectious delta and omicron subvariants shifted to younger age groups. Although children have a lower risk of severe disease, MIS-C and long-term complications are potentially major COVID-19 complications. Long-term symptoms after COVID-19, such as fatigue, dyspnoea and

cognitive symptoms were already described in the adult population, but there was limited knowledge regarding persistent sequelae in children and adolescents (**paper III**) (237). This was partly due to lower infection rates with the ancestral strain in the youngest age groups (238). The long-term symptoms reported in our study after delta infection in younger people, especially in adolescents, were similar to those previously described in adults. Importantly, higher Wuhan spike-specific IgG as well as Wuhan and delta HAT antibodies were associated with persisting symptoms, suggesting a similar underlying cause in both children and adults (238-240). Some of the adolescents (~40%) were vaccinated prior to delta infection (94% primary dose only) and had hybrid immunity. Seventy six percent of the previously immunised adolescents received their second mRNA dose three months post delta infection. COVID-19 vaccination in adults has been associated with reduced infection-mediated long-term symptoms (241), yet vaccination failed to mitigate the long-term symptoms in our adolescent group. However, factors such as the infecting variant, number of mRNA doses and the order of vaccination and infection may impact findings.

COVID-19 vaccines have demonstrated waning protection and reduced vaccine effectiveness against novel VOC in adults (242, 243). In addition to the superior protection offered by hybrid immunity, mRNA vaccines are highly immunogenic in children (>9 years) and adolescents (244). The higher cross-reactive antibody responses achieved by children with hybrid immunity resulted in reduced omicron BA.1/2 break-through infections, compared with unvaccinated subjects (**paper IV**). Regardless, many of these children experienced break-through infections, confirming the reduced cross-neutralisation against omicron subvariants. Furthermore, we found that COVID-19 vaccine protection against omicron BA.1/2 break-through infection was short-lived in children, due to significant immune escape.

5.2 Antibody landscapes

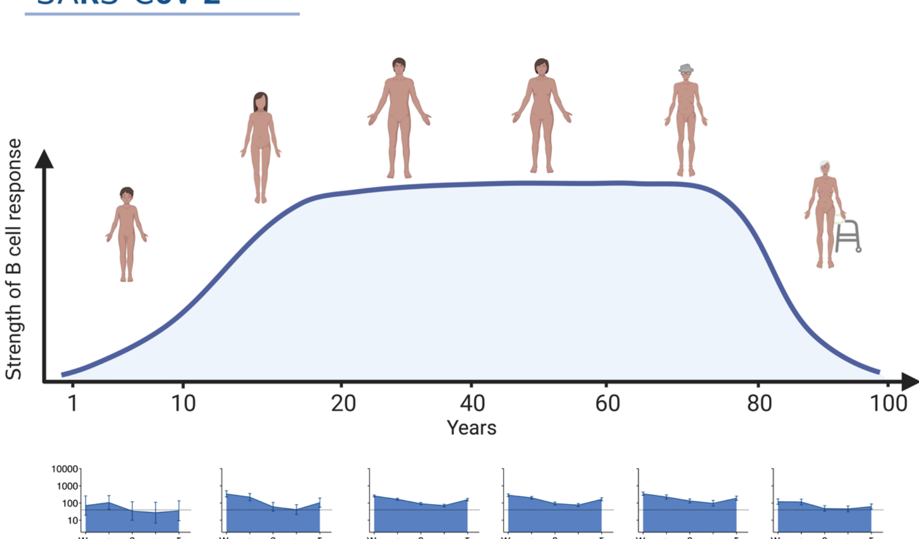
Antibody landscapes are an illustrative approach to present antibody breadth, both pre- and post-vaccination or infection. The concept was introduced by Fonville and

colleagues in 2014 with back-boosting responses after influenza A/H3N2 infection and vaccination (107). The A/H3N2 virus has rapidly drifted with multiple viral clades, creating an ideal opportunity to investigate the breadth of antibody responses to this subtype (**paper I**). The term hybrid immunity has been used to define the increasing pre-existing immunity to SARS-CoV-2 through combinations of infection and vaccination, although it could well be applied to influenza viruses. In **paper I** the A/H3N2 antibody landscapes reveal the breadth of pre-existing antibodies, and how these landscapes may change depending upon the existing antibody landscape, largely influenced by age. Irrespective of age, the vaccine- or infection-induced antibodies often mirrored the pattern of pre-existing antibodies. This agrees with earlier studies, where pre-existing antibodies were found to largely determine the post-vaccination responses (180, 245, 246).

Although SARS-CoV-2 has circulated for a much shorter time than influenza, several variants have already emerged. This enabled the unique opportunity to evaluate SARS-CoV-2 cross-reactive antibody responses (alpha through delta) across groups of infected and vaccinated individuals (**paper II-IV**). In our first study of immune responses after COVID-19 infection and vaccination (**paper II**), the infected individuals were recruited at the start of the pandemic, and the vaccinees one year later in January 2021. This was prior to the widespread circulation of SARS-CoV-2 in Norway due to diligent infection control, therefore only a low number vaccinees were previously infected (n=14) with the homologous SARS-CoV-2 variant (Wuhan) included in the vaccine. As expected, we observed the highest antibody titres against the ancestral strain in both groups, with gradually decreasing antibody titres against the other VOC in the order highest to lowest: alpha, delta, beta and gamma, similar to the cross-reactive patterns reported in other studies from the UK and US (247, 248).

Furthermore, these cohorts provided a unique opportunity to study antibody responses to a novel virus with no pre-existing immunity across different ages (**Figure 20**).

Figure 20: Peak SARS-CoV-2 RBD-specific antibody landscapes in age groups (1-99 years)



The graph illustrates the strength of B cell response (B cell affinity maturation and antibody isotype switching) by age (adapted from (249)). The age span in the two youngest groups are 10 years (1-9 and 11-22 years) and 20 years in the older groups (23-39 years, 40-59 years, and 80-99 years). The antibody landscapes after vaccination (3-5 weeks post 2nd dose) or infection (3-10 weeks) are plotted as geometric mean titres of HAT antibodies with 95% confidence intervals. The landscapes of the youngest groups (1-22 years) are infection responses, the ages between 23-79 represents a mixture of infection and vaccination responses, although a large part of individuals included in the 60-79 age groups are infected, while the oldest 80-99 years are mostly vaccinated.

Similarly to **paper I**, the SARSCoV-2 Wuhan-specific antibody landscapes were plotted as a mixture of infection and vaccination responses from **paper II**, covering the entire age-span (1-99 years, n=630) (**Figure 20**). The hospitalised cases with severe disease (mostly elderly) were excluded due to their higher antibody titres, confounding the interpretation of age. The landscapes therefore largely represent healthy individuals with mild COVID-19 and vaccinees. The youngest age groups (1-22 years, median 16 years) had low infection rates in the Wuhan-wave and were not prioritised for vaccination, hence under-represented in the landscapes as evident by larger confidence intervals (n=32). Conversely, the largest proportion of individuals 80-99 years are vaccinated. Overall, the antibody landscapes clearly illustrate the difference in responses through the ages, with lowest titres in the youngest children (1-9 years) and the oldest adults (80-99 years). This could simply be explained by the decreased B cell

somatic hypermutation and antibody isotype switching in these age groups (**Figure 20**) (249). Another explanation for the reduced antibody responses in the youngest could be that they had lower viral loads and consequently experienced more frequently asymptomatic Wuhan-infections, as evident by seropositivity despite a low number of PCR-detected infections (250).

5.3 Influenza and SARS-CoV-2 antigenic imprinting

Studies and real-world data have demonstrated the possibility of life-long immunity after the primary influenza infection against viruses of the same subtype or phylogenetic group (178, 251) the same virus. The "sin" has referred to the narrow potential of antibody responses. Individuals imprinted with heterosubtypic influenza during epidemics and pandemics may be more susceptible to severe disease compared to those imprinted with the homologous subtype (10, 13, 14, 170). Our findings in **paper I** support the potential of contemporary infections or vaccination to recall memory responses against previously circulating viruses (108). Upon A/H3N2 exposure, the 1967-1976 (H3-primed) birth cohort had the broadest antibody boosting, ranging from the oldest HK68 to the future HK14. The oldest and younger birth cohorts were primarily boosted against the newer A/H3N2 strains (from SY97/CA04). The boosting of pre-existing antibodies provide evidence for the contribution of MBCs. However, antibodies to the oldest strains did not dominate any of the antibody landscapes (OAS or antigenic seniority), agreeing with other recent studies on A/H3N2 antibody landscapes (107, 108). Finally, two aspects must be kept in mind if antibody titres are used to infer real-world disease protection. Firstly, antibody titres are not equivalent but correlated to protection against disease (252). Secondly, higher antibody magnitudes do not necessarily translate to greater protection (253).

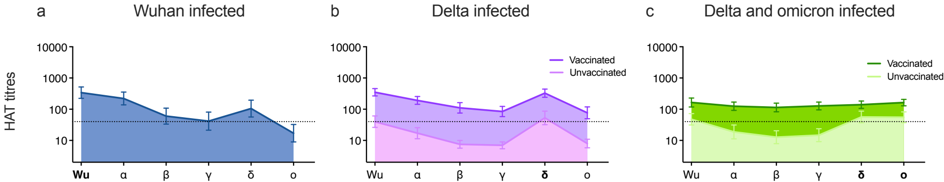
Overall, our study supports the imprinting phenomenon, although not at the expense of *de novo* responses, but as a supplement to contemporary antibody responses. The pre-existing antibodies reflect an accumulation of antibodies to diverse HA head epitopes. The immunological imprinting can be understood as a recall of antibodies mediated by

MBCs during secondary influenza exposures (108). The memory responses are focused on epitopes conserved among several strains and could be beneficial for future protection, although dependent on conservation of these epitopes in drifted strains (254). To protect against infection by drifted strains, MBCs must undergo affinity maturation, or naïve B cells must be recruited to GCs. Based on the competition between naïve and MBCs, imprinting is sometimes referred to as having a cost. Due to their lower activation threshold, MBCs may outcompete the naïve cells that require further stimulation and dominate the antibody landscape. However, a recent paper on primary and secondary B cell responses in mice found that the secondary GCs are dominated by naïve B cells rather than MBCs (255, 256). Only a small fraction of MBCs were found to engage in secondary GCs, and this small but selective set of B cells may be directed against conserved epitopes. The authors hypothesise that imprinting could be explained by a "recycling" of high-affinity MBCs producing antibodies against historic strains (heterologous or cross-reactive antibodies), while the majority of antibodies are derived from naïve B cells, capable of generating homologous antibodies. However, these findings remain to be reproduced in humans, who have a more complicated history of exposure.

The insight of immune responses to influenza being subjected to imprinting led to early suggestions of a possible SARS-CoV-2 imprinting (257). Indeed, both SARS-CoV-2 infection or vaccination have been shown to boost antibodies to the human betacoronaviruses HCoVs OC43 and HKU1 and SARS-CoV-1 (258, 259). As the population is successively exposed to emerging variants and updated vaccines, individuals will have different exposure histories. As with influenza, the SARS-CoV-2 imprinting pattern will depend on the priming variant and the order and number of exposures (232, 258, 260). In our cohort of delta infected children and adolescents (papers **III** and **IV**), none of the subjects who provided blood samples were previously infected by SARS-CoV-2 (n=88). However, 40% were previously vaccinated (one-dose priming) with the mRNA (Wuhan-based) COVID-19 vaccine. The convalescent profile after delta infection in our study is comparable to those reported previously (261). The vaccinated individuals elicited significantly higher antibodies to all SARS-

CoV-2 strains, including delta, compared to the unvaccinated young participants (**Figure 21b**). Our findings demonstrate that higher titres are achieved by hybrid immunity, as previously reported (90). Both groups have similar shaped antibody landscapes, inducing high antibodies to the ancestral strain relative to the infecting strain, with no distinct imprinting in the vaccinees.

Figure 21: SARS-CoV-2 RBD-specific antibody landscapes in Wuhan, delta and omicron BA.1/2 convalescent children and adolescents.



The graphs show geometric mean HAT antibody titres with 95% confidence intervals against Wuhan (Wu) and variants of concern alpha (α), beta (β), gamma (γ), delta (δ), and omicron BA.2 (ο). Panel (**a**) includes Wuhan-infected children (10-22 years, n=21) sampled 1.5 months post-infection, while panel (**b**) and (**c**) represent delta infected children (10-20 years). Serum samples were collected 3 months after delta infection from n=88 (n=35 vaccinated pre-infection) (**b**), and 3 months after omicron BA.1/2 reinfection from n=51 (n=25 with previous vaccinations) (**c**).

To further investigate the different serological profiles induced by infection with specific variants, we compared the delta infected children to the children infected with the Wuhan virus during the first wave of the pandemic (**paper II**, n=21) in two age-matched groups (10-22 years old) (**Figure 21a,b**). Furthermore, 51 delta infected were reinfected with omicron BA.1 or BA.2 (BA.1/2) (**Figure 21c**). Due to the proximity of sampling to Wuhan infection (1.5 months), the ancestral strain appeared to have induced higher delta cross-reactive RBD-specific antibodies than delta infection (at 3 months). The infecting variant generally induce the highest titres to the homologous virus, which we believe also is the case here.

Upon omicron BA.1/2 reinfection, cross-reactivity to all VOC improved. Further mRNA vaccination between infections with delta and omicron BA.1/2 infection changed the overall landscape, with equally high antibody titres to all VOC. It has previously been described that infection with omicron subvariants induced a distinctively different antibody pattern from that induced by a primary Wuhan infection

or vaccination. Some have found reduced epitope diversity with lower nAbs (262). By decreasing diversity, imprinting was suggested to concentrate immune pressure on certain RBD epitopes, giving rise to specific escape mutants with strong evolutionary advantages, called convergent evolution. Convergent mutations have been observed in multiple omicron subvariants independently, suggesting that a collective immunological pressure is driving these mutations. However, affinity maturation might reduce this problem by inducing more cross-reactive antibodies with increased breadth, which are potentially able to neutralise the convergent escape mutants (263). Indeed, others have found that omicron BA.1 break-through infection contributes to broadening of the antibody repertoire (264). Our omicron BA.2 antibody landscape support the latter hypothesis, with an overall improved cross-reactivity, possibly due to several rounds of affinity maturation.

The rapid viral evolution of SARS-CoV-2 required discussions of vaccine updates, as the Wuhan-based vaccines showed reduced effectiveness against all VOC, except Alpha. The question is whether boosting with a new variant in pre-immunised individuals has a cost by focusing the response on conserved epitopes instead of the novel mutated epitopes and eventually side-tracking the immune response. Studies have confirmed imprinting after bivalent booster doses or omicron break-through infection (265, 266). A study of vaccination responses in humans found that the overall MBC responses to mRNA booster doses encoding the spike from the ancestral strain, beta and gamma strain or omicron BA.1, were dominated by cross-reactive antibodies to the original strain, as evidence of imprinting (267). However, there were also *de novo* B cell responses that targeted variant-specific epitopes. These new epitopes were likely not recognised by existing high-affinity antibodies and could therefore stimulate naïve B cells (254).

In summary, imprinting is a phenomenon linked to variable antigens, and has been observed after both influenza and SARS-CoV-2 exposure. To exploit the advantage of influenza imprinting, early life vaccination with several antigens has been proposed (268). However, it remains to be established if vaccination could provide comparably

durable MBCs and T cell responses as observed after infection. In this context, live-attenuated vaccines better mimic a natural infection. The question is if these vaccines could be delivered in time before the first infection. LAIV are approved for children >2 years due to the increased risk of wheezing associated with administration of these vaccines in younger age groups. However, other mucosal vaccine platforms such as viral vectors are currently being researched. Perhaps with novel vaccines, either administered mucosally or intramuscularly, multiple antigens could be included in future vaccines and delivered prior to the priming infection, providing robust and durable cellular and humoral immunity.

5.4 Differences in humoral responses after vaccination and infection

Immune responses after vaccination will differ from infection, based on antigen composition, concentration, duration and site of immunisation. In all papers (**I-IV**), humoral responses after influenza or SARS-CoV-2 infection and vaccination have been evaluated (**Table 5**). During a natural infection, the immune system is exposed to all viral antigens, while vaccines usually contain antigens important for inducing virus-specific antibody responses.

Table 5: Difference in antibody responses after vaccination and infection

Virus	Influenza			SARS-CoV-2		
Immune stimuli	Natural infection	LAIV*	IIV	Natural infection	mRNA vaccine[§]	Hybrid immunity
Serum response	+++	++	+++	+++	++++	++++
Mucosal response	+++	++	(+)	+++	(+)	+++
HA-specific response	+++	++	+++	-	-	-
S-specific response	-	-	-	+++	++++	++++
Longevity	+++	++	+	++	+	+++
Breadth	++	+	+	++	+	+++

*In children
[§]After primary vaccination series and booster

The typical vaccine seroconversion rates measured by HI, defined as a fourfold antibody titre increase, is 20-77%, compared to >70% after infection (146, 269). Both

influenza infection and vaccination may induce cross-reactive antibodies to historical strains in individuals with pre-existing immunity, although the breadth is generally improved after infection. In **paper I**, we found similar HI antibody breadth in adults after influenza A/H3N2 infection and vaccination. While the infection response is more multifaceted, the HA head-specific response in our study was comparable between vaccinees and infected subjects. Another study on cross-reactivity to historic influenza A/H3N2 strains in adults found that infection elicited broader antibody responses compared to vaccination (108). However, the cohort had not been vaccinated during the past 9 years, which is likely very different to the HCWs in our cohort who were recommended yearly vaccination.

COVID-19 mRNA vaccines based on the S-protein have demonstrated higher antibody titres compared to infection (270). We confirm the higher vaccination-induced antibodies in **paper II**, when comparing vaccinees (after two doses) and infected individuals. Vaccinees and convalescent plasma with high titres have high proportions of binding antibodies, while low level convalescent plasma had the highest relative concentration of nAbs (259). Taken together, this might suggest that higher levels of antibody titres are associated with higher proportions of non-neutralising antibodies. Antibodies with high neutralising potency have a more limited breadth, in contrast to antibodies with moderate neutralising potency with ability to cross-neutralise many variants, although perhaps not able to prevent infection.

The children and adolescents included in **paper**s **III** and **IV** had either hybrid immunity (delta infection and vaccination) or delta infection immunity. As described by others, we observed higher antibody levels and improved cross-reactivity to omicron BA.2 in children with hybrid immunity compared to those with delta infection alone (271, 272). The ability of an infection to recall rare, but broadly reactive MBCs, induced by vaccination, is the suggested explanation for the robust antibody titres. The mRNA vaccines have been found to induce robust GC responses lasting several months after vaccination, perhaps due to the prolonged antigen persistence in lymph nodes (273). This increases the chances for further affinity maturation and generation of higher

diversity in the MBC pool. As opposed to significant differences in antibody titres between naïve younger and older vaccinees in **paper II**, the younger and older adults with hybrid immunity in **paper II** (Wuhan infection + vaccination) both had similar antibody responses after the primary vaccination, which is another testimony to the robustness of hybrid immunity. Furthermore, we observed little boosting after the second dose in these individuals, as previously described (274, 275). Antibodies cannot be infinitely boosted, and at some point, the antibody level will reach a threshold level. In **paper III**, we found an association between higher antibodies and persisting symptoms in delta infected children, but not in those with hybrid immunity. Although there was a trend of higher antibody levels in the vaccinated group with symptoms compared to asymptomatic cases, antibody titres were magnitudes higher, restricting any further correlation to symptoms. We believe that the high antibody titres induced by hybrid immunity might mask this potential association with persisting symptoms.

The duration of vaccination responses is of major importance to estimate timely boosters. Influenza infection may induce antibody responses measurable by HI to the homologous strain that are very long-lasting, perhaps lifelong (146). Influenza vaccination-induced antibodies usually wane by 6 months. Our findings in **paper I** confirm this waning also in the broadly induced antibody repertoire. We could not compare the same vaccine types between age groups, as children received LAIV and adults IIV. Although LAIV have been reported to induce more durable antibody responses than IIV (149), we found a similar antibody durability between the two vaccines. Interestingly, repeated IIV improved the durability of cross-reactive antibodies, with measurable antibodies up to 4 years after the first vaccination (**paper I**). Similar kinetics of the antibody response have been observed after SARS-CoV-2 antibody waning following infection is generally slower than after two mRNA vaccine doses (228, 276). Waning of vaccine protection paired with insufficient vaccine-elicited nAbs to drifted strains, results in reinfections. We confirmed the significant viral escape of omicron subvariants in **paper IV**, in which vaccination was estimated to protect against BA.1/2 reinfection for 22 days, corresponding to the peak of the

antibody response. However, the 50% vaccine-mediated protection lasted 95 days, which is similar to previous findings (277).

5.5 Hemagglutination as a tool for measuring antibodies

The HI assay was used to measure antibody responses to 14 distinct A/H3N2 strains in **paper I**. However, the evolution of A/H3N2 viruses in humans has led to a gradual decline in avidity for the avian receptors. The majority the influenza vaccine production still relies on egg-propagation, although growth of virus in eggs has become increasingly difficult. Cell cultures that overexpress the human α-2,6 SA receptor can be utilised to achieve high viral titres, although receptor mutations are frequently introduced. The declining ability of HA to agglutinate cells facilitates the evolution of viruses to mediate agglutination by NA, which has been observed through passage of virus in cell culture (116). The HI protocols for characterisation of A/H3N2 antibodies have therefore included addition of the NA-inhibitory drug oseltamivir in recent years. Furthermore, the loss of ability to agglutinate first chicken RBCs in the 1990s, and recently also turkey RBCs, has required introduction of guinea pig RBCs for HI (115). In **paper I**, we used exclusively egg-grown viruses, and experienced no problem agglutinating turkey RBCs. For the most recent A/H3N2 virus (HK16), the HI assay was conducted with both turkey and guinea pig RBCs, although no differences in antibody titres was observed between the two.

The hemagglutination method is not exclusively used for evaluating influenza-specific antibodies. Historically, agglutination has been used to identify antibodies to blood groups and for evaluating antibodies against other pathogens, including HIV, and recently SARS-CoV-2 (278-280). With the rapid evolution of lab assays for COVID-19, a surrogate neutralisation assay such as the hemagglutination may be more easily implemented in many labs. The HAT assay is essentially a binding assay that correlates with nAbs but does not measure these antibodies directly (281). HAT has been utilised by other research groups to investigate the cross-reactivity to VOC in convalescents and vaccinees (282), and to estimate the seroprevalence of SARS-CoV-2 antibodies in a Sri Lankan population, with a high agreement between HAT and ELISA (283). The

main difference from ELISA is that the RBD antibodies detected must be able to crosslink labelled RBCs.

In **paper II**, the correlation to nAbs was demonstrated for the ancestral strain (Wuhan) and VOC alpha through delta. The majority of Class 1 and 2 monoclonal antibodies that bind either side of the head of the RBD domain are successfully detected in the HAT assay. Greaney *et al.* have shown that class 2 nAbs tend to dominate in polyclonal sera, at least to the Wuhan strain (284). The HAT assay therefore favours the detection of nAbs, but this is not absolute, as non-neutralising antibodies to class 4 epitopes also cross-link, as shown by the positive control EY6A monoclonal antibody (223).

We observed a high cross-reactivity to the alpha variant after Wuhan infection or vaccination in **paper II,** as confirmed by others (203). The mutations in the alpha spike have primarily diminished NTD-specific rather than the RBD-specific nAbs (208). Given the immunodominance of RBD, the modest reduction in HAT-specific antibodies to this variant agreed with our findings. However, the cross-reactive antibodies against the beta and gamma variants were significantly reduced, with the lowest frequency of seroconversion (HAT titre ≥40) to gamma. Other studies measuring beta and gamma cross-reactive binding or nAbs after Wuhan infection have found lowest titres to the beta variant (285, 286). This is in line with the significantly reduced real-world VE to beta (287). We speculate that the single K417N/T RBD amino acid difference between beta and gamma (mainly altering class 1 antibody binding) limited any significant distinction in HAT-specific antibodies between the two.

We found higher cross-reactive HAT antibodies against the delta variant compared to beta and gamma in **paper II**. The delta HAT reagents do not contain the E484K mutation that yields substantial class 2 antibody escape, but the L452R and T478K mutations which enhance affinity for the ACE2 receptor and primarily escape nAbs of class 1 (82). This is in agreement with other studies findings improved cross-reactivity to delta, and preserved real-world VE ~80% to this VOC (203). The reinfections with

this variant could therefore primarily be driven by increased infectivity and not necessarily immune escape. The HAT antibodies measured to the drifted omicron BA.2 subvariant (**papers III** and **IV**) were most likely non-neutralising, reducing the correlation to nAbs. The HAT is still a reliable test for establishing detection of antibodies to the RBD, many of which will be neutralising. Furthermore, the functions and importance of non-neutralising antibodies in mediating protection from disease has become more evident with variants escaping nAbs.

In summary, the advantages of rapid hemagglutination assays are their easy utilisation, simplicity, and low cost, in addition to being quantitative. Whilst technically easy assays, there is a need for international harmonisation of the assay and utilisation of appropriate standards to allow comparison of antibody responses between different vaccines. With limited access to expensive lab equipment, the HAT assay offers a mean to assess large-scale SARS-CoV-2 seropositivity in a population. Furthermore, it can easily be adapted to new VOC, and measure cross-reactive antibodies as demonstrated in **papers II**, **III** and **IV**.

5.6 Vaccination in the face of antigenic drift

Low VE has been a challenge both with A/H3N2 viruses and SARS-CoV-2 VOC. However, the real world experience with vaccines has shown their success in protecting the lower respiratory tract and preventing hospitalisations and deaths. With this in mind, the next sections which will cover aspects that reduce VE and possible interventions to improve it.

Repeated influenza vaccination has been associated with blunted antibody responses to novel viruses and reduced VE, but not always (145, 288). In **paper I**, we found favourable outcomes in terms of higher, broader, and more durable HI antibody responses in the repeatedly vaccinated group. Although clinical information was not collected as part of the study, higher HI antibody levels have been found protective in VE studies (289). Varying VE is tied to antigenic distance between the virus in

circulation and the strain incorporated in the current and previous seasons' vaccine (168). In our repeatedly vaccinated cohort, the first vaccine contained an A/H3N2 strain antigenically similar to the second vaccine A/H3N2 component, which could explain our findings of durable antibodies after repeated vaccination. An increased seropositivity towards several viruses could be favourable in the face of future epidemics of the same subtype, with identical or similar conserved epitopes. The back-boosting results imply that a pre-emptive vaccine update may ensure a broad antibody response covering multiple strains (107). As covered in methodological considerations, influenza VE can also be reduced by egg adaptations of vaccine strains, and further compromised by the 6 months production delay.

The elderly were excluded from the initial COVID-19 vaccine trials due to comorbidities or medications that did not allow inclusion in such clinical trials (200). However, the frequency of polypharmacy in the elderly populations might affect vaccine effectiveness and safety, and their exclusion should be questioned. Much remained to be learned about potential differences in vaccine responses among younger and older adults as the vaccines were rolled out. Real-world experience revealed that older adults required subsequent booster doses to elicit immune responses comparable to younger adults (290, 291).

Surges of reinfections have been associated with the emergence of novel SARS-CoV-2 variants, especially omicron subvariants (292). A recently published test-negative, case-control study in unvaccinated adolescents found that previous infection with variants pre-omicron (ancestral strain or alpha) provided high (>86%) protection against symptomatic delta infection (293). Previous delta infection was highly protective (92.3%) against reinfection with the same variant, but protection against the antigenically distant omicron BA.1/2 variants was significantly lower (52.4%). This is consistent with our findings in **paper III** of young and healthy 10-20 year olds. Despite a new delta wave at the end of 2021, less than 1% had delta reinfections, but 55% experienced omicron BA.1/2 reinfection. Similarly, monovalent COVID-19 VE is drastically reduced against omicron subvariants due to significant antigenic drift.

Nearly all pre-delta infection vaccinated subjects in **paper III** who did not receive any further vaccinations were reinfected with omicron BA.1/2 (93%).

COVID-19 vaccination was recommended 3 months post-infection in Norway, and the majority of vaccinated children were vaccinated between November 2021 and January 2022 (**paper IV**). Higher VE has been observed in individuals with hybrid immunity, both pre- and post-omicron (293-296), possibly explained by the increased magnitude and durability of the antibody responses. This supports the added benefit of vaccination in previously infected individuals (243). Adolescents were primarily recommended the BNT162b2 vaccine (93%) due to the longer experience with the BNT162b2 vaccine and the rare, but increased risk of myocarditis and pericarditis in young males after the mRNA-1273 vaccine (194). Although heterologous vaccine combinations (mixing BNT162b2 and mRNA-1273) might provide higher VE (297), studies have found similar protection against omicron BA.1/2 between homologous BNT162b2 and mRNA-1273 vaccination series, and vaccine protection against omicron BA.1 and BA.2 is comparable (293). Strikingly, 55% of our cohort with hybrid immunity experienced an omicron BA.1/2 break-through infection, although all experienced mild disease. There is consensus that the current vaccines have maintained protection against severe disease, as well as reducing viral titres and shorten viral shedding upon break-through infection, further reducing viral transmission (298). However, epidemiological studies have shown equal risk of transmission in prior vaccinees or convalescent individuals compared to naïve individuals (299). Updating the COVID-19 vaccines with strains matching those in circulation will improve VE.

Other benefits of vaccination beyond preventing infection, is the potential of vaccines to reduce or mitigate post COVID-19 condition is promising (241). Primarily as infection prophylaxis, although also by reducing the severity of disease, which in itself is a risk factor for long-term complications. We found reduced length of sick leave in vaccinees versus unvaccinated individuals with long-term symptoms (unpublished data). Our cohort of delta infected children (**paper III**) experienced mild infections, which could explain why no difference could be found between the vaccinated and

unvaccinated group in acute symptoms or persisting symptoms 3 months post-infection. At 8 months post-infection, it was no longer possible to compare these groups, as all but 22 subjects were vaccinated. Most studies find symptom relief in vaccinated groups (39, 300, 301), although the outcome may change according to which symptoms are measured.

5.7 Infections and long-term complications

The societal impact of post COVID-19 condition may change according to the circulating variant. Our initial hypothesis was that the delta variant would cause more long-term symptoms than earlier circulating variants in children and adolescents, because of the reported higher viral load in the upper respiratory tract (31). Studies directly comparing the persisting symptom burden after infection by different variants are limited, perhaps due to the underestimated number of Wuhan infections in children (302). We had previously recruited an almost complete cohort of SARS-CoV-2 infected individuals during the first pandemic wave (238), of whom 23 were in the correct age range for comparison with our delta infected participants 10-20 years old (**paper III**). When comparing Wuhan and delta infected children, Wuhan infected subjects had a lower number of long-term symptoms 6 months after infection, while the delta infected children had higher frequencies of symptoms, but only significant for dyspnoea.

In **paper III**, the omicron BA.1 and BA.2 reinfections increased the frequencies of some long-term symptoms after delta infection, mainly in children 10-15 years. We observed more respiratory and systemic symptoms, but no change in reported taste/smell distortions and cognitive/neurological symptoms. This could indicate a more limited upper respiratory tract infection, although longer follow-up after omicron BA.1/2 infection is needed to draw conclusions. Certain long-term symptoms are reduced after infection with omicron subvariants compared to previous variants, but the overall post COVID-19 burden could be outweighed by the high number of infections (303, 304). There is also documentation of reinfections associated with

higher risk of post COVID-19 condition (214), which have become a prevalent problem after the emergence of omicron (**paper IV**). Furthermore, newer omicron subvariants escape immunity induced by prior subvariants (305, 306).

We found age <16 years and asymptomatic acute COVID-19 to be associated with protection against long-term symptoms (**paper III**). Adolescents 16-20 years were more likely to report long-term symptoms, especially dyspnoea, fatigue, and cognitive symptoms, which is similar to symptoms found in adults with post COVID-19 condition (307). The association between spike IgG antibodies and persisting symptoms is another similarity to adults. Several studies have found immune system dysfunctions in patients with post COVID-19 condition compared to controls, among them elevated SARS-CoV-2-specific antibodies (238, 239, 308, 309). Interestingly, the age groups at highest risk for persisting symptoms correlate with the expected strength of B cell responses (**Figure 20**). Persisting circulating and intestinal viral antigens have been detected in COVID-19 patients (310, 311), possibly facilitating the prolonged immune responses. Furthermore, hormonal imbalances are detected in patients with post COVID-19 symptoms, such as low level cortisol, which is critical in mediating stress responses (308, 312). Many hypercortisolism symptoms overlap with post COVID-19 condition, such as fatigue. Other factors point to the involvement of sex hormones, as females are at disproportional risk for post COVID-19 condition, consistent with our findings of an association between female sex and long-term dyspnoea. Furthermore, persisting symptoms appear after puberty and are absent in older adults, and have certain similarities to symptoms of menopause (313). Female sex and severe acute infection are similarly risk factors for post-viral fatigue after other viral infections, such as Epstein-Barr virus, dengue virus, and certain influenza viruses (314).

Long-term cognitive symptoms such as memory and concentration problems were more prevalent in adolescents compared to children in our study (**paper III**). Taste/smell impairment, headache, depression, and insomnia were other symptoms reported long-term. Many studies have hypothesised that persisting neuroinflammation

is causing neuronal damage resulting in these symptoms (315). Brain imaging has revealed reduced global brain size and grey matter and tissue damage in the olfactory cortex in patients with post COVID-19 condition (316). Whether the changes in the olfactory cortex are mediated by inflammation or reduced sensory input or represent viral spread through the olfactory nerve is unknown, although studies have not detected signs of classical viral encephalitis (317). Psychiatric and neurological symptoms after COVID-19 were found to differ in children and adults. Children had reduced depression and anxiety in 6 months post-infection, but were at heightened risk for cognitive deficit and insomnia (318).

At the time our study was initiated, a specific definition for post COVID-19 condition in children did not exist and has only recently been formulated. Based on the difference in symptoms between COVID-19 cases and controls, the definition especially emphasise fatigue, altered taste/smell and anxiety as important symptoms. However, due to the (I) evolving understanding of this condition, (II) high heterogeneity between studies, and (III) younger children often presenting atypical symptoms in general, several other symptoms were listed, including cognitive difficulties and dyspnoea (215)

Despite extensive research efforts, the cause of post COVID-19 syndrome is not yet known, which speaks to the complexity of the condition. With the emerging evidence of the pathophysiological mechanisms sustaining symptoms after COVID-19, it is important that this condition is recognised by the society at large. The impact on everyday life is substantial, limiting children's fundamental development, educational performance, physical activity, and social behaviour. Fortunately, there is hope for recovery, perhaps especially in the youngest children, who we found to gradually report fewer symptoms after infection.

6. Conclusions

This thesis has focused on cross-reactive antibody responses across different ages to influenza A/H3N2 and pandemic SARS-CoV-2. As more infectious SARS-CoV-2 VOC arose, we expanded the work and investigated the association of antibodies and risk factors for post COVID-19 condition in a young cohort.

Antibody repertoires towards viruses develop and expand with age, particularly against strains with similar characteristics as the priming virus. In 1968, the pandemic A/H3N2 virus arose and has caused repeated epidemics through rapid antigenic drift. In our influenza cohorts, we found pre-existing antibodies to many historical A/H3N2 viruses, especially in adults, facilitated by memory responses from previous infections and/or vaccinations. We found that vaccination with IIV or LAIV induced cross-reactive A/H3N2 antibody responses to historical viruses back to the individual's year of birth. Irrespective of age, antibodies elicited by the vaccine and infecting strain cross-reacted with drifted strains. Our findings from A/H3N2 imprinting and antibody repertoires after vaccination and infection may guide our future understanding of SARS-CoV-2 imprinting.

Similarly to influenza, nAbs to the SARS-CoV-2 main surface glycoprotein provide protection. To measure these antibodies, we demonstrated that a rapid, simple hemagglutination test could be readily adaptable to emerging VOC. When analysing the antibody responses to the novel mRNA vaccines in an elderly cohort, we observed lower mRNA vaccine immunogenicity than in younger adults. However, after completion of the primary mRNA vaccination series, high homologous and cross-reactive nAbs were elicited. Moreover, by using the HAT assay, we quantified specific antibodies in delta-infected children and adolescents, finding an association between high antibody levels and persisting COVID-19 symptoms. We further characterised post COVID-19 condition in this understudied young age group and found several of the same risk factors and symptoms previously established in adults. Children <16 years mainly recovered from COVID-19 with less reported long-term symptoms.

Although upon omicron BA.1/2 reinfection, children and adolescents >16 years reported similar frequencies of upper respiratory tract symptoms. Overall, we found limited vaccine protection against omicron BA.1/2 infection in adolescents, even with hybrid immunity. Our data suggest a persisting immune stimulation, based on the elevated spike-specific antibodies. Acute symptoms are found to be predictors of long-term complications and whilst vaccination may not prevent infection per se, it attenuates symptomatic illness. Therefore, improved vaccine effectiveness in this age group could reduce the frequencies of post COVID-19 condition.

In conclusion, this work emphasises the similarities and differences of these two respiratory viruses. Similar challenges exist in immunological imprinting and vaccine updates in the face of antigenic drift. However, post COVID-19 condition presented an unexpected challenge, even after mild cases in healthy adolescents. During the COVID-19 pandemic, flexible and practical laboratory assays allowed characterisation of virus specific antibodies to rapidly changing VOC. Vaccination reduces the risk of severe outcomes, while potentially reducing post COVID-19 condition. Overall, our studies may contribute to the development of improved future influenza and COVID-19 vaccines.

7. Future perspectives

The SARS-CoV-2 pandemic has emphasised the necessity of a united global effort to control the virus. The unprecedented pace of scientific advances, particularly in vaccine development, licensure and production will no doubt influence future influenza research. Furthermore, the widespread infection control measures substantially impacted influenza viruses' circulation, possibly extinguishing a viral lineage of influenza B. During the 2022-23 season, influenza viruses re-established their seasonal circulation pattern with a normal range of morbidity and mortality. However, the decreased influenza-specific population immunity and lack of imprinting in children may impact the severity of future influenza epidemics. Undoubtedly, vaccination remains the most effective protective measure. New vaccine technologies may eliminate production delays and egg-associated mutations. In the future, multiple influenza A strains, particularly A/H3N2, may be incorporated into current or next generation vaccines to induce improved cross-reactive immune responses. Future studies should focus on age-specific differences in influenza memory responses, and how tailored vaccines to specific target groups may mimic the advantages of long-term infection memory. Instead of focusing on how to overcome memory responses, their benefits could be used to our advantage. If improved early life vaccines were given prior to the imprinting infection, there is potential to induce durable, broad protection to multiple influenza viruses.

Supporting that imprinting is associated with variable antigens, there is evidence of back-boosting to the priming SARS-CoV-2 strain. However, *de novo* B cell responses are also generated to the new VOC. Furthermore, the substantial affinity maturation observed in the months following mRNA vaccination will ensure a diverse pool of cross-reactive antibodies. It is interesting that COVID-19 vaccination strategies have a similar trajectory as influenza vaccines, with regularly updated vaccine recommendations for high risk groups, such as the elderly. Although the vaccines were initially updated as bivalent, the latest recommendation includes only the XBB.1.5 subvariant. In time, we will know if SARS-CoV-2 will continue circulating similarly

to influenza in the future, requiring annual vaccination to maintain protection. The optimal COVID-19 vaccination formulation and interval is unknown, as protection from severe disease and death have been maintained by current vaccines. It has proven valuable to increase timing between vaccinations to allow waning of antibody responses, not forgetting the important factor of individual motivation to be vaccinated. Future vaccine strategies suitable for high-risk groups and healthy individuals across the ages are important knowledge gaps requiring further research.

As SARS-CoV-2 continues to circulate, population-based immunity is established and will probably be maintained. Despite the wealth of knowledge, there is still much to learn about COVID-19. It is currently uncertain if the burden of post COVID-19 condition is reduced following infection with omicron subvariants on a background of hybrid immunity. Research efforts must be continued to reduce and ultimately end long-term sequela in all age groups, especially in the youngest. Therefore, there is a need for a more standardised approach to studying paediatric post COVID-19 condition to understand the true global burden. This includes utilisation of the updated diagnostic definitions, with confirmed infection, and factoring in the impact on daily life. In the near future, pathophysiological explanations will hopefully inform prophylaxis or treatment options.

Finally, several questions remain: is it necessary to prevent all SARS-CoV-2 infections, even the mild cases? Generally, fewer long-term symptoms are expected after mild or asymptomatic disease, and it is uncertain if all infections must be avoided to reduce the burden of post COVID-19 condition. To mitigate transmission and prevent infection, an improved understanding of mucosal immunity is needed. Since a large proportion of the population have already been infected, the added benefits of mucosal vaccination are debatable. If prior infection fails to provide lasting sterilising immunity, are we expecting too much of COVID-19 vaccines? These are some of the unanswered questions that future research can focus on. The ultimate end-goal for both influenza and SARS-CoV-2 vaccines is developing universal vaccines providing broad B and T cell-mediated protection.

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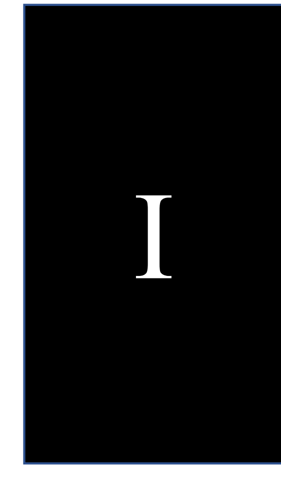
Papers I-IV



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ARTICLE OPEN

Seasonal influenza vaccination expands hemagglutinin-specific antibody breadth to older and future A/H3N2 viruses

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History of influenza A/H3N2 exposure, especially childhood infection, shape antibody responses after influenza vaccination and infection, but have not been extensively studied. We investigated the breadth and durability of influenza A/H3N2-specific hemagglutinin-inhibition antibodies after live-attenuated influenza vaccine in children (aged 3-17 years, $n = 42$), and after inactivated influenza vaccine or infection in adults (aged 22-61 years, $n = 42$) using 14 antigenically distinct A/H3N2 viruses circulating from 1968 to 2018. We found that vaccination and infection elicited cross-reactive antibody responses, predominantly directed against newer or future strains. Childhood H3-priming increased the breadth and magnitude of back-boosted A/H3N2-specific antibodies in adults. Broader and more durable A/H3N2-specific antibodies were observed in repeatedly vaccinated adults than in children and previously unvaccinated adults. Our findings suggest that early A/H3N2 exposure and frequent seasonal vaccination could increase the breadth and seropositivity of antibody responses, which may improve vaccine protection against future viruses.

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INTRODUCTION

Annual influenza epidemics cause 3-5 million cases of severe illness, and 290,000-650,000 respiratory deaths[1], with particularly increased mortality in epidemics dominated by influenza A/H3N2 viruses[2]. Since the COVID-19 pandemic, many countries have reinforced strict public-health measures, which also limit the spread of influenza viruses[3]. However, co-infection with influenza viruses in COVID-19 patients has been reported and is associated with increased disease severity and deaths[4,5]. Vaccination is the most effective way to prevent disease and annual influenza vaccination is recommended for high-risk groups[6]. Currently, there are two main types of seasonal influenza vaccines, inactivated influenza vaccine (IIV) and live-attenuated influenza vaccine (LAIV)[7]. Both vaccines aim to induce immunity against the major viral surface glycoprotein, hemagglutinin (HA)[8]. Antibodies directed against the globular head of HA can be measured in the hemagglutinin inhibition assay (HI), where a level of HI ≥40 is an established correlate of protection[9,10]. Influenza A/H3N2 undergoes more rapid viral drift than influenza A/H1N1 and B, facilitating a continual need for seasonal vaccine updates. Since the A/H3N2 virus appeared in 1968, 29 vaccine updates have taken place[11], versus 15 times for influenza A/H1N1 and 20 times for B[12,13]. Despite frequent vaccine updates, vaccine mismatches due to drifted A/H3N2 viruses during influenza seasons causes low vaccine effectiveness (VE)[14]. Therefore, the correct selection of A/H3N2 strains in seasonal vaccines is critical to improve vaccine-induced protection. However, the vaccine strain selection process has largely ignored the role of human factors, such as pre-existing immunity and repeated annual vaccination, influenced by a lifetime of viral encounters[15].

There are multiple theories of how pre-existing immunity may impact immune responses. Focus has largely been on how early-life influenza infections and repeated vaccination is shaping the immunity. In 1953 Francis launched his theory of the "original antigenic sin" describing an immunological dominance of the first infecting virus over successive influenza infections[16,17], where later infecting viruses elicit antibodies against the priming virus. Similarly, *Lesser et al*. found evidence of "antigenic seniority", where repeated exposure elicited the highest antibodies to "senior" strains from childhood[18]. Antibody cross-reactivity has been modelled to explain an individuals' complex influenza infection history[19]. Other studies focus on how priming or imprinting with influenza A subtypes in different birth cohorts can preferentially impact the antibody response and potentially reduce influenza mortality[20–24]. A recent theory, termed "back-boosting", does not restrict cross-reactive antibody responses after recent infection or vaccination to the primary infecting virus, but rather against all previously encountered viruses of the same influenza A subtype[25–28].

Studies of antibody landscapes against historical and recently circulating viruses are needed to understand how pre-existing immunity and historical exposure affects antibody responses. Furthermore, whether back-boosting responses vary in adults primed with different HAs compared to more naïve children and the effect of repeated vaccination on antibody cross-reactivity are unknown. Our study aimed to provide detailed characteristics of cross-reactive antibody responses in healthy adults and children, using 14 antigenically distinct A/H3N2 viruses which circulated over five decades, from 1968 to 2018. We studied A/H3N2-specific antibodies after recent infection, and single or repeated seasonal vaccination, conducting long-term follow-up[29,30]. We further investigated the extent and maintenance of A/H3N2 HI-antibody "back-boosting", the impact of "original antigenic sin" and childhood priming. Our findings provide insight to cross-reactive antibody responses by increasing age and repeated vaccination. We

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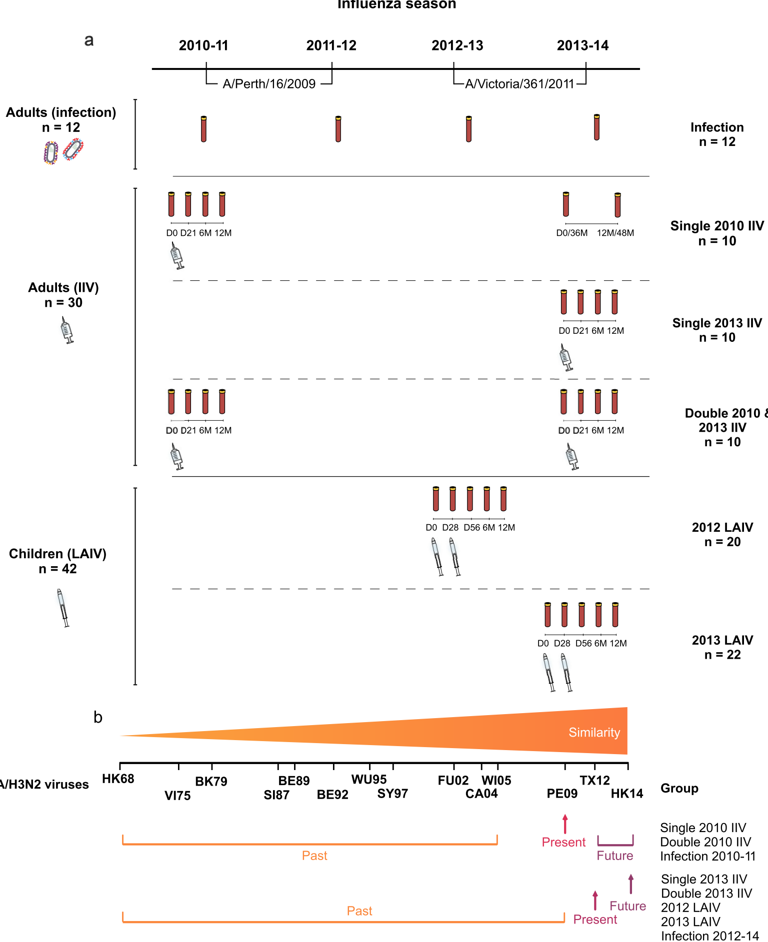

Fig. 1 Study design. During the influenza seasons 2010-14, the A/H3N2 component in the seasonal vaccine changed from A/Perth/16/2009 in 2010-12 seasons to A/Victoria/361/2011 in 2012-14 seasons. **a** A group of unvaccinated adults were infected with circulating influenza A/H3N2 viruses, defined as a fourfold seroconversion in HI titres between pre- and post-season blood samples (infection, $n = 12$). Thirty adults were vaccinated with IIV during the study period, generating three different vaccination groups ($n = 10$ each): single 2010 IIV, single 2013 IIV and double 2010 and 2013 IIV. Five individuals from the single 2010 IIV group provided long-term follow-up blood samples 36 and 48 months after vaccination (equivalent to day 0 and 12 months in the 2013-14 season). The children's cohort were vaccinated with either one (≥9 years old) or two doses (<9 years old) of a live-attenuated influenza vaccine (LAIV) in two different influenza seasons (2012 LAIV ($n = 20$) and 2013 LAIV ($n = 22$)). Blood samples were collected in all vaccinated individuals at day 0 (D0) and post-vaccination day 21/28 (D21/D28), day 56 (D56, only in children), 6 and 12 months (6 M, 12 M). (b) Timeline of A/H3N2 viruses circulated from 1968 to 2018 and the different groups' perspective of past, present and future viruses according to the timeline. Adults vaccinated or infected in 2010-12 with the A/Perth/16/2009 (PE09) had not yet been exposed to A/Texas/50/2012 (TX12) or A/HongKong/4801/2014 (HK14), defined as future strains for these subjects. Vaccinated or infected adults, and vaccinated children, in 2012-14 had not been exposed to the future strain HK14 which circulated from 2015-18.

show that vaccination elicited cross-reactive antibody responses similarly to infection-induced responses, highlighting the value of annual vaccination.

RESULTS

We investigated the breadth and durability of influenza A/H3N2-specific antibodies using 14 antigenically distinct A/H3N2 viruses circulating from 1968 to 2018 in groups of adults (vaccinated or infected) and vaccinated children (Fig. 1, Table 1). Vaccinated adults (aged 22-61 years, $n = 30$) received IIV either in 2010, 2013 or both years. Children (aged 3-17 years, $n = 42$) were vaccinated with LAIV in 2012 or 2013. Blood samples were collected in all vaccinated individuals at day 0 and postvaccination day 21/28, day 56 (only children), 6 and 12 months. The unvaccinated adults provided blood samples in September/October each year in 2010-2014, and natural infection was confirmed by seroconversion in twelve adults. The majority of all adults (32/42) and half of children (20/42) were female. No significant differences in age or sex between the infected or vaccinated adult groups or between

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Table 1. The 14 genetically and antigenically distinct influenza A/H3N2 viruses used in the study.

Influenza season	A/H3N2 vaccine strain[1]	Strains used in study	Abbreviation
1970-72	A/Hong Kong/1/1968-like	A/Hong Kong/1/1968	HK68
1976-80	A/Victoria/3/1975-like	A/Texas/1/1977	VI75
1980-83	A/Bangkok/01/1979-like	A/Bangkok/01/1979	BK79
1988-89	A/Sichuan/02/1987-like	A/Sichuan/02/1987	SI87
1991-93	A/Beijing/353/1989-like	A/Beijing/353/1989	BE89
1993-94	A/Beijing/32/1992-like	A/Beijing/32/1992	BE92
1996-98	A/Wuhan/359/1995-like	A/Wuhan/359/1995	WU95
1998–2000	A/Sydney/5/1997-like	A/Sydney/5/1997	SY97
2004-05	A/Fujian/411/2002-like	A/Wyoming/3/2003	FU02
2005-06	A/California/7/2004-like	A/New York/55/2004	CA04
2006-08	A/Wisconsin/67/2005-like	A/Hiroshima/52/2005	WI05
2010-12	A/Perth/16/2009-like	A/Wisconsin/15/2009	PE09
2012-15	A/Victoria/361/2011-like	A/Texas/50/2012	TX12
2016-18	A/Hong Kong/4801/2014-like	A/Hong Kong/4801/2014	HK14

[1]WHO recommended

The influenza A/H3N2 virus that caused the 1968 pandemic and the 13 predominant circulating A/H3N2 strains until 2018 were selected. All strains were recommended by the World Health Organization (WHO) to be included in seasonal influenza vaccines for the Northern hemisphere. All 14 viruses were egg-grown and inactivated before use in the hemagglutination inhibition assay.

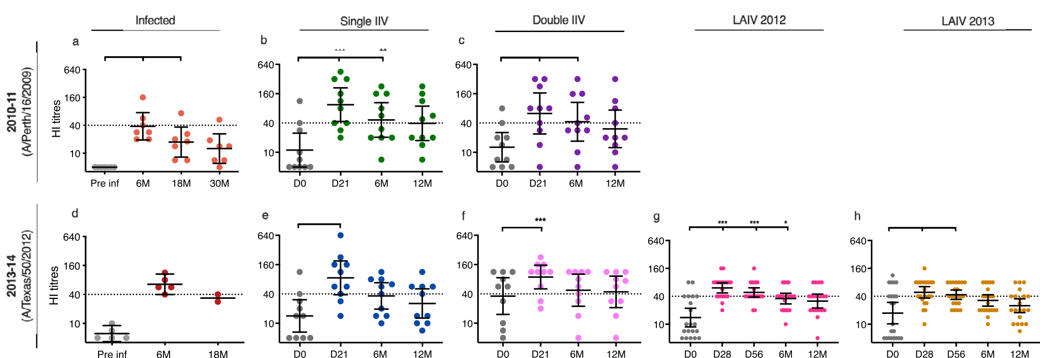

Fig. 2 Homologous hemagglutination inhibition (HI) titres after influenza A/H3N2 infection and vaccination in adults and children. Adults were either infected or vaccinated with seasonal inactivated influenza vaccines (IIVs) and children were vaccinated with seasonal live-attenuated influenza vaccines (LAIV). Serum samples were collected once a year before the start of influenza season in infected adults (September/October) and time post-infection was calculated from the season seroconversion occurred. HI titres against homologous A/Perth/16/2009 (H3N2) or A/Texas/50/2012 (H3N2) are shown in adults pre- and post-infection ($n = 7$ or 5, respectively) (**a**, **b**) and in adults pre- (day 0 (DO)) and post-IIV (day 21 (D21), 6 (6 M) and 12 months (12 M)) ($n = 10$ each) (**c–f**). LAIV was not licensed in Europe and Norway until 2012, therefore we do not have vaccination responses against A/Perth/16/2009 in children. HI responses to the homologous A/Texas/50/2012 (H3N2) vaccine strain pre- (day 0) and post-LAIV (day 21, 56, 6, and 12 months) in 2012 ($n = 20$) (**g**) and 2013 ($n = 22$) (**h**). Each symbol represents an individual HI titre. The horizontal lines show geometric mean HI titres with 95% confidence interval. The dotted line indicates an HI titre of 40. Pre- and post-vaccination HI titres were compared using nonparametric repeated measure Friedman test with Dunn's multiple comparison correction for each vaccination group, except the TX12 infected group due to missing sample for long-term follow-up, which was analysed using non-parametric Wilcoxon matched-pairs signed-rank test for pre- and 6 M post-infection time points. *$P < 0.05$, **$P < 0.01$, ***$P = < 0.001$.

groups of children receiving LAIV were found (Supplementary Table 1).

Infection and vaccination boosted homologous antibodies

We first tested the homologous HI antibody response after infection or vaccination in adults and children. Twelve unvaccinated adults, with no or low pre-existing antibody titres, seroconverted against influenza A/H3N2 viruses during the 4-year period, probably due to infection. These adults had increases in HI titres after infection, 7 individuals against the A/Perth/16/2009 (PE09) virus ($p < 0.001$) between 2010-12, and 5 adults against the A/Texas/50/2012 (TX12) ($p = 0.062$) in 2012-14 (Fig. 2a, d). Antibody titres then waned overtime in the infected individuals.

We found significant increases in HI titres against the homologous vaccine strains after vaccination in all groups (Fig. 2b, c, e–h). Post-vaccination responses were surprisingly comparable to post-infection responses against PE09 and TX12. A higher number of children had pre-vaccination HI titres ≥40 in the LAIV group vaccinated in 2013 than in 2012, however, post-vaccination titres were similar between the two groups (Fig. 2g, h). The double IIV group had higher pre-vaccination titres ($p = 0.0637$) but comparable post-vaccination titres to the single 2013 IIV group (Fig. 2e, f). This double IIV group also had higher titres

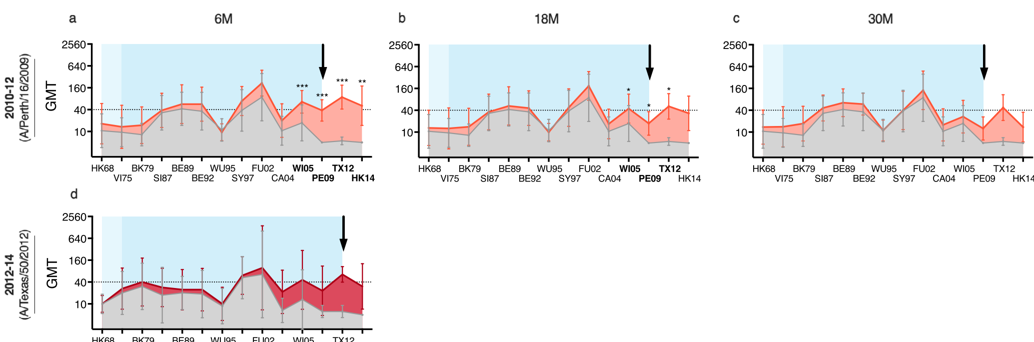

Fig. 3 Hemagglutination inhibition (HI) antibody landscapes after A/H3N2 infection in adults. Antibody landscapes were generated with geometric mean HI titres (GMTs) against 14 historical and future influenza A/H3N2 viruses (see Table 1 for the strains used). Error bars represent the 95% confidence intervals of the GMTs. In Norway, the influenza season usually starts at the end of October and peaks between December and March. Blood samples were collected annually in September/October from 2010 to 2014 from unvaccinated adults. Seroconversion (four-fold increase in HI titres) between two time points against the predominantly circulating A/H3N2 viruses were defined as infection. The time post-infection was calculated from the season seroconversion occurred. All individual landscapes are shown in Supplementary Figure 2a. HI antibody landscapes are shown pre-infection (grey) and at 6-9 months (6 M) (**a**), 18–21 months (18 M) (**b**) and 30-33 months (30 M) (**c**) post-infection with PE09, $n = 7$, and at 6-9 months post-infection with TX12, $n = 5$ (**d**). No landscape was generated at 18-21 months post-TX12 infection because only 2 individuals provided serum samples. The viral exposure period is shown in blue based on the group's median age, and in light blue indicating the oldest individual in the group. Pre- and post-infection HI titres were compared using non-parametric repeated measure Friedman test with Dunn's multiple comparison correction for each infected group. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P = < 0.001$.

than both groups of children pre-vaccination (2012 $p = 0.039$, 2013 $p = 0.085$) and 21/28 days post-vaccination (2012 $p = 0.067$, 2013 $p = 0.0164$) (Fig. 2f, g, h). Notably, only the double IIV group had durable HI titres ≥40 at 12 months post-vaccination while neither the single IIV nor the LAIV children achieved this.

Vaccination and infection increased heterologous antibodies

To investigate the breadth of antibody responses after infection and vaccination, we evaluated HI antibodies against 14 antigenically distinct A/H3N2 viruses spanning from 1968 to 2018 (Fig. 1b, Table 1). Antibody landscapes were generated from the HI titres pre- and post-exposure for each individual (Supplementary figure 2,3) and from the geometric mean titres (GMTs) for each group (Figs. 3–5).

We observed that infected adults had detectable pre-existing antibodies (>10) against a number of past A/H3N2 viruses, but not against the infecting or future viruses circulating in subsequent years (Fig. 3a, d). Following PE09 infection (n = 7), HI antibodies increased significantly ($p < 0.01$) against the infecting (PE09) and future (TX12, HK14) viruses and back-boosted against the closest virus, WI05, while antibodies against the distant historical viruses were maintained above the pre-infection levels (Fig. 3a). HI titres waned over the next two years post-PE09 infection, but remained elevated above pre-infection levels (Fig. 3b, c). A similar trend of antibody responses was observed after infection with TX12, although not statistically significant probably due to a lower number of subjects in this subgroup ($n = 5$) (Fig. 3d).

The IIV adults also had pre-existing antibodies against historical viruses dating back to HK68 (Fig. 4). Unlike the infection group, all IIV groups had pre-vaccination titres against PE09 and TX12. Vaccination elicited significant increases in HI titres ≥40 at day 21 to the vaccine viruses, the previously circulating (back to SY97 or CA04) and the future strain(s) (TX12 and/or HK14) (Fig. 4a–d). In all IIV groups, the GMTs remained above baseline at 12 months post-vaccination against the four strains WI05, PE09, TX12 and HK14.

The LAIV children had pre-vaccination antibodies against recent (SY97-TX12) and future (HK14) viruses, but no antibodies against historical viruses (HK68-WU95) that circulated before they were born (Fig. 5a, b). A significant increase in HI titres at 28 days post-LAIV was observed against the four strains WI05, PE09, TX12 and HK14 in the 2012 LAIV group ($p < 0.001$) and against TX12 and HK14 in the 2013 LAIV group ($p < 0.01$). These vaccine-induced antibodies waned throughout the 12 months post-LAIV, but remained above pre-vaccination levels. The antibody landscape in children was lower in magnitude and breadth after LAIV compared to after IIV in adults, with HI titres ≥40 post-LAIV only elicited against the TX12 vaccine virus in both groups and additionally WI05 in the 2012 LAIV group.

Repeated vaccination in adults maintained cross-reactive antibody responses

We followed vaccinees in the single 2010 IIV group ($n = 5$) up to 48 months and observed a decrease in antibody titres to below baseline against most viruses at 36-48 months after vaccination (Fig. 4a, Supplementary Figure 4). In contrast, the double 2010 and 2013 IIV group ($n = 10$) maintained antibodies at or above baseline levels against all 14 viruses at 36 months after 2010 vaccination (first IIV) (Fig. 4c). HI antibodies against these 4 strains WI05-HK14 were boosted following the second IIV in 2013 ($p < 0.01$, except HK14), and remained elevated above the pre-2010 IIV baseline at the end of the 2013 season (Fig. 4a, d). This suggests an advantage of repeated vaccination in inducing durable cross-reactive antibody responses.

Priming influenced antibody landscapes

To better understand the effect of priming and age on antibody cross-reactivity after infection and vaccination, we divided children into two birth cohorts based on the recommendation of one or two doses of LAIV; 2003-2009 (3-9 years old, $n = 31$) and 1995-2002 (10-17 years old, $n = 11$). Adults were divided into three birth cohorts according to the likelihood of priming with different influenza A subtypes that circulated when they were born[22]: 1948-1966 (H1/H2 primed, $n = 14$), 1967-1976 (H3 primed, $n = 14$) and 1977-1987 (H1/H3 primed, $n = 14$) (Fig. 6). As expected, the antibody landscapes reflect the lifetime experience of A/H3N2 virus encounters. The children had pre-existing antibodies against recent viruses that circulated in the years since

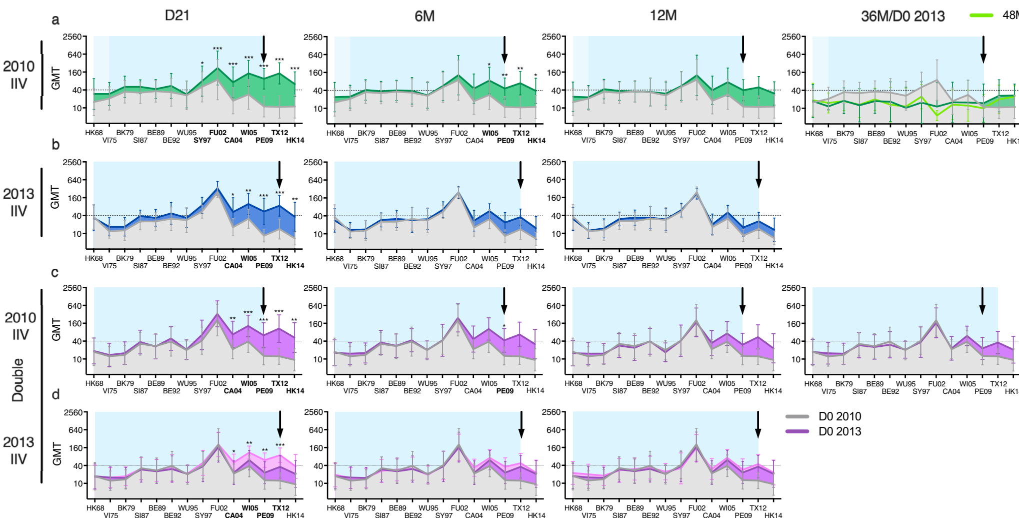

Fig. 4 The A/H3N2-specific hemagglutination inhibition (HI) antibody landscapes after vaccination with inactivated influenza vaccines (IIV) in adults. Antibody landscapes were generated using the groups' geometric mean HI titre (GMT) against 14 antigenically distinct influenza A/H3N2 viruses (see Table 1 for the strains used). Error bars represent the 95% confidence intervals of the GMTs. The pre-vaccination GMTs at day (D)0 are displayed in grey. **a** Antibody landscapes of adults vaccinated with single IIV in 2010, $n = 10$ (single 2010 IIV) at D21, 6 months (M), 12 M, 36 M (equivalent to D0 in 2013) (dark green), and 48 M (equivalent to 12 M in 2013) (light green) post-IIV. **b** Antibody landscapes of adults vaccinated in 2013, $n = 10$ (single 2013 IIV) at D21, 6 M and 12 M post-IIV (dark blue). (**c–d**): Antibody landscapes of adults vaccinated in both 2010 and 2013 (double IIV), $n = 10$, at D21, 6 M, 12 M and 36 M after 2010 IIV (dark purple) (**c**) or after 2013 IIV (light purple) (**d**). The individual vaccinee landscapes can be found in Supplementary Figure 2b–d. The black arrow indicates the vaccination virus, and the dotted line indicates the HI titre of 40. The viral exposure period is shown in blue based on the group's median age, and light blue indicating the oldest individual in the group. Pre- and post-IIV titres were compared using nonparametric repeated measure Friedman test with Dunn's multiple comparison correction for each vaccination group. *$P < 0.05$, **$P < 0.01$, ***$P = < 0.001$.

they were born (Fig. 6a, b). Following vaccination, antibodies were significantly "back-boosted" against these viruses and induced to future virus HK14 ($p < 0.05$). Interestingly, LAIV induced antibodies against the PE09 virus ($p = 0.027$) in the older children born 1995-2002, despite no pre-existing antibodies towards this strain. Adults had higher and broader cross-reactive antibody responses compared to children. There were significant differences between adults and children against viruses that circulated before children were born (HK68-FU02) (Supplementary Table 6). Only the 1967-1976 birth cohort had significantly higher titres than children cohorts post-exposure against all viruses, including the newer strains (CA04-HK14).

Pre-existing titres were similar against recently circulating strains from SY97 to HK14 between the different adult birth cohorts, but differed against historical strains (Fig. 6c–e, Supplementary Table 6). The two older birth cohorts (1948-1966 and 1967-1976) had higher pre-existing titres to HK68 and VI75 than the youngest adults, which circulated before this cohort was born. The 1967-1977 birth cohort had broader antibody responses post-exposure, against 10 of 14 viruses (9 viruses $p < 0.05$ and 1 virus $p = 0.055$), including the oldest HK68 ($p = 0.046$). In contrast, the antibodies in the youngest and oldest adults were boosted primarily against the newer viruses (Fig. 6c–e), back to SY97 or CA04, respectively. The 1967-1976 birth cohort had higher titers post-exposure against HK14, BE92, BE89 and BK79 ($p < 0.1$) compared to the 1948-1966 birth cohort, who did not have the advantage of early childhood H3-priming.

HI-antibody responses were broadened by age and vaccination

To further explore the lifetime impact of influenza A/H3N2-exposure, we calculated the seroprotection rates (percentages of individuals with HI titres ≥40) against the 14 A/H3N2 strains in the five birth cohorts (Supplementary figure 5a–f). We then plotted the cumulative seroprotection rates against the number of viruses pre- and post-exposure (Fig. 7a–c). These seroprotection curves aided in illustrating the breadth of antibody responses against the range of 1-14 A/H3N2-viruses, regardless of when they circulated. We found that the adults had significantly higher seropositivity pre-exposure compared to children, particularly the 1967-1976 birth cohort (Fig. 7a, Supplementary Table 7). The greatest differences pre-exposure between the birth cohorts were observed to 5 viruses, to which ≥43% of adults had protective antibodies, compared to only 6-9% of children. The seroprotection curves were expanded in all birth cohorts after exposure, although the adults had significantly higher percentages of seroprotection than children (Fig. 7b, c, Supplementary Table 7). The 1995-2002 birth cohort had a peak post-exposure seroprotection curve similar to adults up to 7 viruses (Fig. 7b), but did not maintain the seropositivity long-term (Fig. 7c). Importantly, the 1967-1976 birth cohort had broader seroprotection curves after exposure than the 1948-1966 birth cohort (peak $p = 0.049$, long-term $p = 0.078$), and only this H3-primed cohort had protective antibodies to ≥12 viruses (Fig. 7b, c).

Similarly, we investigated the impact of previous vaccination on the breadth of antibody responses by comparing cumulative seroprotection rates against the range of 1-14 A/H3N2 viruses between adults with different previous vaccination status (Fig. 7d–f) (see Supplementary figure 5g–I for seroprotection rates against each A/H3N2 virus). The children were not included since healthy children are not recommended for annual influenza vaccination in Norway (only 2/42 of children had previously been vaccinated). We found that adults with prior influenza vaccination had a significantly broader seroprotection curve than the

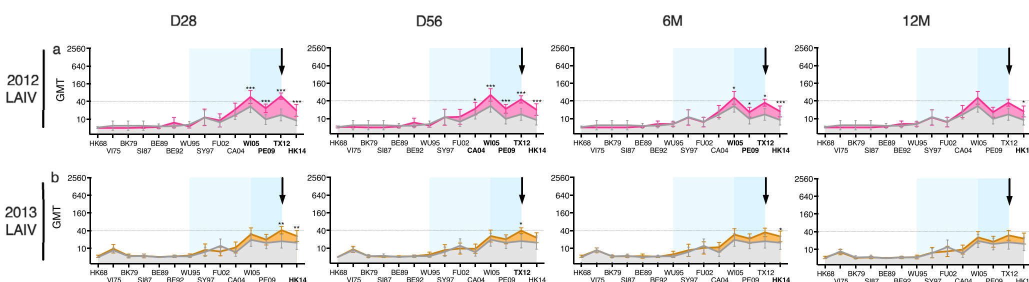

Fig. 5 The A/H3N2-specific hemagglutination inhibition (HI) antibody landscapes after vaccination with live-attenuated influenza vaccines (LAIV) in children. Antibody landscapes were generated using the groups' geometric mean HI titre (GMT) against 14 antigenically distinct influenza A/H3N2 viruses (see Table 1 for the strains used). Error bars represent the 95% confidence intervals of the GMTs. The pre-vaccination GMTs at day (D)0 are displayed in grey. Antibody landscapes of children vaccinated with LAIV in 2012 (2012 LAIV, $n = 20$) (pink) (**a**) or in 2013 (2013 LAIV, $n = 22$) (brown) (**b**) at D28, D56, 6 months (M) and 12 M post-LAIV are shown. Individual landscapes can be found in Supplementary Figure 3. The black arrow indicates the vaccination virus, and the dotted line indicates the HI titre of 40. The viral exposure period is shown in blue based on the group's median age, and light blue indicating the oldest individual in the group. Pre- and post-LAIV titres were compared using non-parametric repeated measure Friedman test with Dunn's multiple comparison correction for each vaccination group. *$P < 0.05$, **$P < 0.01$, ***$P = < 0.001$.

previously unvaccinated or infected adults before exposure (Supplementary Table 7, Fig. 7d). The biggest difference pre-exposure was found against 8 viruses, where 50% of the previously vaccinated adults were seropositive compared to only 8% of the previously unvaccinated or infected adults. No significant differences between the groups were found after exposure (Fig. 7e, f). However, we observed a trend of higher percentages of previously vaccinated adults having protective antibodies long-term post-exposure compared to the previously unvaccinated ($p = 0.068$) or infected ($p = 0.135$) adults (Fig. 7f), suggesting a better maintenance of protective antibodies after repeated vaccination.

DISCUSSION

Since its appearance in 1968, the influenza A/H3N2 has undergone frequent antigenic drift, outpacing vaccine strain selection and reducing seasonal VE. Therefore, studies of antibody landscapes against historical and recent circulating A/H3N2 viruses are important to understand the effect of pre-existing immunity and priming-related differences of antibody responses to current virus exposure. To the best of our knowledge, our study is unique in describing the breadth, magnitude, and durability of antibody responses to 14 historical, recent, and future A/H3N2 viruses after LAIV in children, compared to after IIV or infection in adults. We found that antibody landscapes after vaccination or infection are affected by pre-existing immunity, shaped by priming, prior vaccination status and age. Pre-existing antibodies were found against viruses dating back to the individual's birth year, in line with earlier studies[16,27]. The pattern of antibody responses post-vaccination or infection followed the pre-existing antibodies. However, the greatest antibody increase post-exposure was observed against viruses that circulated within the last 5-8 years, regardless of priming pattern, vaccination or infection status, or pre-existing immunity. Importantly, antibodies were induced to future strains which the individuals had not yet encountered, potentially providing partial immunity toward future A/H3N2 strains. Remarkably, the exclusively H3-primed adult group had increases in antibodies to most viruses post-exposure, indicating that childhood priming increases the breadth and magnitude of antibody cross-reactivity. Overall, the durability of antibody responses was positively impacted by childhood H3-priming and repeated vaccination, as reflected by broader antibody responses. Our study has implications for the development of next-generation influenza vaccines, and ultimately universal influenza vaccines that can provide antibody-based protection against past and future viruses.

Our findings of narrower antibody responses to A/H3N2 viruses in children agreed with previous findings[31,32] since age is associated with greater influenza exposure[33]. Vaccination broadened the antibody responses in children, however, adults had maintained broader antibody responses. Importantly, we showed that higher proportions of adults who were previously vaccinated had protective antibodies pre- and post-exposure against higher numbers of A/H3N2 viruses, compared to adults who had not been vaccinated previously or were infected. This suggests that annual influenza vaccination expanded and maintained the breadth of antibody responses as vaccinated individuals had pre-existing protective antibodies and durable post-vaccination antibodies. Unvaccinated individuals with no or low pre-existing antibodies remain more susceptible to influenza infection in the coming seasons as shown in our infection group.

We found that the reactivity to the oldest viruses increased after vaccination or infection, although non-significantly, and did not change the overall antibody landscape. In contrast to the theories of "original antigenic sin" and "antigenic seniority" we found that the more recently circulating viruses (SY97-HK14) dominated the cross-reactive HI-antibody responses in all birth cohorts with higher titres post-exposure. These viruses are more genetically and antigenically similar to the vaccine or infecting viruses than the oldest viruses (Supplementary Figure 1). Our observations best fit a cross-reactive hypothesis, boosting a homologous response, as well as antibodies directed against conserved HA-head epitopes on other strains[26]. Despite the constant evolution of the A/H3N2-viruses, some conserved epitopes remain in the head region, allowing for cross-reactive antibody responses[34]. Whilst we would expect cross-reactivity against TX12 to be induced by the closely related PE09, antibodies were also elicited against the future drifted HK14 virus that dominated from 2015 to 2018. However future studies are needed to confirm cross-reactive antibody responses to circulating viruses[35,36]. On the other hand, the H3-primed adults back-boosted antibodies against most viruses, even the oldest HK68, thus the cross-reactive hypothesis alone does not fully explain our results. Long-term memory responses are probably influenced by multiple mechanisms, including the order of influenza infections[37]. Our findings agree with previous reports of back-boosting[25,27,28], and further extend the theory by detecting antibodies to an advanced, future strain. More studies of antibody landscapes are needed to better understand the impact of pre-existing immunity on antibody

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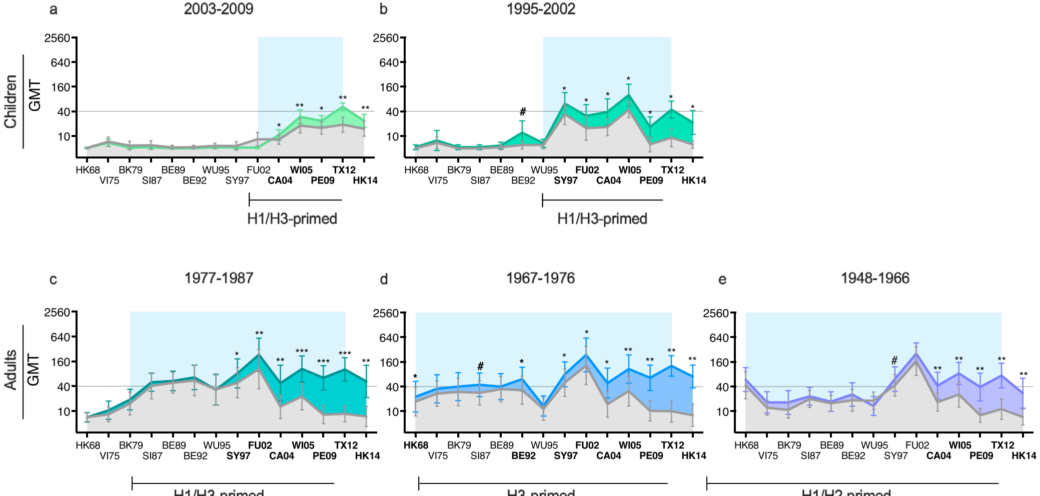

Fig. 6 The A/H3N2-specific hemagglutination inhibition (HI) antibody landscapes by birth-year in adults and children. Antibody landscapes were generated using the groups' geometric mean HI titre (GMT) against 14 antigenically distinct influenza A/H3N2 viruses (see Table 1 for the strains used). Error bars represent the 95% confidence intervals of the GMTs. Children and adults were grouped by their birth year regardless of the year they were vaccinated or infected. The pre-existing HI titres are displayed in grey. Peak titres after vaccination or infection were used (21/28 days post-vaccination or 6 months post-infection). Antibody landscapes post-vaccination of children born between 2003 and 2009 ($n = 31$) in light green (**a**) and children born between 1995 and 2002 ($n = 11$) in dark green (**b**). Antibody landscapes post-vaccination or infection of adults born 1977-1987 ($n = 14$) in ocean green (**c**), adults born 1967-1976 ($n = 14$) in blue (**d**), and adults born 1948-1966 ($n = 14$) in lavender (**e**). The dotted line indicates the HI titre of 40 and the period of viral exposure is highlighted by a light blue background. Pre- and post-vaccination or infection HI titres were compared using non-parametric Wilcoxon matched-pairs signed-rank test with individual ranks computed for each comparison, and Holm-Šídák method for multiple comparisons. $^{\#}P < 0.1$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P = < 0.001$.

responses. It remains to be seen if our findings extend to different populations and with larger sample sizes.

With occasional antigenic shift and continuous drift, adults and children will have different priming patterns to influenza A subtypes, according to their birth cohorts. The children's landscapes reflect their infection history, since healthy Norwegian children are rarely recommended for influenza vaccination (only 0.05% in our cohort). The younger children had pre-existing antibodies against the newer strains PE09-HK14, whereas the older children had pre-existing titres to the older strains SY97-WI05, which circulated in their early childhood. This suggests that older children might not be susceptible to influenza infection with the same subtype for a number of years after their first childhood infection[21]. It is unclear how the children's landscapes will evolve in the future, especially since LAIV-induced immunity is multifaceted and different to antibody-mediated immunity after IIV. We propose that there may be an advantage of childhood A/H3N2 vaccination upon antibody responses in the future. Interestingly, the PE09-unprimed older children clearly demonstrated induction of a cross-reactive antibody response to this strain after LAIV with TX12. This finding supports the vaccine strategy of pre-emptive vaccine updates with an advanced A/H3N2 strain to potentially provide the dual benefit of cross-reactive antibody responses directed to both advanced and previous viruses[25].

The A/H3N2 viruses have been associated with the lowest VE of all vaccine strains[14,38], and reduced VE after repeated vaccination[39,40]. However, we found that vaccination-elicited A/H3N2-specific antibody responses were equivalent to infection, suggesting a robust vaccine response. We further observed that repeated seasonal vaccination induced more durable cross-reactive antibody responses than in singly vaccinated individuals or those without previous influenza vaccination, suggesting a potential advantage of repeated vaccination. This discrepancy could be due to the way VE is measured by the test-negative study design, which is largely biased to symptomatic patients requiring medical attention[41,42]. Whereas our findings suggest that repeatedly vaccinated individuals may be partially protected against severe disease and therefore experience asymptomatic or mild infection not seeking medical care. Our healthy adult cohort was <65 years old, a group with higher vaccine performance than the elderly who often experience the greatest burden of A/H3N2 infections. Our results are in line with previous studies, demonstrating that repeated vaccination did not reduce influenza vaccine protection[43–45] and beneficially induced antibody responses to drifted strains[46]. However, we did not directly evaluate the vaccine protection, but rather measured seroprotection. We suggest that previous vaccination history and priming effects should be accounted for in future VE studies.

Caveats to our study are limited numbers of individuals, although we have included both adults and children, but not the elderly. Furthermore, we used the dominant circulating strains and defined infection by seroconversion at 6-9 months post-infection, since we did not have clinical or laboratory confirmation, limiting the number of subjects in the infection group. We used the HI assay to measure protective antibodies against the head region of HA against 14 A/H3N2 viruses, since only this assay has established correlates of protection. However, IIV-induced antibodies are known to be strain- and HA head-specific, whereas infection and LAIV immunisation elicit a multifaceted response directed against different viral antigens, which are not measured by the HI assay. We cannot exclude all evidence of "original antigenic sin" in our population, as we did not measure HA stalk-targeting antibodies. In addition, detection of traditional HI antibodies is becoming an increasing problem with the most

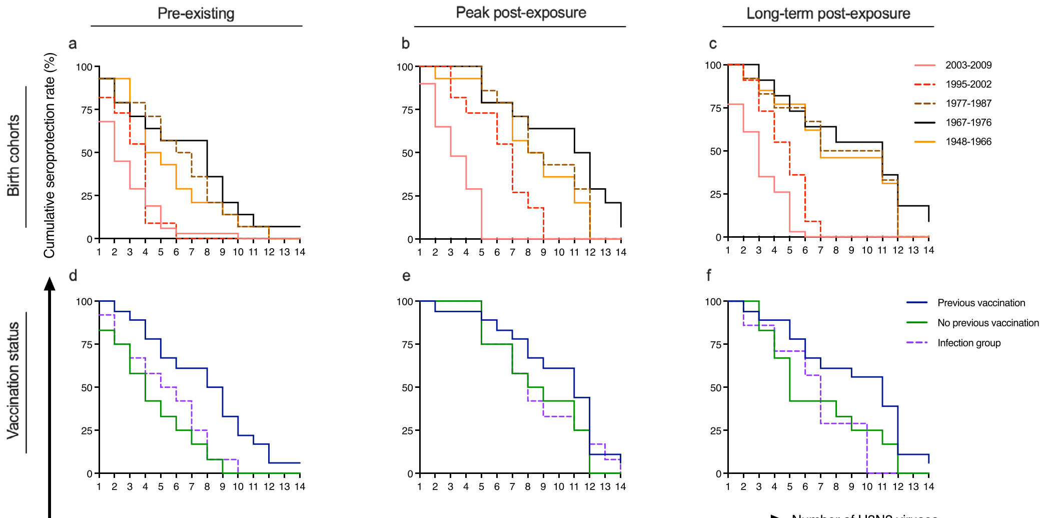

Fig. 7 The impact of priming and previous vaccination on the breadth of hemagglutination inhibition (HI) A/H3N2-specific antibody responses. The cumulative seroprotection rates (cumulative percentages of individuals with HI titres ≥40) against a range of influenza A/H3N2 viruses (total 14 viruses) regardless of the viruses' circulation time pre-exposure (pre-vaccination or infection) ($n = 84$) (**a**), peak responses post-exposure (day 21/28 post-vaccination or 6 months post-infection) ($n = 84$) (**b**), and long-term post-exposure (6 months post-vaccination or 18 months post-infection) ($n = 79$) (**c**). Subjects were stratified by their birth year based on the likelihood of priming with different influenza A subtypes (**a–c**). Children were divided into two birth cohorts: 2003-2009 and 1995-2002 (both H1/H3 primed) and adults were divided into 3 birth cohorts: 1977-1987 (H1/H3 primed), 1967-1976 (H3 primed) and 1948-1966 (H1/H2 primed). The cumulative seroprotection rates against the numbers of influenza A/H3N2 viruses in adults pre-exposure ($n = 42$) (**d**), peak post-exposure ($n = 42$) (**e**), and long-term post-exposure ($n = 37$) (**f**) were stratified by previous vaccination history and compared to the infection group.

recent A/H3N2 strains[47], although we found no differences in HI titres against the newest virus (HK14) using an egg-grown virus and different species' red blood cells (Supplementary Figure 6). Since egg-adapted changes in HK14 vaccine viruses have been reported to alter antigenicity impacting vaccine-induced antibody responses in different age groups[35,36], our results should be interpreted with caution. Further studies are warranted to investigate the clinical impact of cross-reactive antibody responses upon protection from infection.

Our results show that antibody landscapes diversified with increasing age. However, not only age, but multiple factors, such as the order and variability of influenza exposure influences the antibody back-boosting responses. Individual landscapes displayed great diversity and were not simply guided by the first or successive influenza infections or vaccinations, although priming-related factors were observed in different birth cohorts. More durable HI antibodies were detected after repeated vaccination, and a history of multiple vaccinations broadened the HI antibodies and increased the pool of cross-reactive HI antibody responses. These antibodies might provide partial immunity against novel influenza viruses or the recurring problem of influenza A/H3N2 antigenic drift during an influenza season. Vaccination with A/H3N2 strains in childhood and repeated vaccination with advanced drifted strains may improve vaccine protection against this subtype.

METHODS

Study design

Adults (21-61 years old) and children (3-17 years old) ($n = 42$ per group) were included in this study which was approved by the regional ethics committee (Regional Committee for Medical Research Ethics, Western Norway (2009/1224 and 2012/1088) and the Norwegian Medicines Agency (National Institute for Health database Clinical trials.gov (NCT01003288 (adults) and NCT01866540 (children)). All participants provided written informed consent. The study population was retrospectively selected to compare A/H3N2-specific antibody responses after IIV, LAIV and infection (Fig. 1a). The majority of adults (32/42) and half of children (20/42) were female (Supplementary Table 1). Depending upon their seasonal vaccination history, adults were divided into three groups ($n = 10$/group): single vaccination in 2010 or 2013, or double vaccination in 2010 and 2013 IIV[30]. Unvaccinated adults who were infected with circulating A/H3N2 viruses were included for comparison (infection group, $n = 12$). Children were intranasally immunized with seasonal LAIV either in 2012 (2012 LAIV, $n = 22$) or in 2013 (2013 LAIV, $n = 20$)[29].

Vaccines

The trivalent seasonal IIV was either subunit (Influvac, Abbott Laboratories) or split-virion (Vaxigrip, Sanofi Pasteur) containing 15 µg HA per strain. The trivalent LAIV contained 10^7 fluorescent focus units (FFU) of each strain (FLUENZ, AstraZeneca). The A/H3N2 viruses changed between seasons from A/Perth/16/2009 in 2010-11 and 2011-12 to A/Victoria/361/2011 in 2012-13 and 2013-14.

Blood samples

Serum samples were collected pre- and post-vaccination (21 days, 6 and 12 months) in the three IIV adult groups and once a year before the start of influenza season (September/October) in the infected adults. Five adults in the single 2010 IIV group provided long-term follow-up blood samples at 36 and 48 months after vaccination. Plasma samples were collected pre- and post-vaccination (28 and 56 days, 6 and 12 months) in the children (Fig. 1a). Blood samples were aliquoted and stored at −80 °C until used in the HI assay.

Viruses

Fourteen genetically and antigenically different A/H3N2 viruses were included in the study, spanning from 1968 to 2018 (Fig. 1b, Table 1, Supplementary Figure 1). The wild-type A/Hong Kong/1/1968 (HK68) virus was propagated in embryonated hen eggs and used in the HI assay. The

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twelve previously circulating viruses and one future strain A/Hong Kong/4801/2014 (HK14) were inactivated egg-grown viruses, derived from either reassortant vaccine strains or reference wild-type viruses (obtained from the National Institute for Biological Standards and Controls (NIBSC), UK or the International Reagent Resource (IRR), USA).

Hemagglutination inhibition assay

The HI assay was conducted as previously described[30]. Briefly, blood samples were treated with receptor-destroying enzyme (Seiken, Japan) and pre-adsorbed with packed turkey red blood cells (TRBC) before serial dilution from 1/10 in duplicate and incubation with 4 hemagglutinating units of each virus and 0.5% (volume/volume) TRBC. The HI titre was read as the reciprocal of the highest dilution that inhibited 100% hemagglutination. Negative values were assigned a titre of 5 for calculation purposes. GMTs were calculated from duplicates and reported as final titre for each sample. Fourfold or higher increases in HI titres were considered seroconversion.

Statistical analysis

HI data was analysed and visualized in Prism version-9 (GraphPad Software, USA). Missing data at 6 months were interpolated for subjects that had day-21 and 12-month data using linear regression models ($n = 6$). Other missing data at random were interpolated using the group's GMT ($n = 6$). Non-parametric ANOVA Friedman, Wilcoxon test or Kruskal-Wallis test were used as appropriate to compare within or between groups, with multiple comparison correction. Log-rank Mantel-Cox test was used to compare the cumulative seroprotection curves between groups. Exact p-values and other statistical values are reported in Supplementary Tables 2-7. $P < 0.05$ was considered statistically significant.

Reporting Summary

Further information on research design is available in the Nature Research Reporting Summary linked to this article.

DATA AVAILABILITY

The datasets generated during and/or analysed during the current study are available from the corresponding authors on reasonable request. Supplementary Figures 2–3 show associated raw data.

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AUTHOR CONTRIBUTIONS

M.-C.T. and R.J.C. designed the study. K.A.B. and K.G.I.M. collected and processed the LAIV samples. N.U.E., S.L.L. and M.-C.T. conducted the HI assay. N.U.E. analysed the data. N.U.E., M.-C.T. and R.J.C. interpreted the data and wrote the manuscript. S.L.L., K.G.-I.M. and K.A.B. made substantial contributions to the manuscript. All authors have read and approved the submitted version of the paper.

COMPETING INTERESTS

All authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

ADDITIONAL INFORMATION

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Seasonal influenza vaccination expands heamagglutinin-specific antibody breadth to older and future A/H3N2 viruses

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Supplementary file

Supplementary Tables 1-7

Supplementary Figures 1-6

Supplementary Table 1: Demographics of the study population

Characteristics	**Adults**					**Children**		
	Total (n=42)	**Infection** (n=12)	**2010 IIV** (n=10)	**2013 IIV** (n=10)	**2010 & 2013 IIV** (n=10)	**Total** (n=42)	**2012 LAIV** (n=20)	**2013 LAIV** (n=22)
Age (median ± SD)	36.0 ± 9.6	33.5 ± 8.3	34.5 ± 7.7	46.0 ± 11.1	37 ± 9.1	5.0 ± 5.2	5.0 ± 5.5	5.0 ± 4.9
Sex (Female/Male)	32/10	9/3	7/3	9/1	7/3	20/22	8/12	12/10
High-risk condition*	1 (2.4%)	0 (0%)	0 (0%)	0 (0%)	1 (10%)	-	-	-
Previous seasonal vaccination	22 (52.4%)	3 (25%)	6 (60%)	8 (80%)	5 (50%)	-	-	-

*Pregnancy, chronic respiratory diseases, neurological diseases, immunosuppressive diseases, heart diseases and obesity.
- Not applicable for children.
A total of 42 adults and 42 children were included in the study.

Supplementary Table 2: Statistical analysis for Figure 2

Analysis	Multiple comparison		Friedman test	
	Z value	Adjusted p value	Friedman statistics	P value
PE09 infected (n=7), Fig 2a				
Pre inf vs 6M	4.244	**<0.0001**	19.46	**0.0002**
Pre inf vs 18M	2.484	**0.0389**		
TX12 infected (n=5), Fig 2b				
Pre inf vs 6M	-	0.0625*	-	-
Single IIV 2010 (n=10), Fig 2c				
D0 vs D21	4.850	**<0.0001**	27.60	**<0.0001**
D0 vs 6M	3.291	**0.0030**		
Single IIV 2013 (n=10), Fig 2d				
D0 vs D21	3.811	**0.0004**	20.01	**0.0002**
Double IIV 2010 (n=10), Fig 2e				
D0 vs D21	3.897	**0.0003**	20.09	**0.0002**
D0 vs 6M	2.425	**0.0459**		
Double IIV 2013 (n=10), Fig 2f				
D0 vs D21	3.724	**0.0006**	22.07	**<0.0001**
LAIV 2012 (n=22), Fig 2g				
D0 vs D28	5.438	**<0.0001**	50.35	**<0.0001**
D0 vs D56	4.258	**<0.0001**		
D0 vs 6M	2.616	**0.0356**		
LAIV 2013 (n=20), Fig 2h				
D0 vs D28	3.855	**0.0005**	31.67	**<0.0001**
D0 vs D56	3.025	**0.0099**		

*Two-tailed exact p value from Wilcoxon test.

Data was analysed using a matched, repeated measure, non-parametric Friedman's test with Dunn's multiple comparison test, except the TX12 infected group due to missing samples for long-term follow-up. The TX12 infected group was analysed using non-parametric Wilcoxon matched-pairs signed rank test for pre-infection and 6-9M post infection time points.

Supplementary Table 3: Statistical analysis for Figure 3 and 4

Analysis			Multiple comparison		Friedman test	
			Z value	Adjusted p value	Friedman statistics	P value
PE09 infected						
	Fig 3a (n=7)					
	Pre inf vs 6M					
		HK14	3.519	**0.0013**	17.21	**0.0006**
		TX12	4.347	**<0.0001**	20.45	**0.0001**
		PE09	4.244	**<0.0001**	19.46	**0.0002**
		WI05	3.726	**0.0006**	18.78	**0.0003**
	Fig 3b (n=7)					
	Pre inf vs 18M					
		TX12	2.484	**0.0389**	20.45	**0.0001**
		PE09	2.484	**0.0389**	19.46	**0.0002**
		WI05	2.588	**0.0290**	18.78	**0.0003**
TX12 infected						
	Fig 3d (n=5)					
	Pre inf vs 6M					
		TX12	-	0.0625*	-	-
2010 IIV (n=10), Fig 4a						
	D0 vs D21					
		HK14	3.897	**0.0003**	21.48	**<0.0001**
		TX12	4.850	**<0.0001**	28.16	**<0.0001**
		PE09	4.850	**<0.0001**	27.60	**<0.0001**
		WI05	4.590	**<0.0001**	25.84	**<0.0001**
		CA04	3.811	**0.0004**	18.80	**0.0003**
		FU02	3.984	**0.0002**	20.84	**0.0001**
		SY97	2.858	**0.0128**	16.14	**0.0011**
	D0 vs 6M					
		HK14	2.511	**0.0361**	21.48	**<0.0001**
		TX12	3.551	**0.0012**	28.16	**<0.0001**
		PE09	3.291	**0.0030**	27.60	**<0.0001**
		WI05	4.590	**0.0361**	25.84	**<0.0001**
2013 IIV (n=10), Fig 4b						
	D0 vs D21					
		HK14	3.464	**0.0016**	19.64	**0.0002**
		TX12	3.811	**0.0004**	20.01	**0.0002**
		PE09	3.811	**0.0004**	20.93	**0.0001**
		WI05	3.118	**0.0055**	15.64	**0.0013**
		CA04	2.858	**0.0128**	13.88	**0.0031**
Double 2010 IIV (n=10), Fig 4c						
	D0 vs D21					
		HK14	3.204	**0.0041**	16.54	**0.0009**
		TX12	4.070	**0.0001**	21.19	**<0.0001**
		PE09	3.897	**0.0003**	20.09	**0.0002**
		WI05	3.724	**0.0006**	17.23	**0.0006**
		CA04	2.944	**0.0097**	14.10	**0.0028**
	D0 vs 6M					
		PE09	3.897	**0.0459**	20.09	**0.0002**
Double 2013 IIV (n=10), Fig 4d						
	D0 vs D21					
		TX12	3.724	**0.0006**	22.07	**<0.0001**
		PE09	3.377	**0.0022**	18.63	**0.0003**
		WI05	2.944	**0.0097**	14.82	**0.0020**
		CA04	2.858	**0.0128**	13.70	**0.0034**

*Two-tailed exact p value from Wilcoxon test.

Data was analysed using a matched, repeated measure, non-parametric Friedman's test with Dunn's multiple comparison test, except the TX12 infected group due to missing samples. The TX12 infected group was analysed using non-parametric Wilcoxon matched-pairs signed rank test for pre infection and 6-9M post infection time points.

Supplementary Table 4: Statistical analysis for Figure 5

Analysis	Multiple comparison		Friedman test	
	Z value	Adjusted p value	Friedman statistics	P value
2012 LAIV (n=20), Fig 5a				
D0 vs D28				
HK14	4.207	**0.0001**	26.69	**<0.0001**
TX12	5.438	**<0.0001**	50.39	**<0.0001**
PE09	4.720	**<0.0001**	40.63	**<0.0001**
WI05	3.796	**0.0006**	34.72	**<0.0001**
D0 vs D56				
HK14	4.300	**<0.0001**	26.69	**<0.0001**
TX12	4.258	**<0.0001**	50.39	**<0.0001**
PE09	4.001	**0.0003**	40.63	**<0.0001**
WI05	4.617	**<0.0001**	34.72	**<0.0001**
CA04	2.770	**0.0224**	24.10	**<0.0001**
D0 vs 6M				
HK14	3.642	**0.0011**	26.69	**<0.0001**
TX12	2.565	**0.0413**	50.39	**<0.0001**
PE09	2.565	**0.0413**	40.63	**<0.0001**
WI05	2.924	**0.0138**	34.72	**<0.0001**
D0 vs 12M				
HK14	2.514	**0.0478**	26.69	**<0.0001**
2013 LAIV (n=22), Fig 5b				
D0 vs D21				
HK14	3.220	**0.0051**	16.30	**0.0026**
TX12	3.318	**0.0036**	20.61	**0.0004**
D0 vs D56				
TX12	2.830	**0.0186**	20.61	**0.0004**
D0 vs 6M				
HK14	2.879	**0.0160**	16.30	**0.0026**

Data was analysed using a matched, repeated measure, non-parametric Friedman’s test with Dunn’s multiple comparison test.

Supplementary Table 5: Statistical analysis for Figure 6

Analysis		Wilcoxon tests		Summary
		P value	Adjusted p value	
Born 2003-2009 (n=31), Fig 6a				
	HK14	0.0014	**0.0042**	**
	TX12	0.0001	**0.0003**	**
	PE09	0.0233	**0.0310**	*
	WI05	0.0001	**0.0002**	**
	CA04	0.0156	**0.0310**	*
Born 1995-2002 (n=11), Fig 6b				
	HK14	0.0039	**0.0270**	*
	TX12	0.0020	**0.0155**	*
	PE09	0.0039	**0.0270**	*
	WI05	0.0078	**0.0270**	*
	CA04	0.0039	**0.0270**	*
	FU02	0.0156	**0.0310**	*
	SY97	0.0039	**0.0270**	*
Born 1977-1987 (n=14), Fig 6c				
	HK14	0.0005	**0.0024**	**
	TX12	0.0001	**0.0010**	***
	PE09	0.0001	**0.0010**	***
	WI05	0.0001	**0.0010**	***
	CA04	0.0010	**0.0029**	**
	FU02	0.0005	**0.0024**	**
	SY97	0.0137	**0.0272**	*
Born 1967-1976 (n=14), Fig 6d				
	HK14	0.0002	**0.0022**	**
	TX12	0.0001	**0.0012**	**
	PE09	0.0002	**0.0022**	**
	WI05	0.0010	**0.0068**	**
	CA04	0.0020	**0.0117**	*
	FU02	0.0020	**0.0117**	*
	SY97	0.0020	**0.0117**	*
	BE92	0.0156	**0.0461**	*
	SI87	0.0547	0.0547	#
	HK68	0.0234	**0.0463**	*
Born 1948-1966 (n=14), Fig 6e				
	HK14	0.0040	**0.0078**	**
	TX12	0.0005	**0.0029**	**
	PE09	0.0010	**0.0049**	**
	WI05	0.0020	**0.0078**	**
	CA04	0.0020	**0.0078**	**
	SY97	0.0840	0.0840	#

Statistical analyses were performed with non-parametric Wilcoxon matched-pairs signed rank test with individual ranks computed for each comparison, and Holm-Šídák method for multiple comparisons.

Supplementary Table 6: Statistical analysis of group comparisons for Figure 6

Analysis	Pre-exposure		Post-exposure	
	t value	Adjusted p value	t value	Adjusted p value
HK14				
1967-1976 vs. 1948-1966	0.271	ns	2.582	0.0718
1967-1976 vs. 1995-2002	0.642	ns	3.089	**0.0184**
1967-1976 vs. 2003-2009	2.027	ns	3.564	**0.0038**
1995-2002 vs. 2003-2009	2.596	0.0914	0.276	ns
TX12				
1967-1976 vs. 1995-2002	0.243	ns	2.753	0.0528
1967-1976 vs. 2003-2009	2.048	ns	2.921	**0.0351**
PE09				
1967-1976 vs. 1995-2002	1.277	ns	3.547	**0.0041**
1977-1987 vs. 1995-2002	0.705	ns	3.483	**0.0046**
1967-1976 vs. 2003-2009	1.393	ns	3.325	**0.0073**
1977-1987 vs. 2003-2009	2.109	ns	3.245	**0.0084**
1995-2002 vs. 2003-2009	2.745	0.0599	1.022	ns
WI05				
1948-1966 vs. 2003-2009	1.155	ns	3.366	**0.0055**
1967-1976 vs. 2003-2009	1.791	ns	4.163	**0.0003**
1977-1987 vs. 2003-2009	0.757	ns	4.084	**0.0004**
1995-2002 vs. 2003-2009	2.652	0.0783	3.594	**0.0027**
CA04				
1948-1966 vs. 2003-2009	2.317	ns	4.482	**<0.0001**
1967-1976 vs. 2003-2009	1.919	ns	4.961	**<0.0001**
1977-1987 vs. 2003-2009	1.442	ns	4.881	**<0.0001**
1995-2002 vs. 2003-2009	2.080	ns	3.873	**0.0008**
FU02				
1948-1966 vs. 1995-2002	6.115	**<0.0001**	5.430	**<0.0001**
1967-1976 vs. 1995-2002	5.479	**<0.0001**	5.239	**<0.0001**
1977-1987 vs. 1995-2002	4.907	**<0.0001**	5.175	**<0.0001**
1948-1966 vs. 2003-2009	9.643	**<0.0001**	12.54	**<0.0001**
1967-1976 vs. 2003-2009	8.848	**<0.0001**	12.30	**<0.0001**
1977-1987 vs. 2003-2009	8.132	**<0.0001**	12.22	**<0.0001**
1995-2002 vs. 2003-2009	1.828	ns	5.270	**<0.0001**
SY97				
1948-1966 vs. 2003-2009	6.489	**<0.0001**	7.912	**<0.0001**
1967-1976 vs. 2003-2009	6.967	**<0.0001**	8.776	**<0.0001**
1977-1987 vs. 2003-2009	6.887	**<0.0001**	8.870	**<0.0001**
1995-2002 vs. 2003-2009	5.271	**<0.0001**	7.320	**<0.0001**
WU95				
1977-1987 vs. 1967-1976	2.982	**0.0203**	2.446	ns
1948-1966 vs. 1995-2002	2.820	**0.0289**	1.820	ns
1977-1987 vs. 1995-2002	4.410	**0.0001**	4.115	**0.0004**
1948-1966 vs. 2003-2009	3.669	**0.0020**	2.975	**0.0237**
1967-1976 vs. 2003-2009	2.158	ns	2.975	**0.0237**
1977-1987 vs. 2003-2009	5.658	**<0.0001**	5.846	**<0.0001**
BE92				
1967-1976 vs. 1948-1966	1.559	ns	2.379	0.0517
1977-1987 vs. 1948-1966	2.982	**0.0146**	2.582	**0.0453**
1948-1966 vs. 1995-2002	2.884	**0.0159**	1.936	ns
1967-1976 vs. 1995-2002	4.346	**0.0001**	4.167	**0.0002**
1977-1987 vs. 1995-2002	5.681	**<0.0001**	4.358	**0.0001**
1948-1966 vs. 2003-2009	4.000	**0.0004**	5.265	**<0.0001**
1967-1976 vs. 2003-2009	5.830	**<0.0001**	8.057	**<0.0001**
1977-1987 vs. 2003-2009	7.500	**<0.0001**	8.296	**<0.0001**

	1995-2002 vs. 2003-2009	0.360	ns	2.608	**0.0453**
BE89					
	1967-1976 vs. 1948-1966	2.169	0.0882	2.311	0.0618
	1977-1987 vs. 1948-1966	3.117	**0.0093**	3.126	**0.0091**
	1948-1966 vs. 1995-2002	2.763	**0.0231**	2.782	**0.0218**
	1967-1976 vs. 1995-2002	4.797	**<0.0001**	4.949	**<0.0001**
	1977-1987 vs. 1995-2002	5.687	**<0.0001**	5.714	**<0.0001**
	1948-1966 vs. 2003-2009	3.480	**0.0031**	3.988	**0.0004**
	1967-1976 vs. 2003-2009	6.025	**<0.0001**	6.700	**<0.0001**
	1977-1987 vs. 2003-2009	7.139	**<0.0001**	7.658	**<0.0001**
	1995-2002 vs. 2003-2009	0.021	ns	0.466	0.6577
SI87					
	1948-1966 vs. 1995-2002	3.560	**0.0019**	3.790	**0.0008**
	1967-1976 vs. 1995-2002	4.450	**<0.0001**	5.448	**<0.0001**
	1977-1987 vs. 1995-2002	5.404	**<0.0001**	5.766	**<0.0001**
	1948-1966 vs. 2003-2009	3.880	**0.0007**	4.729	**<0.0001**
	1967-1976 vs. 2003-2009	4.993	**<0.0001**	6.803	**<0.0001**
	1977-1987 vs. 2003-2009	6.187	**<0.0001**	7.202	**<0.0001**
BK79					
	1967-1976 vs. 1948-1966	2.779	**0.0329**	2.445	0.0573
	1948-1966 vs. 1995-2002	1.971	ns	2.898	**0.0190**
	1967-1976 vs. 1995-2002	4.577	**<0.0001**	5.191	**<0.0001**
	1977-1987 vs. 1995-2002	2.861	**0.0298**	3.280	**0.0064**
	1948-1966 vs. 2003-2009	1.963	ns	3.685	**0.0017**
	1967-1976 vs. 2003-2009	5.224	**<0.0001**	6.555	**<0.0001**
	1977-1987 vs. 2003-2009	3.077	**0.0170**	4.163	**0.0003**
VI75					
	1967-1976 vs. 1977-1987	3.185	**0.0118**	3.330	**0.0072**
	1967-1976 vs. 1995-2002	3.514	**0.0041**	3.900	**0.0009**
	1948-1966 vs. 2003-2009	1.642	ns	2.892	**0.0270**
	1967-1976 vs. 2003-2009	4.188	**0.0003**	5.365	**<0.0001**
HK68					
	1948-1966 vs. 1967-1976	2.507	0.0600	2.650	**0.0322**
	1948-1966 vs. 1977-1987	4.947	**<0.0001**	5.844	**<0.0001**
	1967-1976 vs. 1977-1987	2.440	0.0600	3.194	**0.0072**
	1948-1966 vs. 1995-2002	5.531	**<0.0001**	6.213	**<0.0001**
	1967-1976 vs. 1995-2002	3.179	**0.0091**	3.726	**0.0012**
	1948-1966 vs. 2003-2009	5.531	**<0.0001**	7.905	**<0.0001**
	1967-1976 vs. 2003-2009	3.905	**0.0007**	4.794	**<0.0001**

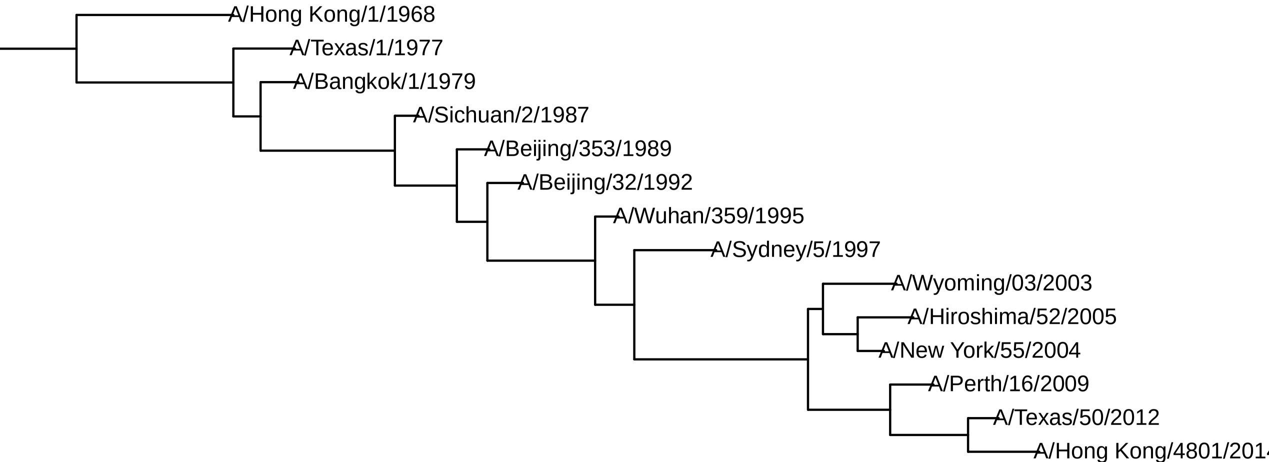
Data was analysed using two-way ANOVA and Holm-Sidak's multiple comparisons test with individual variances computed for each comparison.

ns – not significant p value > 0.05

Supplementary Table 7: Statistical analysis of seroprotection for Figure 7

Analysis	Chi-square	P value	Summary
Figure 7a – Pre-exposure			
1967-1976 vs. 1995-2002	7.189	**0.0073**	**
1948-1966 vs. 2003-2009	8.378	**0.0038**	**
1967-1976 vs. 2003-2009	11.95	**0.0005**	***
1977-1987 vs. 2003-2009	10.45	**0.0012**	**
Figure 7b – Peak post-exposure			
1967-1976 vs. 1948-1966	3.861	**0.0494**	*
1948-1966 vs. 1995-2002	4.616	**0.0317**	*
1967-1976 vs. 1995-2002	9.858	**0.0017**	**
1977-1987 vs. 1995-2002	6.660	**0.0099**	**
1948-1966 vs. 2003-2009	25.28	**<0.0001**	***
1967-1976 vs. 2003-2009	28.45	**<0.0001**	***
1977-1987 vs. 2003-2009	30.21	**<0.0001**	***
1995-2002 vs. 2003-2009	16.05	**<0.0001**	***
Figure 7c – Long-term post-exposure			
1967-1976 vs. 1948-1966	3.102	0.0782	ns
1967-1976 vs. 1995-2002	8.747	**0.0031**	**
1948-1966 vs. 1995-2002	3.956	**0.0047**	*
1977-1987 vs. 1995-2002	7.727	**0.0054**	**
1967-1976 vs. 2003-2009	19.59	**<0.0001**	***
1948-1966 vs. 2003-2009	15.21	**<0.0001**	***
1977-1987 vs. 2003-2009	20.61	**<0.0001**	***
1995-2002 vs. 2003-2009	6.972	**0.0083**	**
Figure 7d – Pre-exposure			
Prev vacc vs no prev vacc	8.904	**0.0028**	**
Prev vacc vs infected	5.526	**0.0187**	*
Figure 7f – Long-term post-exposure			
Prev vacc vs no prev vacc	3.339	0.0677	ns
Prev vacc vs infected	2.234	0.1350	ns

Data was analysed using log-rank Mantel-Cox test.
ns – not significant p value > 0.05



Supplementary Figure 1: Phylogenetic tree of influenza A/H3N2 viruses based on the hemagglutinin (HA) sequence. The HA sequences of 14 influenza A/H3N2 strains circulated from 1968 up until 2018 were obtained from National Center for Biotechnology Information (NCBI, USA, https://www.ncbi.nlm.nih.gov) and Global initiative on sharing all influenza data (GISAID, https://www.gisaid.org). The phylogenetic tree was generated using NGphylogeny.fr, a free online tool from NCBI. HA distances of 0.01 is indicated in the upper left corner.

Supplementary Figure 2: Adult individual landscapes

a) Infection group

YoB	2010	2011	2012	2013	2014	Previously vaccinated*
1965						2006
1972						2007
1976						
1978						
1980						
1981						
1985						
1955						2007
1967						
1973						
1975						
1980						

*Last previous seasonal vaccination.

Geometric mean (GM) haemagglutination inhibition (HI) titers are shown on the Y-axis and the 14 antigenically distinct influenza A/H3N2 viruses from old (1968) to new (2018) along the X-axis. The grey shaded area represents pre-infection titers, orange and red represents post-infection titres at up to three different years. Adults were divided into two groups based on the infecting virus: the first 7 individuals were infected with a PE09-like virus in either 2011 or 2012 (orange post-infection titres), the remaining 5 adults were infected with TX12-like virus in 2013 or 2014 (red post-infection titres). The black arrow indicates the infecting virus, and the dotted line indicates the HI titre of 40. The period of viral exposure is highlighted by a light blue background. Adults were arranged according to birth year (left hand side) within each group.

b) Single 2010 IIV

YoB	D0	D21	6M	12M	Previously vaccinated*
1960					
1963					
1968					2008
1973					
1973					1998
1976					2009
1978					
1978					2008
1982					2003
1983					2009

*Last previous seasonal vaccination.

Geometric mean (GM) haemagglutination inhibition (HI) titers are shown on the Y-axis and the 14 antigenically distinct Influenza A/H3N2 viruses from old (1968) to new (2018) along the X-axis. The grey area represents pre-IIV titers at Day 0 (D0), dark green represents post-IIV titres at 21 days (21D), 6 months (6M) and 12 months (12M). Ten adults were included in this group, arranged according to their birth year (left hand side). The black arrow indicates the vaccination virus, and the dotted line indicates the HI titre of 40. The period of viral exposure is highlighted by a light blue background.

c) Single 2013 IIV

YoB	D0	D21	6M	12M	Previously vaccinated*
1948					2007
1953					
1956					2000
1958					2008
1961					2005
1965					2007
1965					
1975					2004
1977					2007
1982					2008

*Last previous seasonal vaccination.

Geometric mean (GM) haemagglutination inhibition (HI) titers are shown on the Y-axis and the 14 antigenically distinct Influenza A/H3N2 viruses from old (1968) to new (2018) along the X-axis. The grey area represents pre-IIV titers, dark blue represents post-IIV titres at 21 days (21D), 6 months (6M) and 12 months (12M). Ten adults were included in this group, arranged according to their birth year (left hand side). The black arrow indicates the vaccination virus, and the dotted line indicates the HI titre of 40. The period of viral exposure is highlighted by a light blue background.

d) Double 2010 & 2013 IIV

YoB	2010 D0	2010 D21	2010 6M	2010 12M	2013 D0	2013 D21	2013 6M	2013 12M	Previously vaccinated*
1961									2008
1962									2008
1966									2008
1968									
1969									2007
1975									
1975									
1982									
1983									
1987									2008

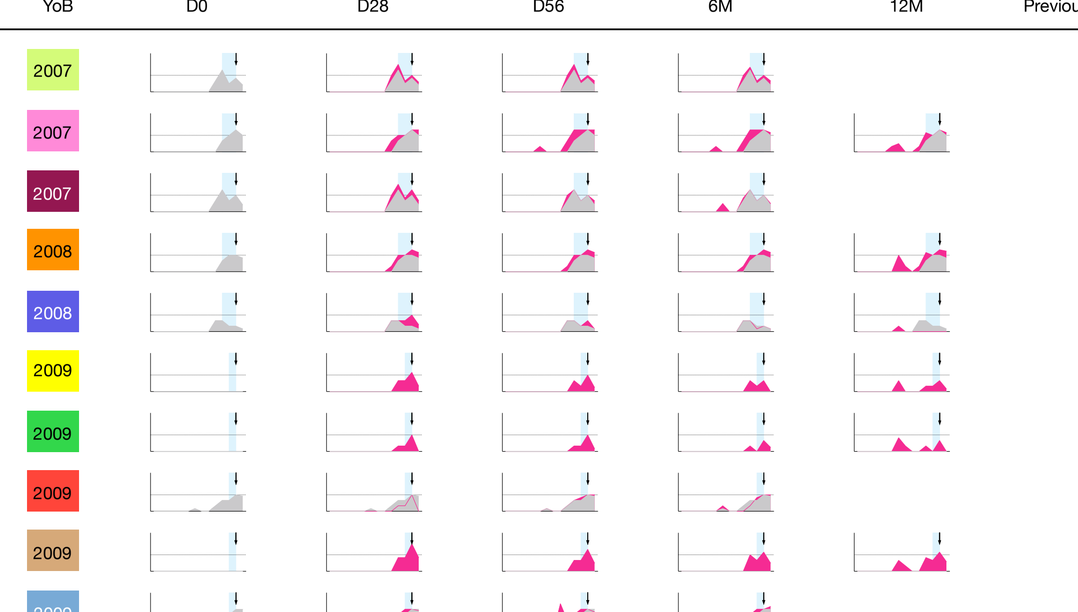
*Last previous seasonal vaccination.

Geometric mean (GM) haemagglutination inhibition (HI) titers are shown on the Y-axis and the 14 antigenically distinct Influenza A/H3N2 viruses from old (1968) to new (2018) along the X-axis. The grey area represents pre-IIV titers at Day 0 (D0) 2010, post-IIV titres in dark purple after 1st vaccination (PE09) 21 days (21D), 6 months (6M) and 12 months (12M).and in light purple after 2nd vaccination (TX12) at D21, 6 and 12M. Ten adults were included in this group, arranged according to birth year (left hand side). The black arrow indicates the vaccination virus, and the dotted line indicates the HI titre of 40. The period of viral exposure is highlighted by a light blue background.

Supplementary Figure 3: Individual landscapes after live attenuated influenza vaccine (LAIV) in children

a) LAIV 2012

*Previous seasonal vaccination.



Geometric mean (GM) haemagglutination inhibition (HI) titers are shown on the Y-axis and the 14 antigenically distinct Influenza A/H3N2 viruses from old (1968) to new (2018) along the X-axis. The grey area represents pre-LAIV titers (Day 0, D0), pink represents post-IIV titres at 28 days (28D) and 56D, 6 months (6M) and 12 months (12M). Twenty children were included in this group (**a**) 10 and (**b**) 10, arranged according to birth year (left hand side). The black arrow indicates the vaccination virus, and the dotted line indicates the HI titre of 40. The period of viral exposure is highlighted by a light blue background.

b) LAIV 2012

YoB	D0	D28	D56	6M	12M	Previously vaccinated*
1996						
1998						
1998						
2000						
2002						
2006						
2007						
2007						
2007						
2008						
2008						

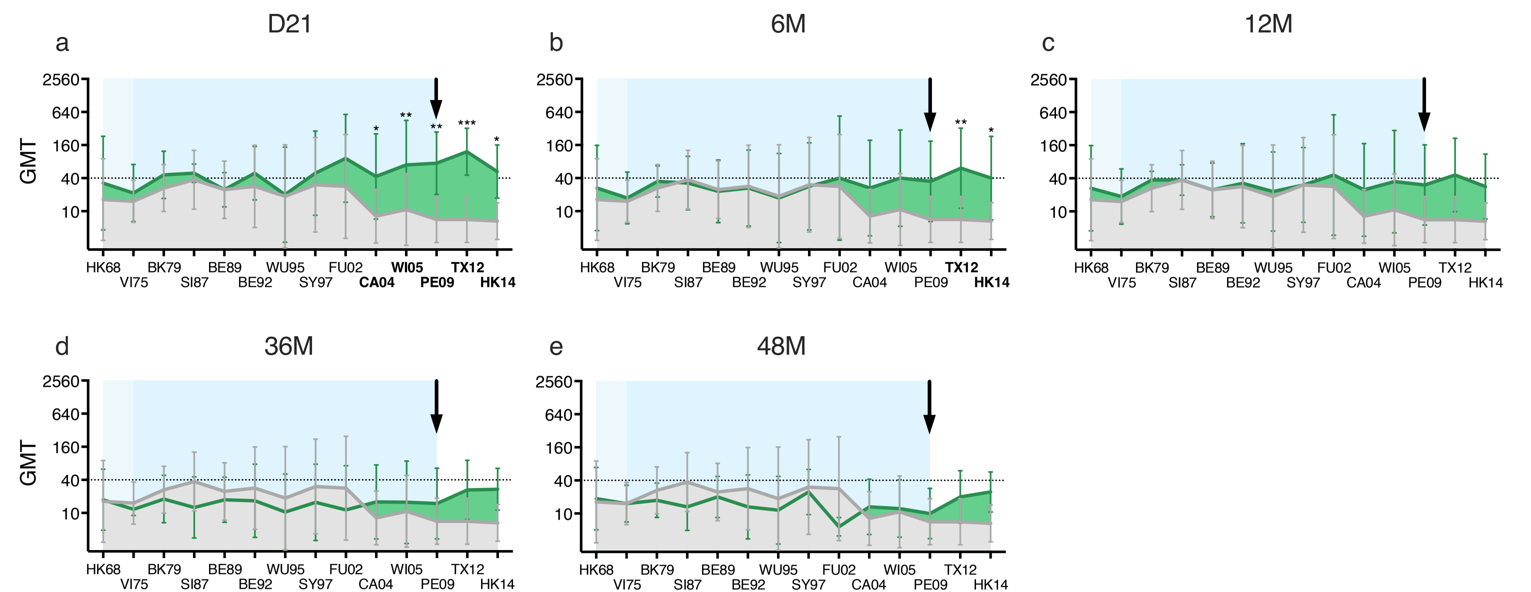
*Previous seasonal vaccination.

YoB	D0	D28	D56	6M	12M	Previously vaccinated*
2009						
2009						x
2009						
2009						
2010						
2010						
2010						x
2010						
2010						
2010						
2011						

*Previous seasonal vaccination.

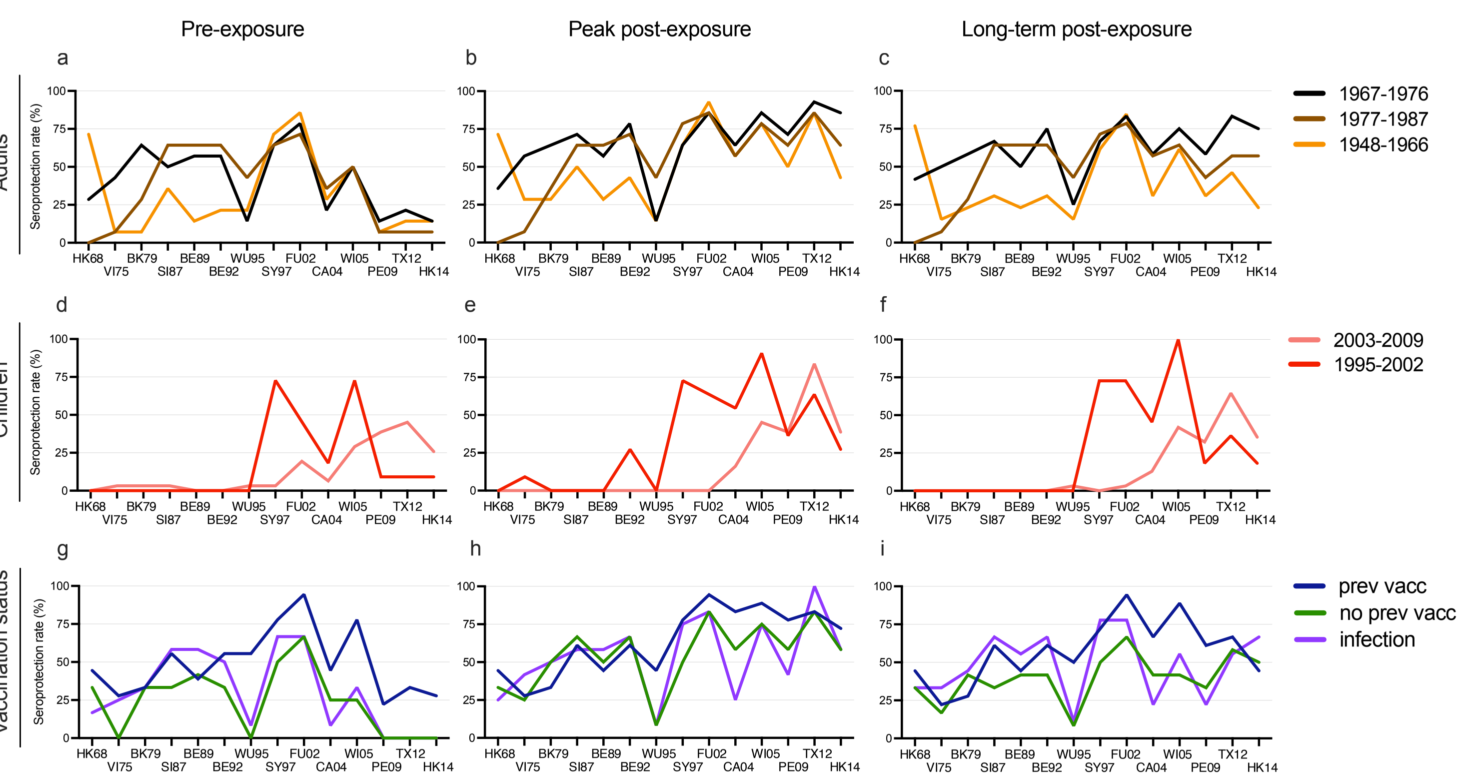
Geometric mean (GM) hemagglutination inhibition (HI) titers are shown on the Y-axis and the 14 antigenically distinct Influenza A/H3N2 viruses from old (1968) to new (2018) along the X-axis. The grey area represents pre-LAIV titers Day 0 (D0), pink represents post-IIV titres at 28 days (28D) and 56D, 6 months (6M) and 12 months (12M). Twenty-two children were included in this group (**a**) 11 and (**b**) 11, arranged according to birth year (left hand side). The black arrow indicates the vaccination virus, and the dotted line indicates the HI titre of 40. The period of viral exposure is highlighted by a light blue background.

Supplementary Figure 4: Landscapes of vaccination long-term responses after inactivated influenza vaccine in 2010 (IIV 2010 group)



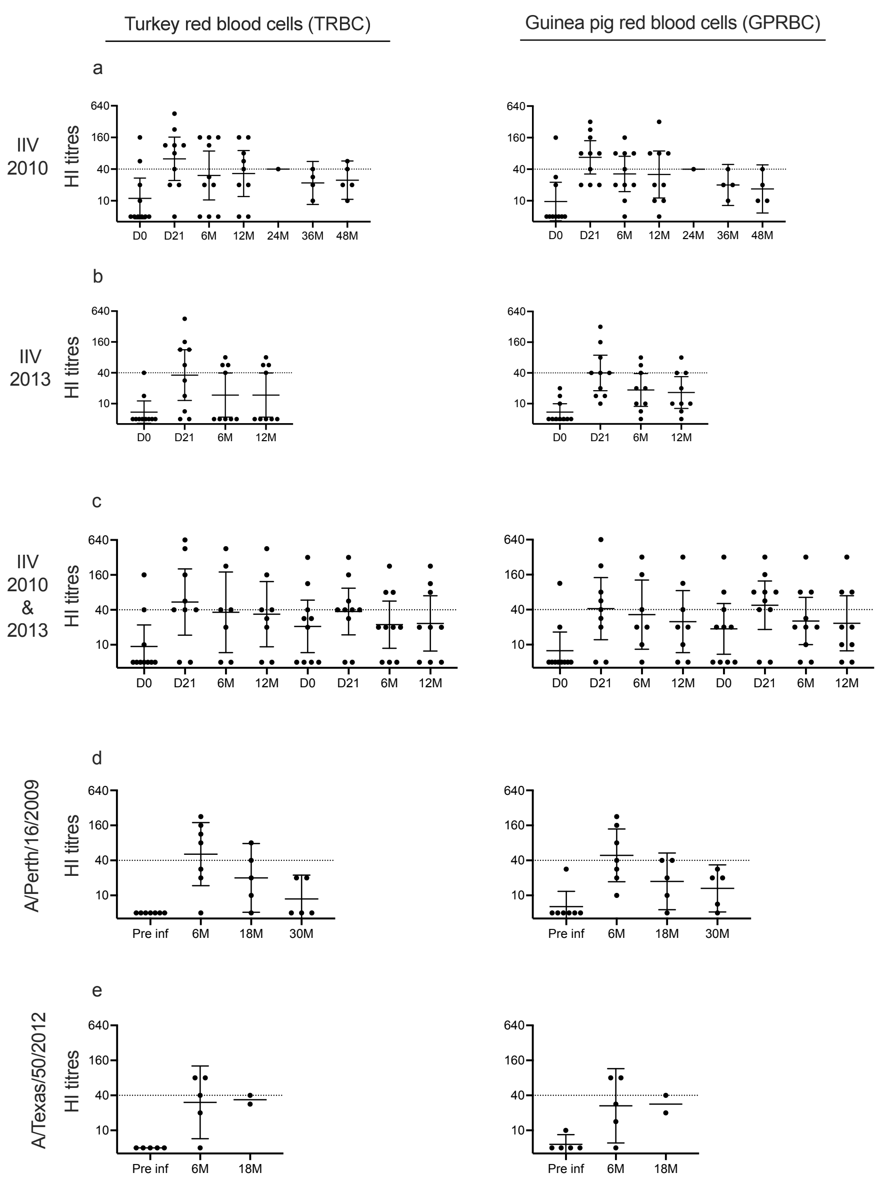
Selected subjects (n=5) from the 2010 IIV group provided blood samples for a long-term evaluation of antibody responses. The figures show geometric mean HI titres (GMTs) against 14 antigenically distinct influenza A/H3N2 viruses. Error bars represent the 95% confidence intervals of the GMTs. Grey pre-IIV titres Day 0 (D0, post-IIV titres in green at time-points day 21 (D21) (**a**), 6 months (6M) (**b**), 12 months (12M) (**c**), 36 months (36M) (**d**) and 48 months (48M) (**e**). The black arrow indicates the vaccination virus. The period of viral exposure is highlighted by a light blue background. Pre and post IIV HI titres were compared using non-parametric repeated measure Friedman test with Dunn's multiple comparison correction. *P <0.05, **P <0.01, ***P =<0.001.

Supplementary Figure 5: The impact of age and previous vaccination on the breadth of heamagglutination inhibition (HI) A/H3N2-specific antibody responses.



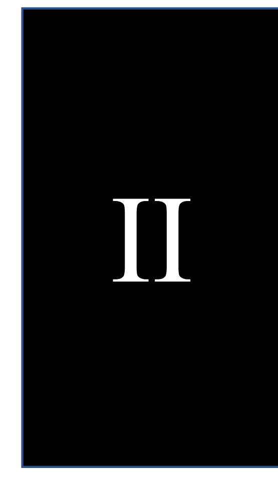
The seropositivity (percentages of individuals with HI titres ≥40) against the different influenza A/H3N2 viruses pre-exposure (pre-vaccination or pre-infection) (**a, d, g**), peak post-exposure (day 21/28 post-vaccination or 6 months post-infection) (**b, e, h**), and long-term post-exposure (6 months post-vaccination or 18 months post-infection) (**c, f, i**). Subjects were stratified by their birth year (**a-f**) or vaccination status (**g-i**). Adults were divided into 3 groups: born 1977-1987 (H1/H3 primed), 1967-1976 (H3 primed) and 1948-1966 (H1/H2 primed) (**a-c**) and children were divided into two groups: born 2003-2009 and 1995-2002 (both H1/H3 primed) (**d-f**). Adults were divided by previous vaccination history and compared to the infection group (**g-i**).

Supplementary Figure 6: Comparison of turkey and guinea pig blood in the haemagglutination inhibition (HI) assay against A/Hong Kong/4801/2014 (HK14)



HI antibodies against HK14 were tested using turkey red blood cells (TRBC) or guinea pig red blood cells (GPRBC) in the different groups of adults, (**a**) inactivated influenza vaccine (IIV) in 2010 **(**single 2010 IIV group), (**b**) single 2013 IIV group, (**c**) double 2010 and 2013 IIV group, (**d**) infection group infected with A/Perth/16/2009 (H3N2), (**e**) infection group infected with A/Texas/50/2012 (H3N2). The procedures of HI assay using TRBC were described in the Methods. Briefly, serum samples were treated with receptor-destroying enzyme (Seiken, Japan) and pre-adsorbed with packed TRBC before serial dilution from 1/10 in duplicates and incubated with 4 hemagglutinating units of virus for 1 hour. The serum-virus mixture was further incubated with 0.5% (volume/volume) TRBC for 30 minutes and the HI titre was read as the reciprocal of the highest dilution of the test sample where complete inhibition of agglutination occurs. The procedures of HI assay using GPRBC were similar to TRBC, except that the serum-virus mixture was incubated with 0.7% GPRBC for 1 hour.

Papers I-IV



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A rapid antibody screening haemagglutination test for predicting immunity to SARS-CoV-2 variants of concern

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Abstract

Background Evaluation of susceptibility to emerging SARS-CoV-2 variants of concern (VOC) requires rapid screening tests for neutralising antibodies which provide protection.

Methods Firstly, we developed a receptor-binding domain-specific haemagglutination test (HAT) to Wuhan and VOC (alpha, beta, gamma and delta) and compared to pseudotype, microneutralisation and virus neutralisation assays in 835 convalescent sera. Secondly, we investigated the antibody response using the HAT after two doses of mRNA (BNT162b2) vaccination. Sera were collected at baseline, three weeks after the first and second vaccinations from older (80–99 years, $n = 89$) and younger adults (23–77 years, $n = 310$) and compared to convalescent sera from naturally infected individuals (1–89 years, $n = 307$).

Results Here we show that HAT antibodies highly correlated with neutralising antibodies (R = 0.72–0.88) in convalescent sera. Home-dwelling older individuals have significantly lower antibodies to the Wuhan strain after one and two doses of BNT162b2 vaccine than younger adult vaccinees and naturally infected individuals. Moverover, a second vaccine dose boosts and broadens the antibody repertoire to VOC in naïve, not previously infected older and younger adults. Most (72–76%) older adults respond after two vaccinations to alpha and delta, but only 58–62% to beta and gamma, compared to 96–97% of younger vaccinees and 68–76% of infected individuals. Previously infected older individuals have, similarly to younger adults, high antibody titres after one vaccination.

Conclusions Overall, HAT provides a surrogate marker for neutralising antibodies, which can be used as a simple inexpensive, rapid test. HAT can be rapidly adaptable to emerging VOC for large-scale evaluation of potentially decreasing vaccine effectiveness.

Plain language summary

The aim of this study was to rapidly investigate the immune responses after SARS-CoV-2 infection and vaccination of younger adults and the elderly. Antibodies are proteins produced by the immune system that are released into the bloodstream and help fight infections. A simple method using red blood cells obtained from blood was developed and used to detect antibodies to SARS-CoV-2. This test was able to measure protective antibodies to several variants of concern. The elderly had lower antibody responses after vaccination. Two vaccinations induced a broader antibody response to viral variants, similar to the response induced following Covid-19. This antibody detection method could be used as a finger prick test to rapidly detect specific antibodies to emerging variants and enable quick identification of individuals who might benefit from a booster vaccination.

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There is increasing evidence that neutralising antibodies to the receptor binding domain (RBD) on the severe acute respiratory syndrome coronavirus-2 virus (SARS-CoV-2) spike protein represent an immunological correlate of protection[1]. SARS-CoV-2 evolution has been rapid with the ancestral virus and emerging variants of concern (VOC) straining global health care systems. These VOC (alpha (B.1.1.7)[2], beta (B.1.351)[3], gamma (P.1)[4], delta (B.1.617.2)[5] and the recent omicron (B.1.1.529)[6,7] show increased transmissibility, can escape pre-existing immunity and reduce vaccine effectiveness[8–10], with breakthrough infections reported in COVID-19 vaccinees with low neutralising antibodies[11]. There is a need for a rapid low-cost surrogate neutralisation assay, which can be used at a low biosafety level. This assay could be used for large-scale screening to identify vaccinees potentially susceptible to emerging VOC and who would benefit from a booster vaccine dose.

The neutralisation assay with live native virus is the gold standard for evaluating antibodies to VOC[1]. However, neutralising assays are difficult to standardise across laboratories, are time consuming, expensive and require high containment. Therefore, antibody binding and pseudotype virus assays are widely used to study antibody responses[12–14], but still require specialised laboratory facilities.

Here, we correlate the low-cost rapid hemagglutination test (HAT)[15] with neutralisation of the ancestral Wuhan-like strain in two large independent cohorts of infected patients. Further, we confirm the correlation between HAT and neutralising antibodies to VOC. In the HAT assay, the RBD domain is linked to a monomeric anti-erythrocyte single domain nanobody. When polyclonal serum antibodies bind to the RBD they cross-link and agglutinate the erythrocytes, which can be read visually after one hour. The HAT has a specificity of >99% for detection of convalescent antibodies after polymerase chain reaction (PCR) confirmed infection[15,16]. For influenza, a correlate of protection (COP) has been defined as a haemagglutination inhibition (HAI) titre of 40 for 50% protection from infection. If a similar COP could be established for HAT, it would allow simple standardised evaluation of susceptibility to SARS-CoV-2 infection and waning vaccine responses to VOC to guide public health policies.

Initially, we establish that HAT titres correlate with neutralising antibodies. We then use the HAT to investigate the antibody responses in 719 individuals consisting of home-dwelling older vaccinees (80–99-year-olds) and younger adults, in both those vaccinated with mRNA (BNT162b2) and in naturally infected individuals to the Wuhan-like virus. With VOC HAT we confirm that HAT titres can be used as a surrogate marker for neutralising antibody titres in vaccinated or infected individuals. Finally, we show that the HAT is readily adapted to finger prick testing.

Methods

Study participants

Norwegian vaccine and infection cohorts. A cohort of convalescents 415 infected individuals was prospectively recruited during the first Wuhan (pre-alpha) and delta pandemic waves in Bergen, Norway to compare the serological assays used in this study as described in[17–19]. For the comparison of vaccine and infection cohorts in Bergen Norway, we prospectively recruited two different age groups (home dwelling older and healthy younger adults) who received two doses of BNT162b2 mRNA COVID-19 vaccine at a 3-week interval during January 2021, and compared them to a group of 307 naturally infected individuals infected (1–89, median 47 years) with the Wuhan-like virus (D614G spike mutation) in February to April 2020[17,19] (Table 1). The older vaccinee group consisted of 96 home-dwelling elderly (80–99 years, median 86), 89 (92.7%) of whom were seronegative and 7 had previous SARS-CoV-2 infection with detectable pre-vaccination antibodies. The younger adult group consisted of 316 vaccinees (23–77 years, median 38) of whom 309 adults had no history of confirmed SARS-CoV-2 PCR test. Four younger vaccinees were not vaccinated on day 21; they received their second vaccination at day 19 ($n = 1$), or day 23 ($n = 2$) or day 24 ($n = 1$). Seven younger individuals had previous SARS-CoV-2 infection and pre-existing antibodies. This study is compliant with all relevant ethical regulations for work with humans and conducted according to the principles of the Declaration of Helsinki (2008) and the International Conference on Harmonization (ICH) Good Clinical Practice (GCP) guidelines. All Bergen subjects provided written informed consent before inclusion in the study, which was approved by the Western Norway Ethics committee (#118664 and #218629, NIH ClinicalTrials.gov Identifier: NCT04706390). Demographics (gender, age), PCR test results and COVID-19-like symptoms were recorded in an electronic case report form (eCRF) in (REDCap® (Research Electronic Data Capture) (Vanderbilt University, Nashville, Tennessee). Clotted blood samples were collected on the day of vaccination, 3 weeks after receiving the first and 3–5 weeks (mean 55 days, standard deviation ± 5 days) after the second vaccine doses or 3–10 weeks after confirmed infection. Sera were separated and stored at −80 °C and heat-inactivated for one hour at 56 °C before use in the serological assays.

UK convalescent cohort. Informed signed consent was obtained from 420 blood donor in the NHS Blood and Transplant cohort for purposes of clinical audit, to assess and improve the services and the research, and specifically to improve knowledge of the donor population. The use of these anonymised donor samples to assess neutralising antibody levels using different assays was approved by the National Blood Supply Committee for Audit and Research Ethics of National Health Service Blood and Transplant Research and Audit Committee (BS-CARE; BSCR20047 and BSCR20051).

Finger-prick and venous blood comparison. For the comparison of finger-prick and venous blood, participants were recruited from Oxford University Hospitals NHS Foundation Trust when they were attending the research clinic with the Oxford Protective T Cell Immunology for COVID-19 (OPTIC) Clinical Team. Written informed consent was obtained from participants with different past infection and vaccination status. Seventy-eight paired finger-prick blood and venous blood in EDTA tubes were taken at the same time and analysed on the same day by the HAT assay. Human study protocols were approved by the research ethics committee at Yorkshire & The Humber-Sheffield (GI Biobank Study 16/YH/0247).

Haemagglutination test (HAT). The haemagglutination test (HAT)[15] was used to investigate the SARS-CoV-2 specific antibodies to the RBD of the ancestral virus (Wuhan-like, pre alpha) and to the VOC alpha (B.1.1.7), beta (B.1.351), gamma (P.1) and delta (B.1.617.2). Briefly, codon optimised IH4-RBD sequences of VOC containing amino acid changes in the RBDs B.1.1.7 (N501Y), B.1.351 (K417N, E484K, N501Y), P.1 (K417T, E484K, N501Y) and B.1.617.2 (L452R, T478K). IH4-RBD were expressed in Expi293F cells and purified by their c-terminal 6xHis tag using Ni-NTA chromatography.

The point HAT was performed in V-bottomed 96-well plate on the same day as the blood was collected. Whole blood was diluted 1 in 40 in Phosphate buffered saline (PBS) 50 µl of dilution was mixed with 50 µl 2 µg/ml IH4-RBD reagent in the test well. Anti-RBD monoclonal antibodies, EY-6A[20] or CR3022[21] (100 ng) were

Table 1 The demographics of the old and healthy adult vaccinees and naturally infected subjects.

Characteristics	Norwegian (Bergen) cohort					UK (PHE) cohort
	Vaccinated		Infected&	Wuhan convalescents§	Delta Convalescents§	Convalescents§
	Old ($n = 96$)	Adult ($n = 316$)	($n = 307$)	($n = 378$)	($n = 37$)	($n = 420$)
Age (median (age range))	86 (80-99)	38 (23-77)	47 (1-89)	45 (1-89)	17 (11-20)	44 (19-65)
Sex (Female)	61 (63%)	214 (68%)	159 (52%)	216 (57%)	21 (57%)	114 (27%)
Comorbidity*	81 (85%)	41 (13%)	136 (44%)	154 (39%)	2 (5%)	-
Immuno-suppression#	14 (15%)	4 (1%)	12 (4%)	11 (3%)	0 (0)	-

*Diabetes, chronic respiratory diseases, chronic heart diseases, neurological diseases, chronic kidney, or liver diseases, dementia, rheumatologic diseases, active cancer.
#Inherent immunosuppressive disease, HIV, organ transplant, chemotherapy, other immunosuppressive treatment/drugs.
§In correlation analysis, Fig. 1.
&In haemagglutination test (HAT) analysis, Fig. 2.
- Information was not available.

positive controls and negative controls were whole blood dilution mixed with PBS. All sera were pre-screened at a dilution of 1:40 in PBS in 96 well V well plates. If HAT positive, serum was double diluted in duplicate from 1:40 in 50 µl PBS giving final dilutions of 1:40 to 1:40,960. Equal volumes of human O negative red blood cells (~1% v/v in PBS)[15] and 2.5 µg/ml IH4-RBD of Wuhan-like or VOC (B.1.1.7, B.1.351, P.1 or B.1.617.2) (125 ng/well) were pre-mixed and 50 µl added per well. Negative controls (PBS) and positive controls (monoclonal antibodies CR3022 and EY-6A) were included in each run. Plates were incubated to allow red blood cells to settle for 1 hr and were read by tilting the plate for 30 s and photographing. Positive wells agglutinated and the HAT titre is defined as the last well in which the teardrop did not form. Partial teardrops were scored as negative.

The IH4-RBD reagents for each VOC were standardised by showing that agglutination of red cells occurred at the same endpoint dilution (~16 ng/well) of the well characterised human monoclonal antibody EY6A[15,20] for each VOC at a working dilution of IH4-RBD of 2 ug/ml (100 ng/well in 50 ul). All the RBDs of the VOC share the conserved class IV epitope recognised by EY6A.

Enzyme-linked immunosorbent assay (ELISA). SARS-CoV-2 antibodies were detected using the ELISA in Bergen, Norway as previously described, but with minor modifications (Supplementary Fig. 1)[13,17,18]. Sera were screened for IgG antibodies against the Wuhan RBD of the SARS-CoV-2 spike protein at a 1:100 dilution and all samples were run in duplicates. The sera were diluted in 1% milk, 0.1% Tween-20 solution in PBS and incubated for 2 h at room temperature in 96 well plates (Maxisorp, Nunc, Roskilde, Denmark) coated with 100 ng/well of the RBD antigen. Plates were washed with PBS containing 0.05% Tween (PBST) between each step. Bound IgG antibodies were detected with a horseradish peroxidase (HRP)-labelled secondary antibody (cat. no.: 2040-05, Southern Biotech, Birmingham, AL, USA) and the addition of the chromogenic substrate 3,3′,5,5′-tetramethylbenzidine (TMB; BD Biosciences, San Jose, CA, USA). Optical density (OD) was measured at 450/620 nm using the Synergy H1 Hybrid Multi-Mode Reader with the Gen5 2.00 (version 2.00.18) software (BioTek Instruments Inc., Winooski, VT, USA).

RBD positive sera were run in an additional ELISA, where the ELISA plates were coated with SARS CoV-2 spike protein (Wuhan, 100 ng/well). Sera were serially diluted in duplicate in a 5-fold dilution, starting from a 1:100 dilution, and the ELISA plates were incubated with diluted serum for 2 h at room temperature. Bound IgG antibodies were detected and measured as described for the RBD screening ELISA.

Positive controls were serum from a hospitalized COVID-19 patient with the pre-alpha virus and CR3022[22], whereas pooled pre-pandemic sera ($n = 128$) were used as a negative control[18]. The mean endpoint titre was calculated for each sample. Samples with no detectable antibodies were assigned a titre of 50 for calculation purposes.

Pseudotype-based neutralisation assay. The pseudotype-based neutralisation assay was performed in biosafety level 2 laboratory in Bergen, Norway. The SARS-CoV-2 pseudotype virus was generated by co-transfection lentiviral vectors pHR'CMV-Luc, pCMVRΔ8.2, and pCMV3 construct encoding the Wuhan or delta spike protein into HEK293T cells as previously described[23]. The protease TMPRSS2 and human ACE2 encoding constructs were transfected into HEK293T to make target cells for the neutralisation assay. The lentiviral vectors and TMPRSS2-encoding constructs were a kind gift from Dr. Paul Zhou, Institute Pasteur of Shanghai, China. The ACE2-encoding construct was a kind gift from Dr. Nigel Temperton, University of Kent, UK. The SARS-CoV-2 Wuhan and delta spike-encoding constructs were purchased from Sino Biological. Serum samples were heat inactivated at 56 °C for 60 min, analysed in serial dilutions (duplicated, starting from 1:10). The SARS-CoV-2 pseudotype viruses corresponding to 20,000 to 200,000 relative luciferase activity (RLA) were mixed with diluted sera in 96-well plates and incubated at 37 °C for 60 min. Afterwards, ACE2-TMPRSS2 co-transfected HEK293T cells were added into 96-well plates and cultured for 72 h. RLA was measured by a BrightGlo Luciferase assay according to the manufacturer's instructions (Promega, Madison, WI, USA). The pseudotype-based neutralization (PN) titres (IC_{50} and IC_{80}) were determined as the reciprocal of the sera dilution giving 50% and 80% reduction of RLA, respectively. Negative titres (<10) were assigned a value of 5 for calculation purposes.

Virus strains. The Wuhan-like strain used in the microneutralisation and virus neutralisation assays in Bergen Norway was the clinical isolate; SARS-CoV-2/Human/NOR/Bergen1/2020 (GISAID accession ID EPI_ISL_541970) and at Public Health England, UK the isolate England/02/2020[24] (GISAID accession ID EPI_ISL_407073). At Oxford, UK[25] the Wuhan-like strain was Victoria/01/2020 (GenBank MT007544.1, B hCoV-19_Australia_VIC01_2020_ EPI_ ISL_ 406844_ 2020-01-25, and alpha (B.1.1.72) virus was the H204820430, 2/UK/VUI/1/2020, the beta (B.1.351) (20I/501.V2.HV001) isolate and delta (B.1.617.2) (sequence identical to virus Genbank ID OK622683.1).

Microneutralisation assay. The microneutralisation (MN) assay was performed on 345 Bergen convalescent sera in a certified Biosafety Level 3 Laboratory in Norway[17–19] against a clinically isolated virus: SARS-CoV-2/Human/NOR/Bergen1/2020. Briefly, serum samples were heat inactivated at 56 °C for 60 min, analysed

in serial dilutions (duplicate, starting from 1:20), and mixed with 100 50% Tissue culture infectious doses ($TCID_{50}$) viruses in 96-well plates and incubated for 1 h at 37 °C. Serum-virus mixtures were transferred to 96-well plates seeded with Vero cells. The plates were incubated at 37 °C for 24 h. Cells were fixed and permeabilized with methanol and 0.6% H_2O_2, and incubated with rabbit monoclonal IgG against SARS-CoV2 NP (Sino Biological). Cells were further incubated with biotinylated goat anti-rabbit IgG (H+L) and horseradish peroxidase (HRP)-streptavidin (Southern Biotech). The reactions were developed with o-Phenylenediamine dihydrochloride (OPD) (Sigma–Aldrich). The MN titre was determined as the reciprocal of the serum dilution giving 50% inhibition of virus infectivity. Negative titres (<20) were assigned a value of 5 for calculation purposes.

The MN assay for the 420 convalescent UK samples was conducted in a certified Biosafety Level 3 as previously described at Public Health England (PHE), UK[24]. using the virus England/02/2020. Sera were heat inactivated at 56 °C for 60 min, before analyses in duplicate serial dilutions (starting from 1:20), and mixed with 100 $TCID_{50}$ viruses in 96-well plates and incubated for 1 h at 37 °C. Then, the cell suspension was added to the virus/antibody mixture[24] and incubated at 37 °C for 22 h. Cells were fixed and permeabilized before staining for NP antibodies, then biotinylated goat anti-rabbit IgG, followed by Extravidin-peroxidase. The reaction was developed with OPD and MN titres calculated, as described above.

At Oxford, UK the detection of antibodies to the Wuhan-like and VOC (alpha, B.1.1.7 and beta, B.1.351) used the method described in[25]. Briefly, quadruplicate serial dilutions of serum were preincubated with appropriate SARS-CoV-2 for 30 min at room temperature, then Vero CCL81 cells were added and incubated at 37 °C, 5% CO_2 for 2 h. A carboxymethyl cellulose-containing overlay (1.5%) was added, monolayers were fixed and stained for the nucleocapsid (N) antigen or spike (S) antigen using EY2A and EY6A monoclonal antibodies, respectively. After development the number of infectious foci were counted by ELISpot reader. Data were analysed using four-parameter logistic regression (Hill equation) in GraphPad Prism 8.3.

Virus neutralisation assay. The virus neutralisation (VN) assay was performed in a certified Biosafety Level 3 facility in Bergen, Norway[18]. Serum samples were tested against a clinically isolated virus: SARS-CoV-2/Human/NOR/Bergen1/2020 as previously described[18]. Briefly, serum samples were heat inactivated at 56 °C for 60 min, analysed in serial dilutions (duplicated, starting from 1:20), and mixed with 100 $TCID_{50}$ viruses in 96-well plates and incubated for 1 h at 37 °C. Mixtures were transferred to 96-well plates seeded with Vero cells. The plates were incubated at 37 °C for 4–5 days, all wells were examined under microscope for cytopathic effect (CPE). The VN titre was determined as the reciprocal of the highest serum dilution giving no CPE. Negative titres (<20) were assigned a value of 5 for calculation purposes.

The delta virus neutralisation assay was performed at the University of Oxford, UK as previously described[26]. Briefly, serial two-fold serum dilutions from 1:20 were incubated with 50 $TCID_{50}$ virus in 96-well plates for 1 h before addition of 20,000 Vero E6 TMPRSS2 cells per well. Plates were incubated for 3 days before staining with amido black and CPE read by eye. Negative titres (<20) were assigned a value of 5 for calculation purposes.

Statistics and reproducibility. The two-tailed Mann–Whitney *U* test with 95% confidence level was used to compare ranks in HAT titres between the older and adult vaccinees. The non-parametric two-tailed Spearman R correlation with 95% confidence interval was used to investigate the correlation between the antibody titres from different serological assays. All analyses were conducted in GraphPad Prism version 9.20.

Results

Previous studies have shown that the HAT titre correlates with SARS-CoV-2 RBD binding and ACE2 blocking antibodies[15,27,28], and identified high titre (>100) neutralising sera with a sensitivity of 76.5%[28]. First, we used the World Health Organisation (WHO) approved human SARS-CoV-2 standards panel to confirm the relationship between spike specific binding and neutralising assay to the HAT assay (Table 2). Second, we investigated the relationship between endpoint HAT titres and neutralising antibodies using three neutralisation assays in convalescent sera from SARS-CoV-2 infected individuals from the first pandemic wave (pre-alpha) and the ongoing delta-wave in Bergen, Norway[17–19]. We then confirmed the results in an independent UK cohort[24].

Correlation of neutralising antibodies and HAT titres. In the Bergen Wuhan convalescents cohort, microneutralization 50% inhibitory concentrations (IC_{50}) titres were significantly associated with HAT titres (Spearman's $R = 0.82$, $p < 0.0001$) (Fig. 1b, Table 1, Supplementary Fig. 2). A HAT titre ≥ 40 detected 99% of samples with MN $IC_{50} \geq 20$, with positive predictive value (PPV) of 94%. A HAT titre > 480 predicted MN titres > 100 with a sensitivity 77% and PPV of 78%.

We extended these results by comparisons to a pseudotype neutralisation (PN) assay, and a classical live virus neutralisation (VN) assay with complete inhibition of Cytopathic Effect (100% CPE) as its endpoint (Fig. 1a, d, e)[18]. The correlation of HAT and PN titres were significant ($p < 0.0001$) at 50% ($R = 0.79$) and 80%

Table 2 Comparison of the haemagglutination test antibody endpoint titres to the neutralisation and binding antibodies in the WHO anti-SARS-CoV-2 international standards.

	WHO antibody standards*			
Antibody test	**High**	**Mid**	**Low S, high N**	**Low S**
HAT	5120	640	5	5
MN titre (IC_{50})	2298	240	55	21
Neutralisation antibodies (IU/mL)#	1473	210	58	44
Anti-Receptor Binding Domain (RBD) (BAU/mL)	817	205	66	45
IgG anti-S1 (BAU/mL)#	766	246	50	46
IgG anti-Spike IgG (BAU/mL)#	832	241	83	53
Anti-N IgG (BAU/mL)#	713	295	146	12

*The WHO anti-SARS-CoV-2 international standards (20/268 NIBSC, UK) contained high (20/150), mid (20/148), low spike (S) and high nucleocapsid (N) (20/144) and low S (20/140) human antibodies. The haemagglutination test (HAT) endpoint titres to the Wuhan-like virus were compared with microneutralisation (MN) titres and #neutralisation titres (IU/ml), and antibody binding (BAU/mL) as reported by NIBSC. Negative HAT tests are given a value of 5 for consistency.

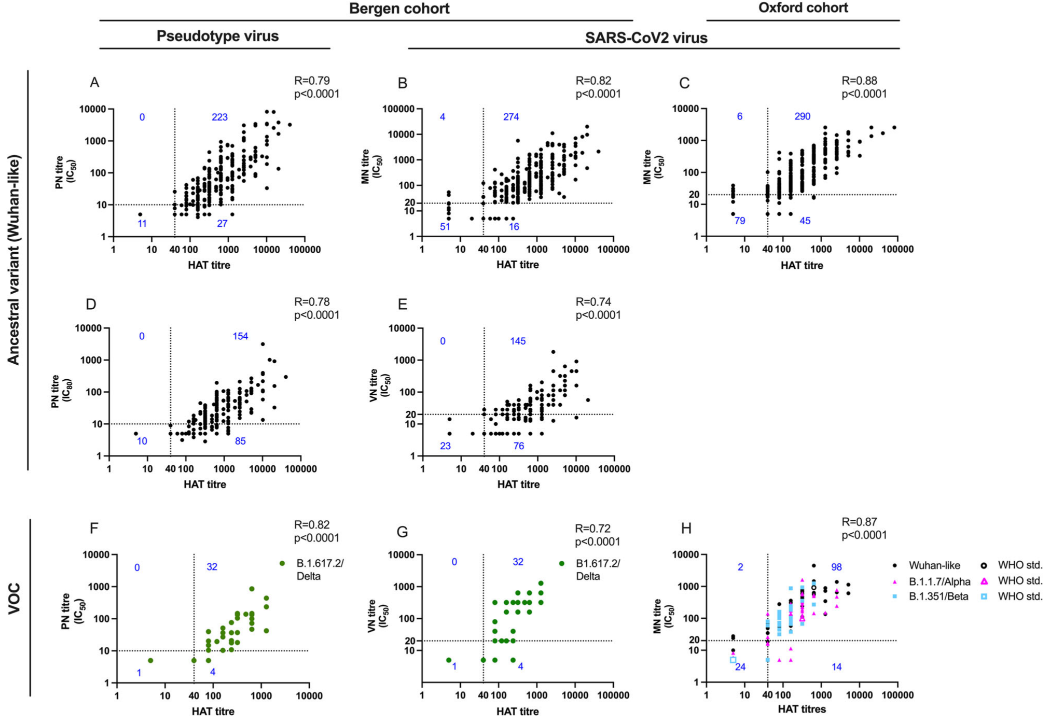

Fig. 1 The correlation between haemagglutination test and SARS-CoV-2 neutralising antibodies. Correlation of endpoint haemagglutination test (HAT) titres with neutralising antibody titres. In a cohort of infected individuals from the first (Wuhan-like) and delta pandemic waves (diagnosis by PCR from nasopharyngeal swabs or serology in Bergen, Norway) (**a**–**b**, **d**–**g**), Wuhan-like neutralising antibodies were measured using the pseudotype (PN) neutralisation assay at 50% (PN_{50}) (**a**) (R = 0.79, 95% confidence interval (CI): 0.73–0.83) and 80% (PN_{80}) (**d**) (R = 0.78, 95% CI: 0.72–0.82) inhibition of pseudotype virus infectivity, the microneutralisation (MN) 50% inhibitory concentration (IC_{50}) (**b**) (R = 0.82, 95% CI: 0.78–0.85) and virus neutralization (VN) 100% inhibition of cytopathic effect (**e**) (R = 0.74, 95% CI: 0.68–0.80) assays. Delta-like neutralising antibodies were measured in the PN assay at PN_{50} (**f**) (R = 0.82, 95% CI: 0.67–0.90) and VN 50% inhibitory concentration (**g**) (R = 0.72, 95% CI: 0.51–0.85). Convalescent sera from 420 infected individuals in UK for whom neutralising antibody and HAT titre were measured (**c**) (R = 0.88, 95% CI: 0.86–0.90). The correlation between the HAT and 50% inhibition of neutralising antibody titres for Wuhan-like, and B.1.1.7 and B.1.351 VOC antibody titres performed at Oxford, UK (**h**) (R = 0.87, 95% CI: 0.82–0.90). HAT titres were measured in a set of donors either infected or vaccinated with one or two doses of the Pfizer BNT162b2 mRNA vaccine who had neutralising antibody levels to the ancestral Wuhan, B.1.1.7, B.1.351 or B1.617.2 live viruses. Open symbols represent the positive anti SARS-CoV-2 WHO standard (20/130). The Spearman R correlations and significant values are shown. In the MN assay, virus infectivity was measured by detecting the amount of nucleoprotein and also spike after 22–24 h incubation in Vero cells. In Bergen (**b**, **e**) the Wuhan-like local D614G virus hCoV-19/Norway/Bergen-01/2020 (GISAID accession ID EPI_ISL_541970) was used in a certified Biosafety Level 3 Laboratory. The dotted lines show the lowest detectable titre in each assay, all negative values were assigned the number 5 for consistency, and the sample size can be derived from adding the blue numbers in the quadrants together.

(R = 0.78) IC (Fig. 1a, d). Confirming our previous results, the VN titres correlated with HAT titres (R = 0.74, $p < 0.0001$) (Fig. 1e). HAT titres ≥ 40 detected 100% of samples with VN titres ≥ 20, but the PPV fell to 54% consistent with the classical VN assay having the more rigorous endpoint.

Confirmation of correlation between neutralisation and HAT titres. As interlaboratory variation has been reported for neutralisation assays, we confirmed the significant correlation between HAT and MN titres (R = 0.88, $p < 0.0001$) in an independent UK collection of 420 convalescent samples (Fig. 1c, Table 1, Supplementary Fig. 2). In close agreement with the Bergen cohort, a positive HAT ≥ 40 detected 98% of samples with MN IC_{50} titre ≥ 20, with PPV of 87%. Similarly, for identification of high titre sera a HAT > 480 identified 75% of sera with MN $IC_{50} > 100$ with PPV 86%. In summary, the HAT titres highly correlated with neutralisation titres in two independent laboratories showing the utility of HAT as a rapid and inexpensive surrogate for the neutralisation test.

Evaluation of HAT antibody responses in older and younger vaccinees. Older adults have carried the burden of COVID-19 throughout the pandemic with increased risk of hospitalizations and death, and are prioritised for vaccination, although most vaccine licensure trials have excluded the oldest (>85 years old)[29]. As proof of principle, we used the HAT to investigate the Wuhan-like antibody responses in seronegative healthy younger adults ($n = 309$, median 37 years) and older home-dwelling adults ($n = 89$, 80–99 years, median 86 years) after the BNT162b2 mRNA COVID vaccine and in individuals naturally infected with the Wuhan-like strain ($n = 307$, median 47 years)[19] (Table 1, Fig. 2). A HAT titre of ≥40 was used as a cut-off to assess the propor of vaccine responders to the Wuhan-like virus[15]. Only 31% of older subjects responded after the first vaccination

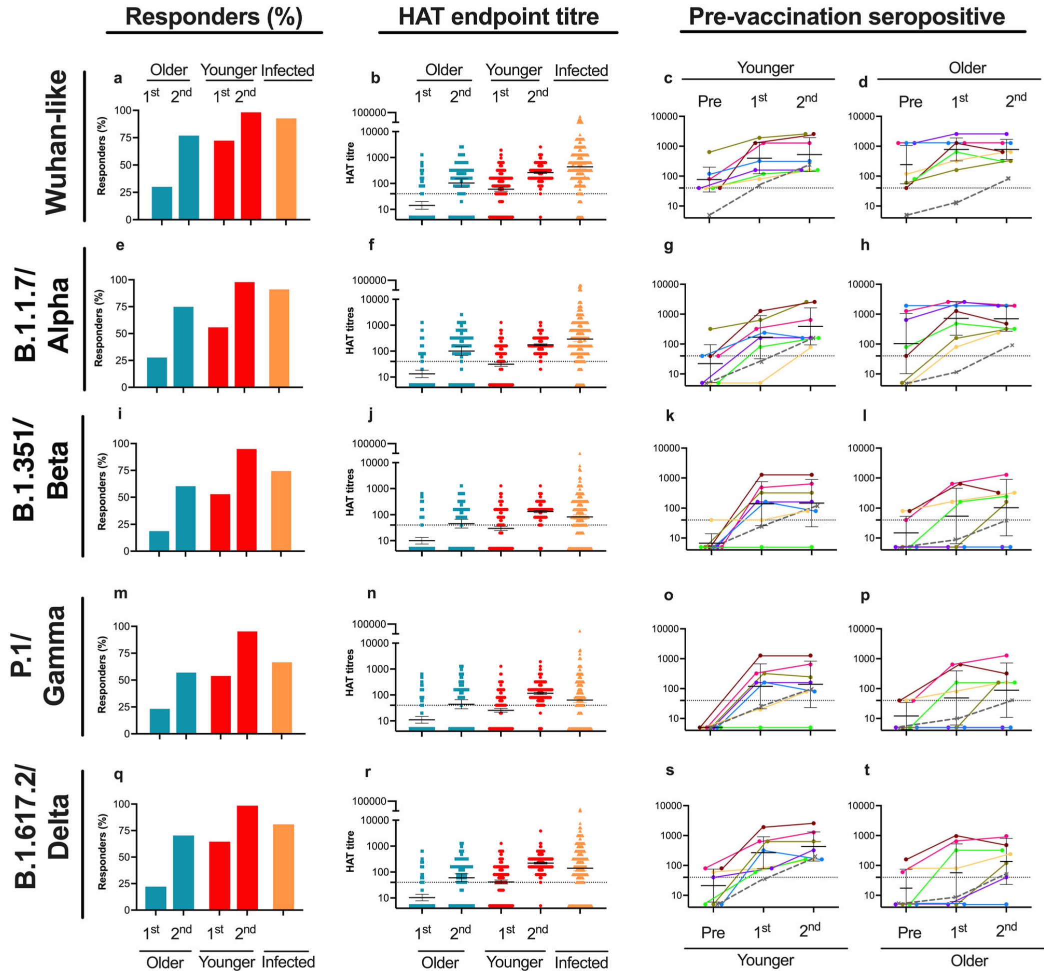

Fig. 2 Haemagglutination test antibodies to the ancestral Wuhan SARS-CoV-2 virus and variants of concern in older and younger adult vaccinees and after natural infection. Haemagglutination test (HAT) antibodies to the SARS-CoV-2 virus receptor binding domains of homologous founder virus (**a**–**d**: Wuhan-like) and variants of concern (**e**–**h** B.1.1.7 alpha; **i**–**l**: B.1.351 beta; **m**–**p**: P.1 gamma and **q**–**t**: B.1.617.2 delta). Endpoint HAT titres are presented in (**b**–**d**, **f**–**h**, **j**–**l**, **n**–**p**, **r**–**t**). The percentage of responders with haemagglutination test titre ≥ 40 (**a**, **e**, **i**, **m**, **q**) and endpoint HAT titres (**b**, **f**, **j**, **n**, **r**) in seronegative older ($n = 89$) and seronegative adults ($n = 309$) post 1st dose (3 weeks) and post 2nd dose (6–8 weeks after 1st dose) mRNA BNT162b2 COVID-19 vaccination. In infected individuals, convalescent serum was collected 3–10 weeks after SARS-CoV-2 confirmed infection (infected, $n = 307$) with D614G virus during the first pandemic wave (**a**–**b**, **e**–**f**, **i**–**j**, **m**–**n**, **q**–**r**). HAT endpoint titres to Wuhan-like and VOC in previously infected older individuals ($n = 7$) and adults ($n = 7$) who were vaccinated are shown in different colours, with the grey dashed line showing comparison of the geometric mean HAT titres for the corresponding seronegative (not previously infected) old ($n = 89$) and adult ($n = 309$) vaccinees (**c**–**d**, **g**–**h**, **k**–**l**, **o**–**p**, **s**–**t**). For endpoint HAT titres (**b**–**d**, **f**–**h**, **j**–**l**, **n**–**p**, **r**–**t**), negative values were assigned a value of 5. The geometric mean titres (GMT) and error bars with 95% confidence intervals are shown in black and each symbol represents one subject (**b**, **f**, **j**, **n**, **r**).

compared to 74% of younger vaccinees (Fig. 2a, Table 3). After the second dose, 78% of the older vaccinees had HAT titre of ≥40 compared to 94% of infected individuals and 99% younger vaccinees. Older people also had a significantly lower magnitude of response than younger adult vaccinees after both the first and second vaccine doses, with the exception after second dose against alpha (Table 4). In summary, the older adults had a blunted response after one dose of mRNA vaccine and required the second dose to increase the magnitude of the response.

Development of variant of concern reagents for HAT. Variants of concern have amino acid changes in their spike protein, and importantly in their RBD which may allow escape from neutralising antibodies. The alpha variant rapidly became the dominant strain in early 2021[2] with beta and gamma dominating in some geographical areas, and was subsequently replaced by the highly transmissible delta variant in 2021[30]. We developed HAT reagents for the VOC as they arose and confirmed a strong correlation between the alpha ($R = 0.79$, $p < 0.0001$) and beta

Table 3 Comparison of Wuhan-like and variant of concerns haemagglutination test responders after one and two doses of mRNA vaccine in younger and older adults, as well as in previously infected individuals with SARS CoV-2.

Virus#	Responders n/N (%)				
	Vaccinated				Infected[3]
	Older[1]	Younger[2]	Older[1]	Younger[2]	
	Post 1st dose		Post 2nd dose		
Wuhan-like	28/89 (31)	228/309 (74)	70/89 (78)	308/309 (100)	289/307 (94)
Alpha, B.1.1.7	26/89 (29)	177/309 (57)	68/89 (76)	307/309 (99)	284/307 (92)
Beta, B.1.351	18/89 (20)	168/309 (54)	55/89 (62)	298/309 (96)	233/307 (76)
Gamma, P.1	22/89 (25)	171/309 (55)	52/89 (58)	299/309 (97)	209/307 (68)
Delta B.1.617.2	21/89 (24)	204/309 (66)	64/89 (72)	309/309 (100)	253/307 (82)

[1]89 seronegative older vaccinees 3 weeks after 1st and 3–5 weeks after 2nd dose of mRNA vaccine.
[2]309 younger adult vaccinees 3 weeks after 1st and 3–5 weeks after 2nd dose of mRNA vaccine.
[3]307 SARS-CoV-2 infected individuals with convalescent sera collected 4–6 weeks after infection.
#The viruses tested are the ancestral virus (Wuhan-like) and variant of concern (B.1.1.7 alpha; B.1.351 beta; P.1 gamma and B.1.617.2 delta) viruses. The data is presented as the subjects with HAT titre over 40 against the different variants, *n/N* and as percentage (%) of the whole group.

Table 4 The haemagglutination test (HAT) antibody response to the Wuhan-like virus and variants of concern after one and two doses of mRNA vaccine and after SARS-CoV-2 infection in seronegative younger and older adults.

	Vaccinated										Infected[3]
	Older[1]		Younger[2]		Older vs. younger	Older[1]		Younger[2]		Older vs. younger	
	Post 1st dose					Post 2nd dose					
Virus#	GM*	Fold-change$	GM	Fold-change	P value#	GM	Fold-change	GM	Fold-change	P value#	GM
Wuhan-like	14	2.8	60	11.9	**<0.0001**	104	7.3	262	4.4	**<0.0001**	438
Alpha	13	2.7	31	6.2	**<0.0001**	101	7.6	175	5.6	0.3323	292
Beta	10	2.0	30	5.9	**<0.0001**	45	4.5	133	4.5	**<0.0001**	82
Gamma	11	2.2	25	5.1	**<0.0001**	44	4.0	116	4.6	**0.0036**	64
Delta	10	2.0	41	8.3	**<0.0001**	59	5.7	223	5.4	**<0.0001**	141

[1]89 seronegative older vaccinees 3 weeks after 1st and 3–5 weeks after 2nd dose of mRNA vaccine.
[2]309 younger adult vaccinees 3 weeks after 1st and 3–5 weeks after 2nd dose of mRNA vaccine.
[3]307 SARS-CoV-2 infected individuals with convalescent sera collected 4–6 weeks after infection.
#The viruses tested are the ancestral virus (Wuhan-like) and variants of concern (B.1.1.7 alpha; B.1.351 beta; P.1 gamma; and B.1.617.2 delta) viruses.
*The data is presented as the geometric mean (GM) of the HAT titres. Negative values were assigned a value of 5 for calculation purposes.
$The fold change is shown in the vaccinated individuals from pre to post 1st dose and from post 1st to post 2nd vaccine dose. All individuals were seronegative (HAT < 40) at baseline.
#Two-tailed Mann–Whitney *U* test with 95% confidence level was used to compare ranks of HAT titres between the adults and the older vaccinees, with $P < 0.05$ considered significant. Statistically significant *P* values are in bold.

($R = 0.89$, $p < 0.0001$) in a UK set of naturally infected and vaccinated donors[25] (Fig. 1h). As the delta variant dominates in Norway, we collected convalescent sera from from 37 infected individuals to confirm the relationship between HAT and pseudotype and virus neutralisation assays. A good correlation was observed for both pseudotype ($R = 0.82$, $p < 0.0001$) and virus neutralisation assays ($R = 0.72$, $p < 0.0001$) (Fig. 1f, g).

HAT antibodies to variants of concern in older vaccinees. We then investigated the breadth of the VOC response in vaccinees and infected subjects. Older vaccinees had the lowest number of responders and lower cross-reactivity after both one and two vaccinations. The second vaccination boosted the number of responders in older adults, from 20–29% to 58–76% to VOC (Fig. 2e, i, m, o). In older and younger vaccinees that responded to VOC, there was good cross-reactivity to alpha and delta, but less so to the beta and gamma in all groups after two doses of vaccine or infection. A similar but higher response pattern to different VOC was observed in infected individuals, with 92% to alpha, 82% to delta, 75% to beta and 68% to gamma compared to responses in 96–100% of younger adult vaccinees. In summary, two doses of mRNA vaccine or natural infection induced higher responses in younger adults to VOCs than in older vaccinees.

Vaccine response in previously infected younger and older individuals. Natural infection induces higher titres of SARS-CoV-2 specific antibodies in older individuals than in younger adults[17]. Previously infected older subjects ($n = 7$, median age 87 years), none of whom had been hospitalised, had higher pre-vaccination HAT titres to the Wuhan-like virus than previously infected younger adults ($n = 7$, median age 38 years). Previously infected older and younger adults developed high Wuhan-like and alpha cross-reactive antibody titres after one vaccine dose (Fig. 2c, d), although lower responses to other VOC. Cross-reactive titres were boosted in some of the older and healthy vaccinees after the second vaccination (Fig. 2g, h, k, l, o, p, s, t). In summary, previously infected older individuals develop high antibody titres after one vaccine dose comparable to healthy younger adults, which contrasts with the suboptimal antibody responses in SARS-CoV-2 naïve older vaccinees.

The use of HAT as a point of care fingerprick test. For the HAT to be implemented at low biosafety level and in resource limited settings, a fingerprick test using autologous patient erythrocytes could be used to rapidly identify populations with low titres to Wuhan-like and VOC SARS CoV-2 viruses. As advised by the Infectious Diseases Society of America guideline on serological

Wuhan-like

a

Whole blood titre	Finger-prick HAT+	Finger-prick HAT-	Total
Venous HAT+	44	3	47
Venous HAT-	2	29	31
	46	32	78

b

Whole blood titre	Finger-prick HAT+	Finger-prick HAT-	Total
Venous HAT+	39	2	41
Venous HAT-	0	22	22
	39	24	63

c B.1.1.7/Alpha

Whole blood titre	Finger-prick HAT+	Finger-prick HAT-	Total
Venous HAT+	35	0	35
Venous HAT-	1	26	27
	36	26	62

d B.1.351/Beta

Whole blood titre	Finger-prick HAT+	Finger-prick HAT-	Total
Venous HAT+	27	0	27
Venous HAT-	0	35	25
	27	35	62

e P.1/Gamma

Whole blood titre	Finger-prick HAT+	Finger-prick HAT-	Total
Venous HAT+	24	1	25
Venous HAT-	0	29	29
	24	30	54

f B.1.617.2/Delta

Whole blood titre	Finger-prick HAT+	Finger-prick HAT-	Total
Venous HAT+	28	1	29
Venous HAT-	0	24	24
	28	25	53

Fig. 3 Correlation of finger-prick and venous blood samples using the haemagglutination test on Wuhan and variants of concern. Correlation of paired finger-prick and venous blood samples collected from vaccinated healthcare workers. HAT titres are shown by a symbol that can represent on or more blood samples. Correlations were analysed by linear regression shown in graphs and tables. **a** The point haemagglutination test (HAT) showing the correlation between finger-prick and venous whole blood samples ($n = 78$). Haemagglutination was scored as shown in the contingency table. "HAT+" samples with haemagglutination and "HAT−" refers to no haemagglutination or endpoint titres <5. **b–f** Endpoint HAT titres of paired finger-prick and venous blood samples. Diluted finger-prick or venous whole blood samples (1 in 40 in phosphate buffered saline (PBS)) were centrifuged, and the supernatant was titrated in the HAT assay using IH4-RBD-reagents and autologous red blood cells (RBC) (washed and diluted 1 in 40 in PBS). **b** Wuhan-like ($n = 63$), **c** B1.1.7/Alpha ($n = 62$), **d** B.1.351/Beta ($n = 62$), **e** P1/Gamma, ($n = 54$), and **f** B.1.617.2/Delta ($n = 53$).

testing[31], we confirmed the comparability of fingerprick and venous blood for reactivity to all VOC (Fig. 3). The HAT can thus be rapidly adapted to test for antibodies to emerging VOC for large-scale screening of fingerprick blood samples with autologous erythrocytes.

Discussion

The rapid evolution of SARS-CoV-2 VOC, particularly delta and omicron, with increased transmissibility and the possibility of escape from vaccine induced immunity, represents a considerable threat. There is a need for a low-cost rapid serological assay which can be used for large-scale screening globally without requiring specialised laboratory equipment to rapidly identify populations susceptibility to VOC. HAT is simple to perform, requires no special equipment, and can be done at the point-of-care in virtually any setting using a fingerprick sample. The HAT IH4-RBD VOC reagents are freely available for research[15]. Inter-laboratory comparability can be guaranteed by including HAT titrations on WHO approved standard sera (as shown in Table 2).

We demonstrate the versatility of the HAT in analysing susceptibility to VOC in home-dwelling older vaccinees showing the importance of two vaccine doses to achieve good cross-reactive antibody titres in older adults who have not been previously infected. Older and high-risk individuals were prioritised in the very first rounds of vaccination early in 2021 in Europe and America. Depending upon the decay in antibody titres over time, the HAT could be used to rapidly identify individuals who may need a booster vaccine dose to mount efficient antibody responses to VOC. We found the oldest age group had a decreased breadth of cross-reactive antibodies to VOC after the first vaccine dose, particularly to the beta and gamma viruses, in agreement with escape from neutralising antibodies[10]. Although vaccination induced cross-reactive antibodies against delta, milder break through infection with this variant in vaccinated subjects is becoming an increasing problem[11]. Reports of very high viral load during delta infections[32,33] may necessitate higher antibody titres to provide sterilising immunity and prevent infection.

In previously infected adults, only one dose of vaccine seems to be required to produce high levels of cross-reactive antibodies against the VOC[16,27]. Extending these findings, we found that in previously SARS-CoV-2 infected older adults, only one dose of vaccine was required to mount strong anamnestic responses, similar to younger vaccinees[34–40].

Caveats to our study are that most convalescent blood samples from our naturally infected cohort were collected during the first SARS-CoV-2 Wuhan-like wave, but we did include a small subset ($n = 37$) of delta infected individuals. Strengths are that we have confirmed the relationship between HAT and several neutralisation assays in two large cohorts in independent laboratories, showing that the relationship holds for VOCs, and included 719 individuals either infected and/or vaccinated, aged up to 99 years old. To our knowledge, this is the first study reporting antibody cross-reactivity to four VOC in this older age group.

Neutralising titres of between 10 and 30 in humans[1], depending upon the assay, have been reported to predict 50% protection from symptomatic infection, and much lower levels to protect against severe infection. Although the absolute HAT titres correlating with protection are not yet known, we demonstrated that the HAT titres correlated with neutralisation titres, and thus provide a surrogate test for neutralising antibodies. We suggest that a positive HAT titre of 40–80, equivalent to 1:40 dilution of whole blood obtained by fingerprick, would correlate with neutralising titres 10–30, and would predict protection. A prospective study to test this predicted relationship between HAT titres and protection is now warranted. The HAT may also aid in evaluating and licensing of new COVID vaccines.

We predict that the lower HAT titres against VOC will lead to a more rapid decline in protective efficacy against variants, thus requiring booster vaccinations. The emergence of the highly infectious and transmissible delta and more worrying omicron VOC which have caused breakthrough infections in vaccinees highlights the importance of real-time cross-reactivity studies. Monitoring of population susceptibility of both previously infected subjects and vaccinees to VOC with increased transmissibility through simple serological assays can guide public health policy.

Data availability

Source data behind figures are available in the Supplementary Data file.

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Author contributions

R.J.C., N.L. and A.T. designed the study, and together with N.U.E. and J.X. analysed the results and wrote the paper. N.U.E., J.X. and S.L. conducted the HAT assays. F.Z. and N.U.E. ran the neutralisation assays in Bergen, Norway. H.S., S.L.L., L.H., M.S., L.H., A.M., K.G.I.M., E.F., J.S.O. and K.A.B. recruited and followed up 345 SARS CoV-2 positive individuals and 412 vaccinees in Bergen and ran the lab assays. T.K.T., P.R., and L.S. developed and standardised the VOC HAT. S.D. and A.J. recruited and collected fingerprick and venous blood samples. D.R., W.S.J., D.N. and A.C.H. recruited the infected subjects and vaccinees in Oxford and ran the neutralisation assays for the VOC. H.H. and M.Z. collected and tested 420 convalescent samples at Public Health England Colindale UK for neutralising antibodies. SØ recruited and vaccinated the older patients. All authors reviewed the manuscript and approved the final version for publication.

Competing interests

The authors declare no competing interests.

Additional information

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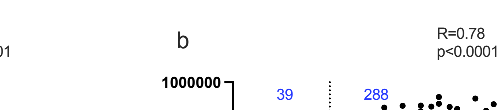
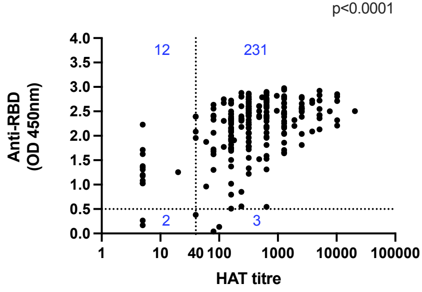
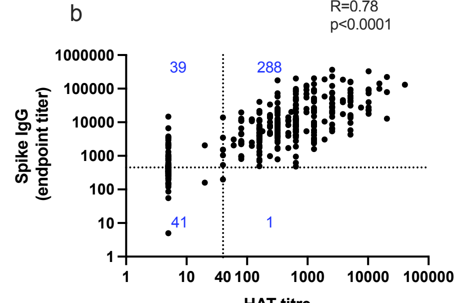
Supplementary Figure 1. Correlations between haemagglutination test antibody endpoint titres and binding antibodies (anti-IgG spike and anti-IgG RBD)

Binding antibodies

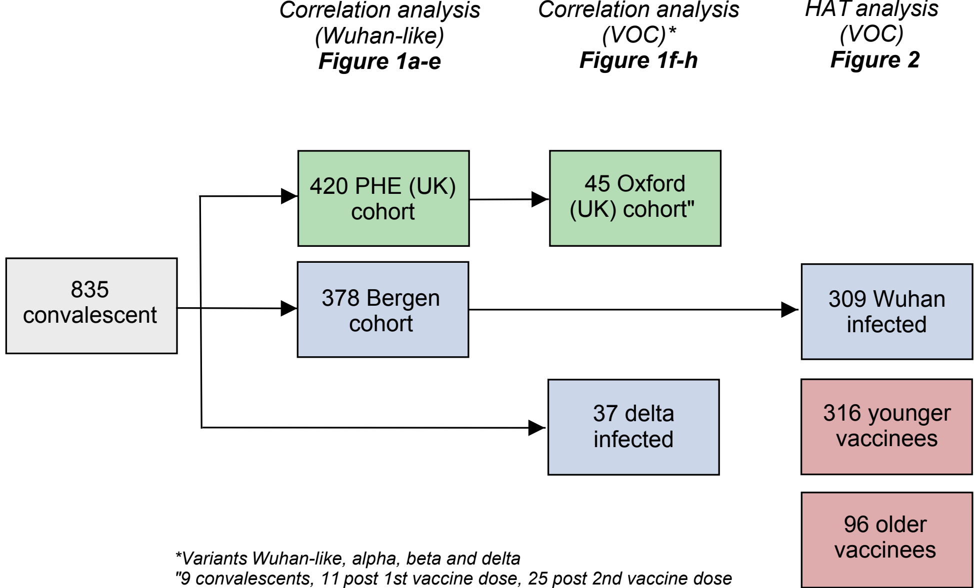






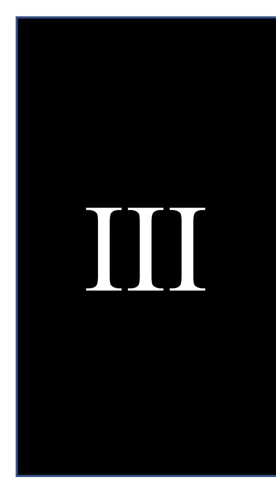
Correlation of endpoint HAT titres with binding antibody titres from a cohort of infected individuals from the first pandemic wave (diagnosis by PCR from nasopharyngeal swabs or serology in Bergen). Wuhan antibodies were measured by ELISA; **a** receptor binding domain (RBD) and **b** spike endpoint titres. The dotted lines show the lowest detectable titre in each assay and the numbers in blue are the number of samples in each quadrant. Samples size can be derived from adding the numbers together.

Supplementary Figure 2. Flow chart showing the cohorts included in the study.



A schematic figure showing the number of individuals used in the Bergen, Public Health England (PHE) and Oxford cohorts used in this study in different analysis and figures.

Papers I-IV



Post COVID-19 condition after delta infection and omicron reinfection in children and adolescents

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Summary

Background The burden of COVID-19 in children and adolescents has increased during the delta and omicron waves, necessitating studies of long-term symptoms such as fatigue, dyspnoea and cognitive problems. Furthermore, immune responses in relation to persisting symptoms in younger people have not been well characterised. In this cohort study, we investigated the role of antibodies, vaccination and omicron reinfection upon persisting and long-term symptoms up to 8 months post-delta infection.

Methods SARS-CoV-2 RT-PCR positive participants (n = 276, aged 10–20 years) were prospectively recruited in August 2021. We recorded the major symptoms of post COVID-19 condition and collected serum samples 3- and 8-months post delta infection. Binding antibodies were measured by spike IgG ELISA, and surrogate neutralising antibodies against Wuhan and delta variants by the hemagglutination test (HAT).

Findings After delta infection, persisting symptoms at 3 months were significantly associated with higher delta antibody titres (OR 2.97, 95% CI 1.57–6.04, p = 0.001). Asymptomatic acute infection compared to symptomatic infection lowered the risk of persisting (OR 0.13, 95% CI 0.02–0.55, p = 0.013) and long-term (OR 0.28 95% CI 0.11–0.66, p = 0.005) symptoms at 3 and 8 months, respectively. Adolescents (16–20 years) were more likely to have long-term symptoms compared to children (10–15 years) (OR 2.44, 95% CI 1.37–4.41, p = 0.003).

Interpretation This clinical and serological study compares long-term symptoms after delta infection between children and adolescents. The association between high antibody titres and persisting symptoms suggest the involvement of an immune mechanism. Similarly to adults, the dominant long-term symptoms in children are fatigue, dyspnoea and cognitive problems.

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Keywords: Post COVID-19 condition; SARS-CoV-2 infection; Antibody; Delta variant; Omicron variant; Children and adolescents

Introduction

Infection with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) can cause long-term symptoms, commonly known as post COVID-19 condition or long COVID.[1] This condition has predominantly been characterised in adults and fewer studies have focused on

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Research in context

Evidence before this study

We searched PubMed, MEDLINE and preprint repositories from May 9th, 2022, to January 5th, 2023, for publications on long-term symptoms after COVID-19 in children and adolescents without any language restrictions. Search terms were "COVID-19"or "SARS-CoV-2" adjacent to long COVID or post COVID-19 condition synonyms, combined with various search terms for children and adolescent and age filters (10–20 years). We further extended this search to include key words for available COVID mRNA vaccines, comprising preprint repositories. Finally, we searched for "immun*" or "serolog* or antibod* after COVID infection or vaccination in the relevant age group. We also found relevant references via citation searching. The major paediatric long-COVID studies are large, cross-sectional epidemiological studies that investigate persisting symptoms after SARS-CoV-2 infection with the ancestral or alpha strain, using online apps for data collection. However, prospective systematic studies including immune responses are largely lacking. The general conclusion drawn from current research is that long-term symptoms in young children are rare, but more common in adolescents. Other risk factors are reported to be female sex and acute SARS-CoV-2 symptoms. We could not find any published articles on how vaccination impacted symptoms in a young cohort.

Added value of this study

In our observational paediatric study, we have characterised risk factors for long-term symptoms, using the WHO post COVID-19 condition definition, after delta infection and omicron reinfection. The study was designed to compare symptoms between children and adolescents. We collected blood samples from a subgroup, providing a unique opportunity to connect persisting symptoms and reinfections with immunological data. We found that acute symptomatic infection and higher antibody titres correlated with post COVID-19 condition. Adolescents were more likely to report long-term symptoms than children, including fatigue (44% vs 19%), dyspnoea (25% vs 16%) and cognitive symptoms (36% vs 16%), which often persisted over time. Omicron infection resulted in increased respiratory and systemic symptoms, particularly in children.

Implications of all the available evidence

While mortality and severe acute illness is uncommon in children and adolescents, persisting symptoms may be a major risk in these young people. The societal impact may be further escalated by emergence of new variants with increased infectivity, as observed by high rates of omicron reinfection in our study. To mitigate long-term COVID sequela, more studies are needed on pathophysiological mechanisms in vulnerable groups, including children and adolescents.

children and adolescents.[2,3] Consequently, no paediatric long COVID definition has been determined, but a recent initiative suggested the following definition: at least one persisting symptom with a minimum 3-month duration, which impacts everyday functions.[4] Our evolving understanding is that risk factors for paediatric long-term symptoms are acute symptoms, post-puberty age and female sex.[5]

Knowledge about long-term symptoms comprises mostly the ancestral Wuhan-Hu-1 (Wuhan) and alpha strain, while data on long-term symptoms after delta and omicron infection is limited.[6] Significantly higher upper respiratory tract viral loads were associated with the delta variant.[7] Previous studies with earlier variants have found low prevalence of long-term symptoms in children <16 years compared to adults[8,9] or uninfected controls.[10,11] Younger people have experienced the burden of new infections with delta and omicron, and therefore the risk of post-COVID complications may have increased in this age group.[12] Although COVID vaccines were licensed, adolescents were often not vaccinated before the delta wave due to prioritised vaccine roll-out, partially explaining high infection rates in this population.[13] It is unknown if COVID vaccination decreases persisting symptoms in younger age groups, but consensus of a protective vaccine effect in adults is growing.[14]

Previous studies on post COVID-19 condition have found an association between the SARS-CoV-2 spike IgG antibodies and persisting symptoms in adults,[15] even after mild disease.[8,9] We have previously shown that a rapid, low-cost receptor-binding domain (RBD)-specific hemagglutination test (HAT) is highly correlated with neutralising antibodies, including antibodies to delta, and may be used as a correlate for neutralising antibodies.[16]

We hypothesised that the higher viral load reported after delta infections would impact persisting symptoms in children and adolescents. We investigated if an association between immune responses and long-term symptoms could be found in children, similarly to adults. In this study, we addressed these knowledge gaps by following a prospectively recruited cohort of children after mild SARS-CoV-2 delta infection.

Methods

Study design

During the first delta wave in Bergen municipality, children and adolescents (n = 276, 40% of eligible participants) aged 10–20 years who had SARS-CoV-2 infection confirmed by RT-PCR in a nasopharyngeal or oral swab were recruited, from August 1st to September 16th, 2021 (Supplementary Table S1). Local

recommendations for testing were symptoms of acute respiratory infection or close contact with a confirmed SARS-CoV-2 positive person. Questionnaires were answered at recruitment (baseline), 3 and 8 months after initial delta infection (Supplementary Fig. S1). Serum samples were collected at 3- and 8-months post infection from n = 88 and n = 87 participants, respectively.

Ethical considerations

The study was approved by the Regional Ethics Committee of Western Norway (#118664) and registered in ClinicalTrials.gov (NCT04706390). All individuals provided written or digital informed consent. For children aged 15 years or younger, parents or legal guardians signed the informed consent.

Clinical data collection

Baseline data were collected by telephone interviews of children or their parents (children <16 years) and stored in electronic case report forms (eCRFs) in the Research Electronic Data Capture database (REDCap®), Vanderbilt University, Nashville, Tennessee. The first follow-up at 3 months post-infection (median 104 days, interquartile range (IQR) 62–109 days) involved a subgroup of participants (n = 89) who were willing to come to the study outpatient clinic and donate a blood sample (n = 88). The second follow-up at 8 months post-infection (n = 204, median 245 days, IQR 205–254 days), was a combination of online responses, telephone interviews and in-person at our hospital's outpatient clinic. All participants were invited to answer a follow-up online questionnaire. A link to the eCRF was sent by SMS to participants ≥16 years or to their parents if less than 16 years. If they were unable to attend the clinic, participants (51%) were interviewed by telephone with the same questionnaire.

Questionnaires at baseline, 3 and 8 months recorded demographic information, comorbidities, medication, COVID-19 vaccination, any reinfection, and up to 18 COVID-19 related symptoms (see Supplementary Table S2). The WHO-definition of post COVID-10 syndrome,[1] where fatigue, dyspnoea and cognitive symptoms are highlighted, were utilised when defining persisting (3 month) and long-term (8 month) symptoms. Alternative diagnoses were excluded by collecting information on comorbidities, and specifically asking if the children had been diagnosed with any new medical condition during the last 8 months at the 8-month follow-up. If a participant reported any persisting or long-term symptoms, not explained by any other diagnosis, the participant was considered to fulfil the criteria for post COVID-19 condition. All participants answered dichotomized yes/no questionnaires with questions about persisting COVID-19 related symptoms at follow-up visits. At 3 months, the validated 11-questionnaire Chalder Fatigue Scale (CFS)[9] with graded responses was used to assess physical and mental fatigue in participants aged ≥16 years, while a modified shortened version of CFS was answered by all participants at the 8-month follow-up.

Up to February 2022 a positive SARS-CoV-2 antigen test result was confirmed by RT-PCR, but later the national health authorities accepted rapid antigen tests as a verified case of COVID-19, and this is reported as a positive test in our cohort. From December 30th, 2021, all recorded reinfections were defined as omicron infections, based on surveillance data from the Norwegian Institute of Public Health. Participants were not screened for omicron reinfections, but actively asked if they had had SARS-CoV-2 infection. During this period, active testing was conducted by the municipality with free lateral flow tests provided to all Norwegians and positive tests confirmed by centralised SARS-CoV-2 PCR testing. During recruitment, the national health authorities recommended COVID-19 vaccination for all age groups 16 years and older. Subjects vaccinated <14 (n = 7) and <3 (n = 4) days pre-infection were considered unvaccinated when analysing the impact on symptoms, and antibody responses, respectively. The two different cut-offs were based on immunological responses. Vaccination after a priming stimulus (or infection followed by vaccination) may recall memory B cells within 3–5 days. The cut-off was therefore set at three days. However, when it comes to symptoms, we chose a minimum of 14 days to give sufficient time for the antibody responses to reach a plateau, before measuring its potential effect on symptom outcomes.

Source data are available upon reasonable request to the corresponding author.

Serum samples

Serum samples were collected at follow-up visits at 3 and 8 months (Supplementary Fig. S1). At the first follow-up, 88 participants provided serum samples, and 87 did so at the second visit. Clotted blood was centrifuged and sera separated, aliquoted, and stored at –80 °C. Samples were heat-inactivated for 1 h at 56 °C before running in the hemagglutination test (HAT) and enzyme-linked immunoassay (ELISA).

Hemagglutination test (HAT)

The hemagglutination test (HAT)[16] was used to investigate the SARS-CoV-2 specific antibodies to the RBD of the ancestral Wuhan-like, delta/B1.617.2 (L452R, T478K) and omicron BA.2 variants using codon optimised IH4-RBD sequence (see sequence alignment in Supplementary Fig. S2). Three additional mutations outside the antibody epitopes, Y365F, F392W and V395I, were included in the BA.2 RBD sequence to improve yield and stability (Supplementary Fig. S2). Sera were double diluted from 1:40 to 1:640 with 120ng/well of IH4-RBDs and equal volume of human 0-negative blood (~1% v/v in PBS). Negative (PBS) and

positive (EY6A) controls were included in each run. Red blood cells were allowed to settle for 1 h and positive wells agglutinated red blood cells. The HAT titre is defined as the last well in which a teardrop did not form.

ELISA
The SARS-CoV-2 spike protein from the Wuhan virus was purified in house and used as coating antigen in the ELISA to detect spike specific IgG antibodies as previously described.[9]

Statistical analysis
Data analysis and visualisation were performed in R version 4.1.3 (R Foundation for Statistical Computing, Vienna, Austria) and GraphPad Prism version 9.5.0 (USA). Illustrations were created with BioRender (Supplementary Fig. S1).

Questions about fatigue, concentration, and memory problems (from the Chalder questionnaire) and depression, originally assigned four categories in questionnaires 3- and 8-months post-infection (0 = less/better than usual, 1 = not more/worse than usual, 2 = more/worse than usual and 3 = much more/worse than usual), were converted to binary categories (0–1 vs 2–3).

The 95% CI in Fig. 1 and Table 1 were calculated by applying prop. test function in R with default settings (null hypothesis stating the two groups had the same proportions and two-sided Z test with Yates' continuity correction and with Wilson's score method). In Fig. 2, the percentages of symptoms are shown with 95% confidence intervals (CI) for proportions in this figure were calculated by Wald interval. Crude risk differences were calculated as an excess percentage of symptoms in one group compared to another group, and significant differences are shown by p values in the figure.

Univariable and multivariable binomial logistic regression were used for analyses of binary outcome variables. The variables were selected both based on a priori hypothesis as explained above and suspicion of confounding (especially age and COVID vaccination). Variables were evaluated by a direct acyclic graph and selected based on multiple published studies showing that age, sex, baseline symptoms and vaccination affect long-term symptoms. The linearity assumption is checked both for univariable and multivariable analysis by using Box–Tidwell test (using car package with boxTidwell function). All of the interactions were insignificant.

IgG and HAT antibody titres in Figs. 3 and 4 and Table 1, Supplementary Tables S3 and S4 were log(10)-transformed to correct for skewness of distribution. Mann–Whitney U test was used to compare untransformed continuous variables in Figs. 3 and 4.

Role of funding sources
Funders had no role in study design, data collection, data analyses, interpretation, or writing of report.

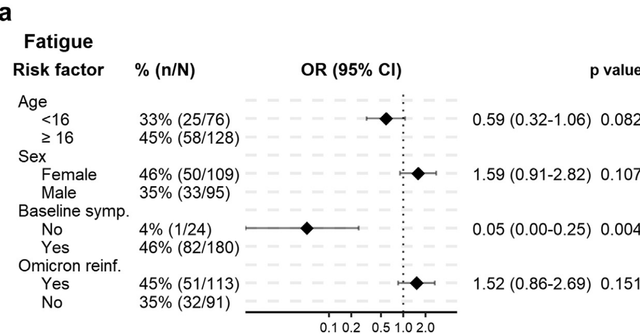
Results
Cohort descriptive
The cohort consisted of 276 children and adolescents (BMI range 15.8–30.1), with mean age of 16.5 years (range 10–20 years), 54% were females (Supplementary Table S1). The most common comorbidities were seasonal allergies (12%) and asthma (9%). All participants had mild, self-limiting delta SARS-CoV-2 infection, not requiring hospitalisation.

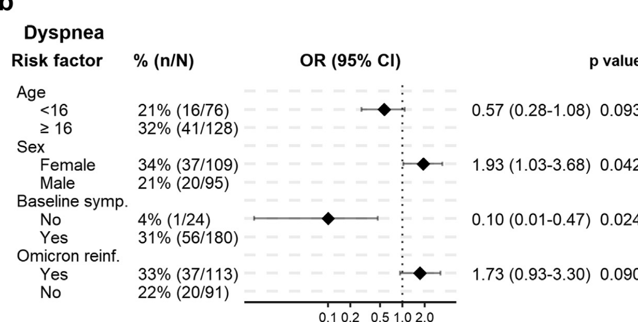
A total of 103 participants (37%), all 16 years or older (mean age 19.0 years), had received their priming COVID mRNA-vaccine dose (primarily Comirnaty (BioNTech/Pfizer)) in June and July 2021, on average 1.5 months prior to the positive RT-PCR test (Supplementary Table S1, Supplementary Fig. S1). Based on the difference in vaccine recommendations, the cohort was divided into groups over and under 16 years.

Acute symptoms impacted persisting and long-term symptoms
Participants were RT-PCR tested for SARS-CoV-2 mainly due to acute symptoms (89%) (Supplementary Table S1). All participants with available serum samples 3 months post-infection (n = 88) had spike-specific IgG antibodies (end point titres >485), and seropositivity was 85% for HAT antibodies (titres ≥40) (surrogate neutralisation titres). The most common acute symptoms in symptomatic cases were fatigue (68%), distorted taste and smell (59%), fever (58%), cough (57%) and headache (57%) (Supplementary Table S2). The median duration of acute symptoms (n = 221) was 4 and 7 days for children and adolescents, respectively. Twenty-five participants had ongoing symptoms after the acute phase.

Eleven percent (30/276) of the participants had asymptomatic acute infection and were RT-PCR tested mostly due to close contact with a SARS-CoV-2 positive. Cases who were asymptomatic at baseline were less likely to have symptoms both at 3 (OR 0.13, 95% CI 0.02–0.55) and 8 (OR 0.28, CI 95% 0.11–0.66) months post-infection (Table 1, Fig. 1, Supplementary Table S3). Individuals reporting dyspnoea or fatigue during acute illness, were more likely to report these symptoms long-term at 8 months (OR 4.21, 95% CI 2.19–8.22 and OR 3.16, 95% CI 1.67–6.21, respectively) (Table 1). Experiencing headache during the acute illness was associated with cognitive dysfunction (impaired memory and concentration) (OR 2.48, 95% CI 1.33–4.76) 8 months post-acute infection (Table 1).

Young age (<16 years) was associated with reporting fewer symptoms. Adolescents (16–20 years) more frequently experienced acute symptoms (fatigue, dyspnoea, headache and taste-smell distortion) and long-term symptoms ("any symptoms", fatigue) (Fig. 2). In the group eligible for vaccination (adolescents ≥16 years), there was no association between vaccination and frequency of symptoms (Supplementary Fig. S3).





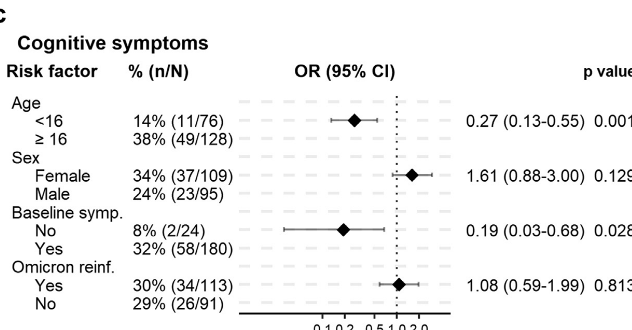


Fig. 1: **Risk factors for long-term symptoms 8 months post delta infection.** Forest plots show odds ratios (OR) with 95% confidence intervals (CIs) and p-values calculated by univariable binary logistic regression models using relevant symptoms. The outcomes are **a** fatigue, **b** dyspnoea and **c** cognitive symptoms at 8 months and relevant risk factors are age (<16 or ≥16 years), sex, symptoms at baseline and omicron reinfection status. The number and percentage (n/N, %) of individuals in each risk factor are shown with the relevant outcomes in the table and forest plot.

At 3 months post delta infection (median 104 days, IQR 62–109 days), we followed a subgroup of 89 participants with questionnaires and serum samples (n = 88). We found that 56% of the cohort reported persisting symptoms at 3 months, although with a lower symptom burden than during the acute phase (mean number of symptoms 1.8 versus 4.0 in the acute phase). The most prominent symptoms were fatigue (36%), impaired concentration (27%) and headache (21%), especially in adolescents (Supplementary Table S2).

	N	OR (CI) P Unadjusted	OR (CI) P Adjusted
Any persisting symptoms			
Female sex	204	1.48 (0.84–2.61) 0.173	1.22 (0.66–2.23) 0.524
Age ≥16 years	204	2.44 (1.37–4.41) 0.003	2.33 (1.28–4.28) 0.006
Reinfection	204	1.43 (0.81–2.53) 0.214	1.41 (0.78–2.56) 0.254
Asymptomatic at baseline	204	0.28 (0.11–0.66) 0.005	0.35 (0.13–0.88) 0.029
Dyspnoea			
Female sex	204	1.93 (1.03–3.68) 0.042	1.61 (0.83–3.18) 0.161
Age ≥16 years	204	1.77 (0.92–3.51) 0.093	1.3 (0.63–2.71) 0.484
Reinfection	204	1.73 (0.93–3.3) 0.090	1.67 (0.86–3.3) 0.135
Dyspnoea baseline	204	4.21 (2.19–8.22) <0.001	3.63 (1.82–7.34) <0.001
Cognitive symptoms[a]			
Female sex	204	1.61 (0.88–3) 0.129	1.37 (0.72–2.65) 0.338
Age ≥16 years	204	3.67 (1.82–7.95) 0.001	3.36 (1.64–7.37) 0.001
Reinfection	204	1.8 (0.59–1.99) 0.813	1.05 (0.55–2.01) 0.881
Headache baseline	204	2.48 (1.33–4.76) 0.005	2.09 (1.08–4.12) 0.030
Neurological symptoms[b]			
Female sex	204	1.33 (0.62–2.92) 0.464	1.14 (0.52–2.56) 0.750
Age ≥16 years	204	2.97 (1.23–8.32) 0.023	2.72 (1.11–7.67) 0.039
Reinfection	204	1.4 (0.49–2.26) 0.915	1.02 (0.47–2.26) 0.962
Headache baseline	204	2.2 (1.01–5.11) 0.055	1.92 (0.85–4.58) 0.125
Fatigue			
Female sex	204	1.59 (0.91–2.82) 0.107	1.47 (0.82–2.66) 0.200
Age ≥16 years	204	1.69 (0.94–3.08) 0.082	1.5 (0.81–2.8) 0.202
Reinfection	204	1.52 (0.86–2.69) 0.151	1.43 (0.79–2.61) 0.233
Fatigue baseline	204	3.16 (1.67–6.21) 0.001	2.82 (1.47–5.62) 0.002

Age was used as a categorical variable to compare symptom prevalence in adolescents ≥16 years to children <16 years as a reference. Associated factors were reported as odds ratios (OR) with 95% confidence intervals (CIs) and p-values. In the multivariable analysis, adjustment was done for factors listed as predictors in the table. [a]Cognitive symptoms include memory and concentration difficulties. [b]Neurological symptoms include numbness, dizziness and sleeping problems.

Table 1: **Predictors for long-term symptoms 8 months post delta infection.**

Older age (≥16 years) was associated with persisting symptoms (Supplementary Table S3).

Convalescent antibody levels were associated with persisting symptoms

In unvaccinated participants, persisting symptoms at 3 months were associated with higher delta ($p = 0.042$) and Wuhan-specific HAT antibody titres ($p = 0.008$) (Fig. 3a) as well as Wuhan-specific spike IgG ($p = 0.048$) (Mann–Whitney U test) (Fig. 3b). Convalescent IgG spike antibodies were significantly correlated with persisting symptoms (Supplementary Table S3). Overall, vaccinated individuals had significantly higher convalescent antibodies (Fig. 4) than unvaccinated subjects, but had no difference in antibody titres in respect to persisting symptoms (Fig. 3).

Long-term symptoms post delta infection

When assessing long-term symptoms after delta infection, omicron reinfected participants were excluded. At the 8-month follow-up (median 245, IQR 205–254 days), the three most prevalent symptoms were fatigue (35%), cognitive problems (29%) and dyspnoea (22%) (Supplementary Table S2). Children <16 years reported less frequent symptoms, while adolescents had unchanged proportions of fatigue, dyspnoea, neurological or cognitive symptoms (Supplementary Table S2, Fig. 2). While long-term dyspnoea and fatigue were not associated with age, composite and specific symptoms such as cognitive dysfunction were associated with age (OR 3.67, 95% CI 1.82–7.95) (Table 1, Fig. 1). Females more frequently had long-term dyspnoea (OR 1.93, 95% CI 1.03-3.68) but no other specific symptoms (Fig. 1b). Depression was recorded only at 8-month follow-up, and 15% of our cohort reported feeling more sad or depressed than usual, of whom 75% were female (Supplementary Table S2). At 8 months, the data did not show evidence of an association between vaccination status and symptom prevalence in participants over 16 years, but the number of unvaccinated participants was low (n = 22) (Supplementary Table S4).

During our follow-up, we recorded symptom-impact on daily functioning, specifically school and/or work absenteeism and the ability to participate in extracurricular activities. At the 8-month follow-up, only 9 asymptomatic individuals (16%) were absent from work and/or extracurricular activities. In the individuals reporting absence, we found that 60% reported fatigue,

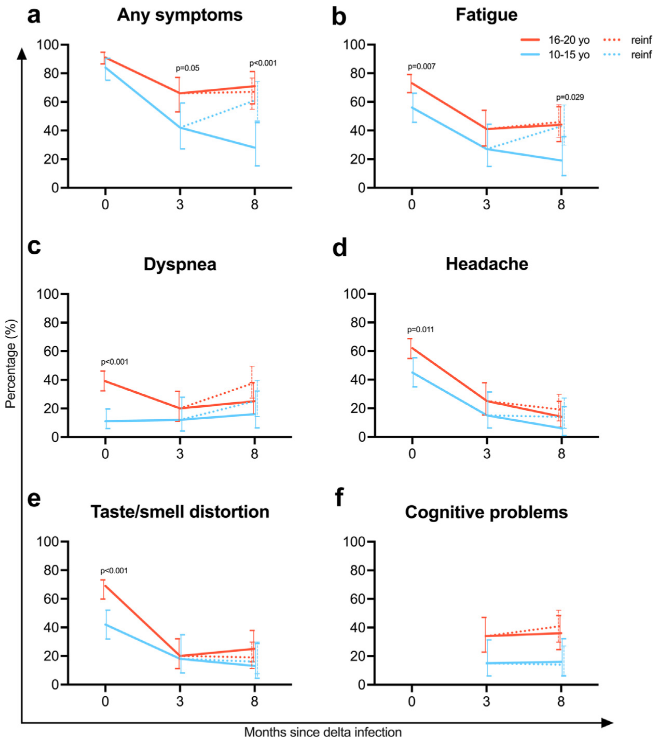


Fig. 2: **Dynamics of long-term symptoms after delta infection by age group and omicron reinfection status.** Symptoms were recorded 0- (acute), 3- and 8-months post-infection. The percentage of symptoms after delta infection are shown by continuous lines for participants 10–15 (orange) and 16–20 (light blue) years, **a** any symptom, **b** fatigue, **c** dyspnoea, **d** headache, **e** taste/smell distortion and **f** cognitive impairment. The dashed line indicates the percentage of symptoms reported at 8 months by omicron reinfected individuals. Crude risk differences were calculated between age groups after delta infection.

compared to 33% fatigue in those without absence (OR 3.1, 95% CI 1.6–5.9) (Supplementary Table S5).

The impact of omicron on long-term symptoms

The omicron reinfected group reported more long-term respiratory and systemic symptoms than those not reinfected, who had only experienced delta infection (Fig. 1, Supplementary Table S2), particularly in participants under 16 years (Fig. 2). There was, however, no difference in symptoms such as taste/smell, cognitive and neurological symptoms compared to delta infection alone (Fig. 1, Supplementary Table S1). After delta infection, the adolescent group had more long-term symptoms compared to children. Interestingly, adolescents reported no change in symptoms after omicron reinfection. This effect reduced the overall difference in symptoms reported by children and adolescents at 8 months (Fig. 2).

Discussion

In our prospective paediatric study, we showed that older age, having acute symptoms, and higher antibody titres are associated with long-term symptoms after SARS-CoV-2 delta infection. The association between antibody titres and long-term symptoms previously described in adults,[8,9,15] can in this study be extended to children. Hence, an immune dysfunction may also be involved in maintaining symptoms in children,[17] as observed in adolescents after other viral infections.

The three most common long-term symptoms after delta infection in our cohort were fatigue, dyspnoea and cognitive impairment, with higher frequencies in adolescents than in children. These three were recently confirmed as the common clusters of long-term COVID symptoms,[18] and frequently reported as persisting paediatric symptoms.[3,11,19,20] We observed that adolescents more frequently reported persistent and long-term

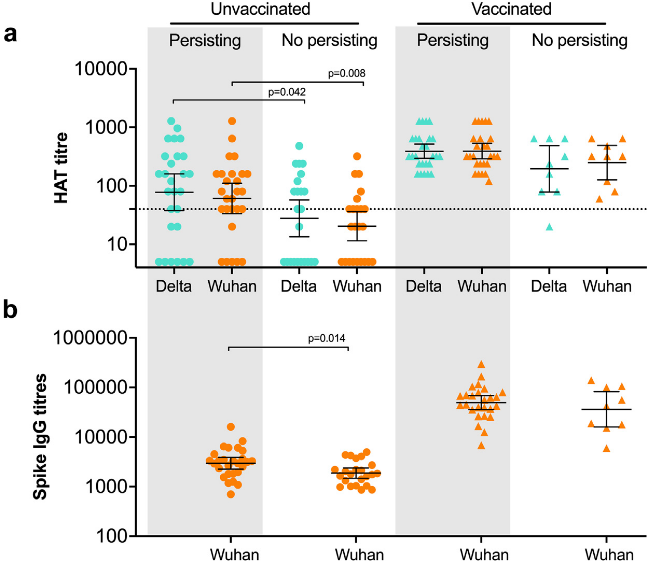


***Fig. 3:* The relationship between SARS-CoV-2 antibodies and persisting symptoms**. Hemagglutination test (HAT)-specific and binding (spike IgG) antibodies and persisting symptoms 3 months post-infection. The geometric mean antibody titres (GMT) with 95% confidence intervals are shown in black. **a** Delta (turquoise) and Wuhan (orange) antibodies and **b** Wuhan spike specific IgG antibody titres (orange) in individuals with persisting (grey background) and no persisting symptoms (white background). Vaccination status is shown by circles (unvaccinated) and triangles (vaccinated). Each symbol represents one individual. The dotted line indicates a positive test (HAT titre of ≥40), associated with neutralising antibodies. Negative values were assigned a value of 5. The differences between antibody titres in groups with persisting and no persisting symptoms were compared by the Mann Whitney U test.

symptoms than children, confirming the association between symptoms and age post-puberty,[5] despite high vaccination rates in adolescents. When comparing groups of vaccinated and unvaccinated adolescents, we found no significant differences in reported acute and persistent symptoms, although the unvaccinated group at 3 months was small. Many studies have found a reduced risk of long COVID symptoms after vaccination in adults,[14] and this is yet to be confirmed in younger age groups.

The course of long COVID can be fluctuating and protracted with worsening symptoms over time.[8,21,22] When investigating the individuals' symptoms over time, we observed that most symptoms improved, while others worsened, and some reported new symptoms during the study period. This fluctuation of symptoms is an essential characteristic of post COVID-19 condition, as defined by the WHO, and has been documented by other studies.[3] Furthermore, the post COVID-19 condition comprehend any impact on daily life, such as absence from school, work, or extracurricular activities. In 40% (49/124) of the young people in our study, symptoms were associated with absenteeism, compared to only 11% absence in the asymptomatic group.

Our study did not detect a higher frequency of depression or sadness than the Norwegian national average in 13–19-year-olds.[23] A recent study in children and adolescents reported that mental health long-term symptoms were more prevalent in cases compared to controls.[24] COVID-associated anxiety and mood disorders have been found to gradually subside over time.[25] In agreement with this, our 8 months data cannot confirm an association between COVID infection and long-term depressive symptoms. A recent large Norwegian study found limited increase in healthcare utilisation among adolescents 6 months after COVID-19.[26] However, concentration and memory problems may go unnoticed by the health care system due to their non-specific nature.

We confirm that acute symptoms and increasing age are factors associated with persisting symptoms.[3] A paediatric Australian study, with a very low median age of 3 years and high proportion of asymptomatic infections, found that children had fully recovered 3–6 months post-Wuhan infection.[27] In our cohort, children were more often asymptomatic than adolescents. Although most symptoms generally improved over time, 28% of children and 71% of adolescents in our

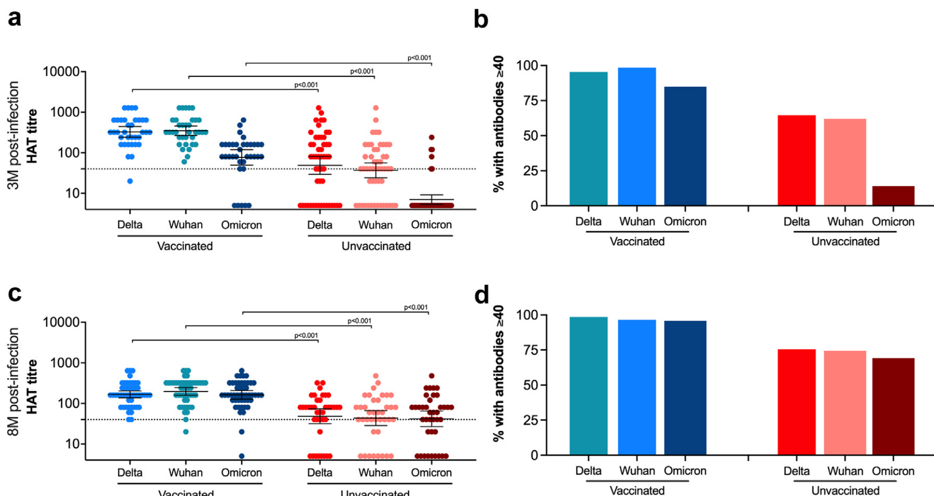


Fig. 4: **Antibody titres in vaccinated and unvaccinated individuals**. Hemagglutination test (HAT)-specific antibodies to the delta, Wuhan and omicron SARS-CoV-2 receptor binding domain (RBD), HAT titres (**a,c**) and percentage (%) with titres ≥40 (**b,d**). Sera were collected at 3 (**a,b)** and 8 months **(c,d)** post delta infection. At 3 months, individuals were divided based on pre delta infection vaccination status: vaccinated (blue, n = 35, sampled mean 76.1 days) and unvaccinated (red, n = 53, sampled mean 87.9 days). At 8 months, individuals who had never been previously COVID vaccinated were defined as unvaccinated (red, n = 39, sampled mean 147.0 days), and individuals with any previous vaccination, n = 48 (blue, n = 48, sampled mean 95.4 days). At 8 months, the percentage of reinfected individuals was comparable (54.5% in vaccinated and 66.7% in unvaccinated). Each individual is indicated by a circle. The dotted line indicates a positive test (HAT titre of ≥40), and negative values were assigned a value of 5. The geometric mean titres (GMT) with 95% confidence intervals are shown in black by horizontal lines. Vaccinated individuals were compared to unvaccinated by a Mann Whitney U test for each variant. Significant differences were maintained after adjusting for days since infection or vaccination at both time points.

cohort reported one or more long-term symptoms at 8 months. However, our study design does not allow inference about the contribution of COVID-19 to these symptoms, and any comparison should solely be made between these two age groups.

Our study is unique in combining immunological results with detailed long-term symptoms, largely based on personal interviews. Further strengths are long-term follow-up for 8 months after delta infection, including omicron reinfection rates. In contrast to other larger epidemiological studies, we have prospectively recruited participants shortly after infection, ensuring limited recall bias of acute symptoms. Our dropout rate was low, with 74% responding at 8 months. A strength of the study was that children and adolescents could be compared in the same geographic area, time period, with identical variant exposure, enabling exploration of the relative differences in long-term symptomatology.

Caveats to our study are the lack of a COVID negative control group which may overestimate the incidence of persisting symptoms attributable to SARS-CoV-2 infection, although our study was not designed to assess prevalence. Nevertheless, our findings are similar to studies with case control design.[24,28] Inclusion in this study was after delta infection, and we could therefore not control for pre-infection physical and mental health. Methodological limitations to the study are potential unmeasured confounding factors, and indirect data collection by parents of participants <16 years, although these children actively participated in the interviews. A modest sample size increases the risk of sparse data bias as evident by large effect size and confidence limits. The ongoing vaccination campaign during the study period skewed the vaccination coverage exclusively to adolescents, with only a few adolescents (n = 22/187) unvaccinated at 8 months, limiting interpretation of the impact of vaccination on long-term symptoms. Omicron reinfection increased the heterogeneity and hampered the long-term evaluation of delta-only infection. We were not able to investigate the relationship between antibody titres and 8 months symptoms due to antibody boosting after vaccinations and omicron reinfections leading to small groups available for comparison.

With emerging variants, hybrid immunity and fluctuating nature of post COVID-19 syndrome, it is difficult to diagnose and study paediatric long-term symptoms during an evolving pandemic. Our findings emphasise the importance of reducing the COVID-19 burden in young people and may shed light on the underlying pathophysiology of this syndrome.

Contributors

N.L. and R.J.C. designed the study. N.U.E, K.K, E.B.F, and K.H recruited the participants and followed them up. A.I. supplemented and distributed the electronic questionnaires. P.R. developed the HAT reagents. N.U.E and J.S.O conducted laboratory analysis. T. Ö, B.B, C.J.B, E.B.F., R.J.C, N.L. and N.U.E. analysed the data and conceptualised the analyses. N.U.E, N.L and R.J.C wrote the first version of the manuscript, and verified the underlying data. B.B. and A.I. critically revised the manuscript. Members of the Bergen COVID-19 research group contributed to the study follow-up, data collection, and laboratory assays. All authors read and approved the final version of the manuscript.

Data sharing statement

The R code used to generate all results in this paper is publicly available on GitHub https://github.com/turkulerc/DeltaKids. Source data are available upon reasonable request to the corresponding author.

Declaration of interests

All authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.ebiom.2023.104599.

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Supplementary material

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Supplementary Table 1. Cohort demographics.

	All	10-15 years old	16-20 years old
Number (n)	276	89	187
Mean age, years (SD)	16.5 (2.8)	13.2 (1.7)	18.1 (1.5)
Sex (%)			
Female	150 (54)	44 (49)	106 (57)
Male	126 (46)	45 (51)	81 (43)
Mean body mass index, BMI (SD)	21.6 (2.6)	19.9 (2.3)	22.3 (2.5)
Vaccination[a]	103 (37%)	0[b]	103 (55%)
1st dose	98/103 (95%)	0	98/103 (95%)
2nd dose	5 (5%)	0	5 (5%)
Comirnaty (BioNTech/Pfizer)	97 (94%)	0	97 (94%)
Spikevax (Moderna)	6 (6%)	0	6 (6%)
Comorbidities	47/193 (24%)	15 (22%)	32 (26%)
Seasonal allergies	24 (12%)	5 (7%)	19 (15%)
Asthma	17 (9%)	7 (10%)	10 (8%)
Gastrointestinal disease	4 (2%)	1 (2%)	3 (2%)
Neurological disease	4 (2%)	1 (2%)	3 (2%)
Chronic cardiovascular disease	1 (1%)	1 (2%)	0 (0)
Rheumatological disease	1 (1%)	1 (2%)	0 (0)
Medication	47/191 (25%)	15/67 (22%)	32/124 (26%)
Antihistamine	6 (3%)	2 (3%)	4 (3%)
Contraceptives	5 (3%)	0 (0)	5 (4%)
Inhalation steroids	3 (2%)	2 (3%)	1 (1%)
Immunosuppression	3 (2%)	1 (2%)	2 (2%)
Stimulants (ADHD medication)	2 (1%)	0 (0)	2 (2%)
Hormone substitution	1 (1%)	0 (0)	1 (1%)
Migraine medication	2 (1%)	1 (2%)	1 (1%)
Preventative cardiovascular medication	1 (1%)	0 (0)	1 (1%)
Other[c]	24 (13%)	9 (13%)	15 (12%)

[a]Vaccination prior to delta infection
[b]Vaccination not recommended at inclusion time point
[c]Missing specific information

Supplementary Table 2. Symptoms in children and adolescents up to 8 months post delta infection.

	Delta									Omicron reinfection*		
	Acute phase symptoms			3 months post-infection			8 months post-infection			2 months post-reinfection		
Age (years)	All	10-15	16-20	All	10-15	16-20	All	10-15	16-20	All	10-15	16-20
Number	276	89	187	89	33	56	91	32	59	113	44	69
Asymptomatic	30 (11%)	14 (16%)	16 (9%)	40 (45%)	19 (58%)	19 (34%)	40 (44%)	23 (72%)	17 (29%)	40 (35%)	17 (39%)	23 (33%)
Any symptoms	246 (89%)	75 (84%)	171 (91%)	51 (57%)	14 (42%)	37 (66%)	51 (56%)	9 (28%)	42 (71%)	73 (65%)	27 (61%)	46 (67%)
Any general symptoms	225 (82%)	68 (76%)	157 (84%)	39 (44%)	11 (33%)	28 (50%)	36 (40%)	6 (19%)	30 (51%)	55 (49%)	20 (45%)	35 (51%)
Fever	161 (58%)	49 (55%)	112 (60%)	5 (6%)	1 (3%)	4 (7%)	1 (1%)	0 (0)	1 (2%)	8 (7%)	2 (5%)	6 (9%)
Fatigue	187 (68%)	50 (56%)	137 (73%)	32 (36%)	9 (27%)	23 (41%)	32 (35%)	6 (19%)	26 (44%)	51 (45%)	19 (43%)	32 (46%)
Headache	156 (57%)	40 (45%)	116 (62%)	19 (21%)	5 (15%)	14 (25%)	10 (11%)	2 (6%)	8 (14%)	19 (17%)	6 (14%)	13 (19%)
Gastro-intestinal symptoms	45 (16%)	14 (16%)	31 (17%)	7 (8%)	2 (6%)	5 (9%)	8 (9%)	0 (0)	8 (14%)	4 (4%)	1 (2%)	3 (4%)
Muscle/joint pain	81 (29%)	17 (19%)	64 (34%)	-	-	-	2 (2%)	0 (0)	2 (3%)	8 (7%)	2 (5%)	6 (9%)
Palpitations	-	-	-	7 (8%)	2 (6%)	5 (9%)	5 (5%)	1 (3%)	4 (7%)	5 (4%)	1 (2%)	4 (6%)
Any respiratory symptoms	216 (78%)	68 (76%)	148 (79%)	19 (21%)	7 (21%)	12 (21%)	29 (32%)	5 (16%)	24 (41%)	59 (52%)	20 (45%)	39 (57%)
Dyspnoea	83 (30%)	10 (11%)	73 (39%)	15 (17%)	4 (12%)	11 (20%)	20 (22%)	5 (16%)	15 (25%)	37 (33%)	11 (25%)	26 (38%)
Cough	158 (57%)	38 (43%)	120 (64%)	-	-	-	0 (0)	0 (0)	0 (0)	13 (12%)	4 (9%)	9 (13%)
Congested nose/sore throat	64 (23%)	26 (29%)	38 (20%)	6 (7%)	5 (15%)	1 (2%)	4 (4%)	1 (3%)	3 (5%)	8 (7%)	5 (11%)	3 (4%)
Chest pain	-	-	-	-	-	-	6 (7%)	2 (6%)	4 (7%)	6 (5%)	1 (2%)	5 (7%)
Smell/taste	162 (59%)	37 (42%)	125 (69%)	17 (19%)	6 (18%)	11 (20%)	19 (21%)	4 (13%)	15 (25%)	20 (18%)	7 (16%)	13 (19%)
Any neurological symptoms	-	-	-	19 (21%)	8 (24%)	11 (20%)	14 (15%)	1 (3%)	13 (22%)	18 (16%)	5 (11%)	13 (19%)
Numbness	-	-	-	0 (0)	0 (0)	0 (0)	3 (3%)	0 (0)	3 (5%)	5 (4%)	1 (2%)	4 (6%)
Dizziness	-	-	-	12 (14%)	7 (21%)	5 (9%)	9 (10%)	1 (3%)	9 (14%)	12 (11%)	3 (7%)	9 (13%)
Sleeping problems	-	-	-	10 (11%)	2 (6%)	8 (14%)	7 (8%)	0 (0)	7 (12%)	8 (7%)	2 (5%)	6 (9%)
Depression	-	-	-	-	-	-	15 (16%)	4 (13%)	11 (19%)	17 (15%)	2 (5%)	15 (22%)
Any cognitive symptoms	-	-	-	24 (27%)	5 (15%)	19 (34%)	26 (29%)	5 (16%)	21 (36%)	34 (30%)	6 (14%)	28 (41%)
Impaired memory	-	-	-	14 (16%)	3 (9%)	11 (20%)	19 (21%)	4 (13%)	15 (25%)	19 (17%)	3 (7%)	16 (23%)
Impaired concentration	-	-	-	21 (24%)	5 (15%)	16 (29%)	20 (22%)	5 (16%)	15 (25%)	25 (22%)	4 (9%)	21 (30%)
Other problems	17 (6%)	4 (5%)	13 (7%)	11 (12%)	1 (3%)	10 (18%)	4 (4%)	1 (3%)	3 (5%)	9 (8%)	3 (7%)	6 (9%)

Data presented as numbers (percentage, %). *n=2 individuals were delta reinfected

Supplementary Table 3. Predictors for persisting symptoms 3 months post delta infection.

	N	OR (CI) P Unadjusted	OR (CI) P Adjusted
Any persisting symptoms			
Female sex	89	0.99 (0.42-2.3) 0.973	0.53 (0.18-1.51) 0.249
Age ≥16 years	89	2.64 (1.1-6.52) 0.031	1.7 (0.47-6.26) 0.416
Asymptomatic baseline	89	0.13 (0.02-0.55) 0.013	0.08 (0.01-0.45) 0.01
Antibodies (IgG) Wuhan[#]	88	2.97 (1.57-6.04) 0.001	3.65 (1.53-9.83) 0.006
Vaccination	89	0.47 (0.18-1.15) 0.105	2.23 (0.55-10.04) 0.274

Age was used as a categorical variable to compare symptom prevalence in adolescents ≥16 years children <16 years as a reference. Associated factors were reported as odds ratios (OR) with 95% confidence intervals (CIs) and p-values. In the multivariable analysis, adjustment was done for factors listed as predictors in the table.
*Cognitive symptoms include memory and concentration difficulties.
[#]Spike IgG are log-transformed and used as a continuous, independent variable

Supplementary Table 4. Factors associated with long-term symptoms 8 months post delta infection in adolescents (≥16 years old)

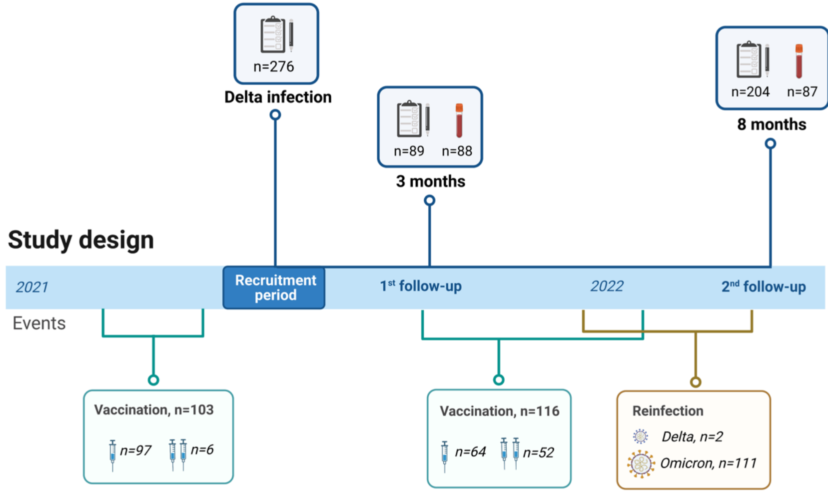
	N	Estimate (CI) P Unadjusted	Estimate (CI) P Adjusted
Any persisting symptoms			
Female sex	128	1.6 (0.75-3.41) 0.223	1.5 (0.66-3.38) 0.331
Age	128	0.82 0.63-1.06) 0.131	0.77 (0.57-1.01) 0.064
Reinfection	128	0.81 0.38-1.71) 0.583	0.8 (0.37-1.73) 0.572
Any symptoms at baseline	128	1.29 (0.32-4.54) 0.703	1.32 (0.3-5.31) 0.697
Vaccination	128	1.67 (0.63-4.29) 0.286	1.98 (0.7-5.57) 0.191
Dyspnoea			
Female sex	128	1.88 0.88-4.15) 0.107	1.59 (0.69-3.71) 0.227
Age	128	0.93 (0.72-1.2) 0.566	0.88 (0.66-1.16) 0.366
Reinfection	128	1.77 0.84-3.86) 0.14	1.83 (0.81-4.22) 0.149
Dyspnoea baseline	128	4.35 (2-9.74) <0.001	4.23 (1.91-9.69) <0.001
Vaccination	128	1.31 (0.49-3.93) 0.6	1.34 (0.44-4.51) 0.62
Cognitive symptoms*			
Female sex	128	1.93 (0.93-4.09) 0.079	1.58 (0.72-3.55) 0.258
Age	128	0.77 (0.6-0.99) 0.043	0.7 (0.53-0.91) 0.011
Reinfection	128	1.24 (0.6-2.55) 0.563	1.22 (0.57-2.66) 0.605
Headache baseline	128	2.44 (1.16-5.33) 0.022	2.59 (1.16-6.04) 0.023
Vaccination	128	1.82 (0.69-5.41) 0.248	2.67 (0.9-8.98) 0.09
Neurological symptoms[§]			
Female sex	128	1.12 (0.47-2.73) 0.798	1.01 (0.4-2.57) 0.989
Age	128	0.83 (0.61-1.11) 0.208	0.81 (0.59-1.09) 0.168
Reinfection	128	0.82 (0.34-1.96) 0.655	0.81 (0.34-1.97) 0.647
Headache baseline	128	1.43 (0.59-3.65) 0.432	1.52 (0.6-4.05) 0.387
Vaccination	128	1.18 (0.39-4.39) 0.785	1.53 (0.47-6.15) 0.511
Fatigue			
Female sex	128	1.87 (0.92-3.84) 0.086	1.9 (0.88-4.17) 0.104
Age	128	0.84 (0.66-1.07) 0.158	0.74 (0.56-0.96) 0.027
Reinfection	128	1.1 (0.55-2.22) 0.794	1.07 (0.5-2.28) 0.866
Fatigue baseline	128	3.92 (1.68-10.08) 0.003	4.85 (1.98-13.09) 0.001
Vaccination	128	1.56 (0.62-4.2) 0.357	2.03 (0.72-6.16) 0.191

Age was used as a continuous variable. Associated factors were reported as odds ratios (OR) with 95% confidence intervals (CIs) and p-values. In the multivariable analysis, adjustment was done for factors listed as predictors in the table.
*Cognitive symptoms include memory and concentration impairment.
[$]Neurological symptoms include numbness, dizziness and sleeping problems.

Supplementary table 5: The impact of long-term symptoms on absenteeism from work and extracurricular activities at 8 months post delta infection.

	Absenteeism (n/N, %)		OR (CI)
	No	Yes	
N	146	58	
Age (years)			
<16	54 (37%)	22 (38%)	1.0 (0.5-2.09)
≥16	92 (63%)	36 (62%)	
Sex			
Female	75 (51%)	34 (59%)	1.4 (0.7-2.5)
Male	71 (49%)	24 (41%)	
Asymptomatic			
Baseline	21 (14%)	3 (5%)	0.3 (0.1-1.0)
8 months	71 (49%)	9 (16%)	0.2 (0.1-0.4)
Symptoms 8M			
Fatigue	48 (33%)	35 (60%)	3.1 (1.6-5.9)
Dyspnoea	31 (21%)	26 (45%)	3.0 (1.6-5.8)
Cognitive symptoms	41 (28%)	19 (33%)	1.2 (0.6-2.4)
Depression	15 (10%)	17 (29%)	3.6 (1.6-8.0)

Supplementary figure 1. Study design.



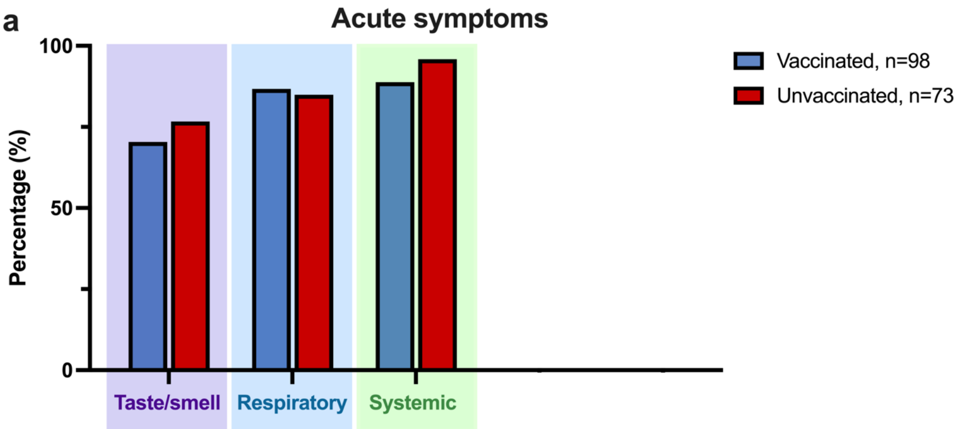
276 delta infected children and adolescents 10-20 years old were recruited after RT-PCR-confirmed SARS-CoV-2 infection, from August 1st to September 16th, 2021. Questionnaires about acute, persisting, and long-term symptoms were answered at recruitment (baseline) (n=276), 3 (n=89) and 8 months (n=204) after delta infection. Serum samples were collected at 3 (n=88) and 8 months (n=87) post infection from a subset of participants. Questionnaires and health care records were used to provide information about COVID vaccination and SARS-CoV-2 reinfection throughout the study period. A total of 110 participants (>16 years old) were vaccinated pre delta infection, of whom n=103 had received 1st dose and n=7 the 2nd dose within the defined criteria of vaccination (≥14 days prior to infection). At the 8-month follow-up, 116 participants (>15 years) were vaccinated, n=88 post delta infection, and n=28 only pre delta infection. SARS-CoV-2 reinfections, confirmed by RT-PCR or antigen tests, identified n=2 delta reinfections in Oct-Nov 2021 and n=111 omicron reinfections from 30th Dec 2021 to April 2022. The illustration was created with BioRender.

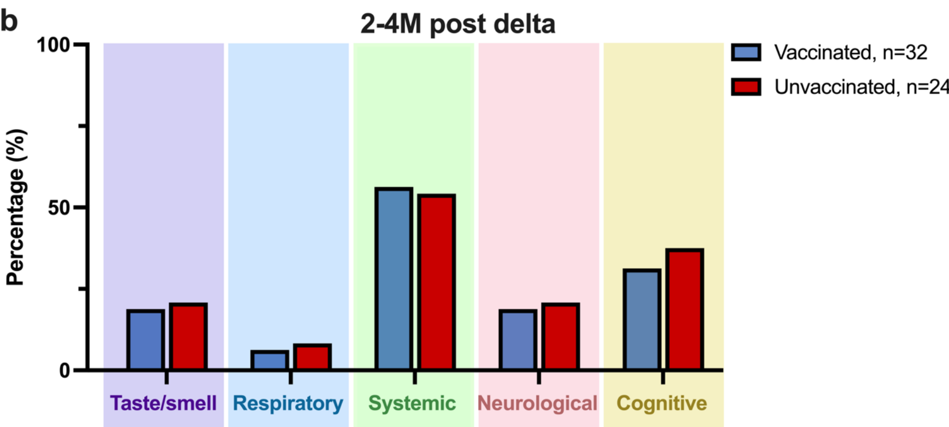
Supplementary figure 2: Sequence alignment in the SARS-CoV-2 receptor binding domain (RBD) regions of Wuhan, delta and omicron (BA.1 and BA.2).

Variant	Amino acids	aa
Wuhan	N I T N L C P F G E V F N A T R F A S V Y A W N R K R I S N C V A D Y S V L Y N S A S F S T F K C Y G V S P T K L N D L	390
Delta	. . . .	390
BA.1	. . . . D . . . . L . P . F	390
BA.2	. . . . D . . . . F . P . F A	390
Wuhan	C F T N V Y A D S F V I R G D E V R Q I A P G Q T G K I A D Y N Y K L P D D F T G C V I A W N S N N L D S K V G G N Y N	450
Delta	. . . .	450
BA.1	. . . . N . . . . K . . . . . S	450
BA.2	. . . . N . . S . . . . N . . . . K	450
Wuhan	Y L Y R L F R K S N L K P F E R D I S T E I Y Q A G S T P C N G V E G F N C Y F P L Q S Y G F Q P T N G V G Y Q P Y R V	510
Delta	. R . . . . K	510
BA.1	. . . . N K . . . . . A . . . . K . . S . R . . Y . . . H	510
BA.2	. . . . N K . . . . . A . . . . R . . . . R . . Y . . . H	510
Wuhan	V V L S F E L L H A P A T V C G P K K	529
Delta	. . . .	529
BA.1	. . . .	529
BA.2	. . . .	529

Amino acids (aa) that match the reference (Wuhan) are marked with dots.
Three additional mutations outside the antibody epitopes were introduced in the BA.1 and BA.2 RBD sequence to improve yield and stability (Ellis D, Brunette N, Crawford KHD, Walls AC, Pham MN, Chen C, et al. Stabilization of the SARS-CoV-2 Spike Receptor-Binding Domain Using Deep Mutational Scanning and Structure-Based Design. Front Immunol. 2021;12:710263.)

Supplementary figure 3. Acute and persistent symptoms in vaccinated and unvaccinated adolescents.





Proportion of acute a and persisting symptoms 3 months (3M) b post delta infection divided by vaccination status. Respiratory symptoms include dyspnoea, cough, congested nose/sore throat and chest pain. Systemic symptoms include fever, fatigue, headache, gastro-intestinal symptoms, muscle/joint pain and palpitations. Neurological symptoms include numbness, dizziness and sleeping problems. Cognitive symptoms include impaired memory and concentration. Vaccinated subjects are indicated in blue, and unvaccinated subjects in red. Questions about cognitive and neurological symptoms were not asked at baseline (acute symptoms).

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