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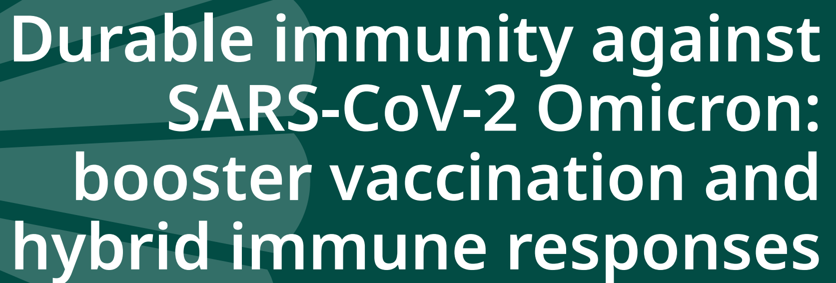

Durable immunity against SARS-CoV-2 Omicron: booster vaccination and hybrid immune responses

Milja Belik

TURUN YLIOPISTON JULKAISUJA – ANNALES UNIVERSITATIS TURKUENSIS
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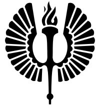

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To my daughter

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Faculty of Medicine
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ABSTRACT

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) emerged in late 2019, causing the coronavirus disease 2019 (COVID-19) pandemic with profound global impacts, prompting urgent vaccine development that achieved high efficacy against initial strains. Nevertheless, the virus's antigenic evolution led to variants capable of immune evasion, particularly Omicron in 2021 and its sublineages, which fueled widespread infections through numerous spike protein mutations that impair neutralizing antibody binding. Elucidating the long-term durability of immunity against Omicron is crucial for advancing understanding of sustained defense and refining public health vaccination strategies.

COVID-19 vaccines elicit adaptive immune responses, including humoral immunity via binding and neutralizing antibodies and cell-mediated immunity through memory T and B cells. In this study, we longitudinally monitored the immune responses of vaccinated Finnish healthcare workers over more than three years. We used enzyme immunoassay (EIA) to analyze binding immunoglobulin G (IgG) antibodies, a microneutralization test (MNT) to quantify neutralizing antibodies against Omicron variants, and an activation-induced marker (AIM) assay with flow cytometry to measure $CD4^+$ and $CD8^+$ T cell activation. In addition, we developed an ELISpot assay to quantify the number of memory B cells.

The results indicate differences between vaccine types and regimens. However, in all cases, antibody levels gradually waned after each dose but were effectively restored by following boosters, though Omicron variants exhibited partial escape from neutralization. Hybrid immunity sustained elevated antibody levels and broader cross-neutralization against variants. T cells were durable and cross-reactive against Omicron, memory T cell subsets persisted, and functional memory B cells were maintained. These findings underscore the persistence of T cells and hybrid immunity against Omicron variants, simultaneously emphasizing the value of booster vaccinations in maintaining protection.

KEYWORDS: SARS-CoV-2, Omicron, COVID-19, vaccines, hybrid immunity, booster dose, koronavirus, koronavirustauti

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TIIVISTELMÄ

Koronavirus SARS-CoV-2 ilmaantui loppuvuodesta 2019, aiheuttaen koronavirustauti COVID-19-pandemian, jolla oli huomattavia globaaleja vaikutuksia. Tämä johti myös kiireelliseen rokotteiden kehitykseen. Rokotteilla oli hyvä teho aiempia variantteja vastaan, mutta antigeenisen evoluution myötä kehittyi immuniteettia väistämään kykeneviä, uusia variantteja. Erityisesti Omicron, joka ilmaantui 2021 lopulla, sekä sen alavariantit saivat aikaan laajoja infektioaaltoja, välttäen neutraloivien vasta-aineiden sitoutumisen lukuisten piikkiproteiini-mutaatioidensa ansiosta. Omicronia vastaan säilyvän pitkän aikavälin immuunisuojan selvittäminen on ratkaisevan tärkeää ymmärryksen lisäämisessä immuunisuojan kestävyyteen liittyvistä tekijöistä sekä suunniteltaessa julkisen terveydenhuollon rokotusstrategioita.

Koronavirustauti COVID-19 aktivoi hankittua immuniteettia sekä humoraalisen immuniteetin ja siihen kuuluvien sitoutuvien ja neutraloivien vasta-aineiden kautta, että soluvälitteisen immuniteetin ja T- ja B-solujen kautta. Tässä väitöskirjassa pitkäaikaisseurattiin suomalaisten rokotettujen terveydenhuollon työntekijöiden immuunivasteita yli kolmen vuoden ajan. Seurannassa käytettiin EIA-vasta-ainetestiä sitoutuvien immunoglobuliini G (IgG) -vasta-aineiden analysoimiseksi, mikroneutralisaatiotestiä neutraloivien vasta-aineiden mittaamiseksi Omicron-variantteja vastaan, sekä aktivaatiomääritystä ja virtaussytometriaa $CD4^+$ ja $CD8^+$ T solujen aktivaation mittaamiseksi. Lisäksi pystytimme ELISpot-vasta-ainetestin muisti-B-solujen mittaamiseksi.

Tulokset osoittavat, että käytetyillä rokotetyypeillä ja rokotusohjelmilla on keskinäisiä eroja. Joka tapauksessa vasta-ainetasot laskivat asteittain jokaisen rokotusannoksen jälkeen, mutta seuraavat tehosteannokset palauttivat ne tehokaasti jälleen korkealle tasolle, vaikkakin neutralisaation havaittiin osittain heikkenevän Omicron-virusvarianttia vastaan. Hybridi-immuniteetti ylläpiti korkeita vasta-ainetasoja ja laajaa varianttien ristiinneutralisaatiota. T-solut säilyttivät pitkäkestoisen ristisuojan Omicronia vastaan ja muisti-T- sekä B-solujen aktivaatio säilyi hyvin. Tulokset korostavat T-solujen ja hybridi-immuniteetin kestävyyttä, sekä samalla tehosterokotusten tärkeyttä pitkäkestoisen immuunisuojan ylläpidossa.

AVAINSANAT: SARS-CoV-2, Omicron, COVID-19, vaccines, hybrid immunity, booster dose, koronavirus, koronavirustauti

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Abbreviations

ACE2	angiotensin-converting enzyme 2
Ad	Adenovirus
AIM	activation-induced marker
APC	antigen presenting cell
ARDS	acute respiratory distress syndrome
ASC	antibody-secreting cell
BCR	B cell receptor
BSL-3	biosafety level 3
CDC	U.S. Centers for Disease Control and Prevention
CFR	case-fatality ratio
ChAd	chimpanzee adenovirus
COVID-19	coronavirus disease 2019
cTfh	circulating follicular T helper cell
DMEM	Dulbecco's modified eagle medium
DMSO	dimethyl sulfoxide
dsDNA	double-stranded deoxyribonucleic acid
E	envelope protein
E1	early region 1
E3	early region 3
E4	early region 4
ECDC	European Centre for Disease Prevention and Control
EIA	enzyme immunoassay
ELISpot	enzyme-linked immunospot
EMA	European Medicines Agency
EMEM	Eagle's minimum essential medium
FBS	Fetal bovine serum
FDA	U.S. Food and Drug Administration
GC	germinal centre
GDP	gross domestic product
GM	geometric mean
hCoV	human coronavirus

HCW health care worker
HIV human immunodeficiency virus
HPV human papillomavirus
HRP horseradish peroxidase
HUS Helsinki University Hospital
ID50 inhibitory dose of 50%
IFN interferon
Ig immunoglobulin
IL interleukin
KRAR kansallinen rokotusasiantuntijaryhmä
M membrane protein
MERS Middle East respiratory syndrome coronavirus
MHC major histocompatibility complex
MNT microneutralization test
mRNA messenger ribonucleic acid
N nucleoprotein
NaN_3 sodium azide
NASEM National Academies of Sciences, Engineering and Medicine
NBT/BCIP Nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate
NK natural killer cell
NSP non-structural protein
NTD N-terminal domain
OD optical density
ORF open reading frame
PAMP pathogen-associated molecular patterns
PBMC peripheral blood mononuclear cell
PBS phosphate-buffered saline
PRR pathogen recognition receptor
RBD receptor binding domain
RdRp RNA-dependent RNA polymerase
RTC replicase-transcriptase complex
RT-PCR reverse transcription polymerase chain reaction
RT-qPCR quantitative reverse transcription polymerase chain reaction
S spike protein
S1 spike protein subunit 1
S2 spike protein subunit 2
SARS-CoV severe acute respiratory syndrome coronavirus
SARS-CoV-2 severe acute respiratory syndrome coronavirus 2
SD standard deviation
SI stimulation index

ssRNA	single-stranded ribonucleic acid
STM	Ministry of Social Affairs and Health
$TCID_{50}$	fifty-percent tissue culture infective dose
TCR	T cell receptor
TCM	central memory T cell
TEM	effector memory T cell
TEMRA	terminally differentiated effector memory cell
Tfh	follicular helper T cell
Th	T helper cell
THL	Finnish Institute for Health and Welfare
TMB	3,3’,5,5’-tetramethylbenzidine
TMPRSS2	transmembrane serine protease 2
TNF	tumor-necrosis factor
TYKS	Turku University Hospital
UTU	University of Turku
VeroE6	African green monkey kidney cells clone E6
VLP	virus-like particle
VOC	variant of concern
VOI	variant of interest
VUM	variant under monitoring
WHO	World Health Organization

List of Original Publications

This dissertation is based on the following original publications, which are referred to in the text by their Roman numerals:

I Belik, M., Jalkanen, P., Lundberg, R., Reinholm, A., Laine, L., Väisänen, E., Skön, M., Tähtinen, P.A., Ivaska, L., Pakkanen, S.H., Häkkinen, H.K., Ortamo, E., Pasternack, A., Ritvos, M.A., Naves, R.A., Miettinen, S., Sironen, T., Vapalahti, O., Ritvos, O., Österlund, P., Kantele, A., Lempainen, J., Kakkola, L., Kolehmainen, P., Julkunen, I. Comparative analysis of COVID-19 vaccine responses and third booster dose-induced neutralizing antibodies against Delta and Omicron variants. *Nature Communications*, 2022; 13: 2476.

II Belik, M., Liedes, O., Vara, S., Haveri, A., Pöysti, S., Kolehmainen, P., Maljanen, S., Huttunen, M., Reinholm, A., Lundberg, R., Skön, M., Österlund, P., Melin, M., Hänninen, A., Hurme, A., Ivaska, L., Tähtinen, P.A., Lempainen, J., Kakkola, L., Jalkanen, P., Julkunen, I. Persistent T cell-mediated immune responses against Omicron variants after the third COVID-19 mRNA vaccine dose. *Frontiers in Immunology*, 2023; 14: 1099246.

III Belik, M., Reinholm, A., Kolehmainen, P., Heroum, J., Maljanen, S., Altan, E., Österlund, P., Laine, L., Ritvos, O., Pasternack, A., Naves, R.A., Iakubovskaia, A., Barkoff, A.M., He, Q., Lempainen, J., Tähtinen, P.A., Ivaska, L., Jalkanen, P., Julkunen, I., Kakkola, L. Long-term COVID-19 vaccine- and Omicron infection-induced humoral and cell-mediated immunity. *Frontiers in Immunology*, 2024; 15: 1494432.

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1 Introduction

Coronavirus disease 2019 (COVID-19), caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), led to a pandemic declared in March 2020, causing millions of deaths and economic burden.[1,2] In Finland, vaccinations began in December 2020, with priority given to health care workers (HCWs) and other high-risk groups, and were gradually expanded to the general population in 2021.[3] SARS-CoV-2 has shown rapid antigenic evolution through mutations and recombination, leading to variants with enhanced transmissibility and immune evasion.[4–6] The variants that present the greatest risk to global public health have been classified as variants of concern (VOC) by the World Health Organization (WHO).[7] A pivotal change in the pandemic occurred in late 2021 with the emergence of a new VOC, Omicron. It had over 30 mutations in the spike protein, allowing it to partially evade immunity developed in the population through initial vaccinations and SARS-CoV-2 infections by earlier variants.[8–11] Although the Omicron variant was characterized by high transmissibility, it showed reduced severity partly due to acquired immunity in the populations.[12–14] Over the years, the original Omicron BA.1 has evolved into many sublineages, including BA.2, BA.4, BA.5, and XBB.1.5, all of which have, in turn, become highly prevalent worldwide.[15,16]

Humoral immune responses involve B cells producing antibodies, primarily immunoglobulin G (IgG), against SARS-CoV-2 antigens, such as the spike protein.[17] Binding antibodies indicate overall humoral reactivity, while neutralizing antibodies block viral entry, both peaking after infection or vaccination but waning over subsequent months.[18] Complementing these humoral responses, cell-mediated immunity involves T cells. Helper $CD4^+$ T cells aid antibody production and $CD8^+$ T cell activation, while cytotoxic $CD8^+$ T cells eliminate infected cells, and their activity has been shown to correlate with the protection against severe disease.[19–21] These activated T cells produce cytokines, such as interferon-gamma (IFN-γ), which enhance antiviral defenses; Th1-biased responses, in particular, promote durable protection.[22,23]

COVID-19 vaccines are designed to elicit humoral and cell-mediated immune responses by presenting the SARS-CoV-2 spike protein antigen, thereby mimicking natural infection without causing disease.[24,25] These vaccines include mRNA types,

such as BNT162b2/Comirnaty and mRNA-1273/Spikevax, and adenovirus vector vaccines, such as ChAdOx1/Vaxzevria. All of the aforementioned have been administered in Finland during the pandemic.[3,26] On the other hand, hybrid immunity, which develops from combined immune responses triggered by COVID-19 vaccination and a SARS-CoV-2 infection occurring before or after vaccination, has been shown to elicit more effective immune responses against SARS-CoV-2 than either infection or vaccination alone.

This thesis focused on characterizing the durability and extent of humoral and cell-mediated adaptive immune responses, especially against Omicron variants. These responses were studied in Finnish HCWs following COVID-19 vaccination with different vaccine types and intervals, and with or without SARS-CoV-2 breakthrough infections, through longitudinal follow-up from initial vaccination to several booster doses and hybrid immunity.

2 Review of the Literature

2.1 SARS-CoV-2

SARS-CoV-2 is an RNA virus belonging to the family *Coronaviridae* and in the subfamily *Orthocoronavirinae*. This subfamily includes 52 viruses in four genera: alpha-, beta-, gamma-, and deltacoronavirus.[27] Viruses in these genera widely infect mammals and birds, often causing only mild respiratory or gastrointestinal infections.[28] Only alpha- and betacoronaviruses are found in humans: human coronavirus HKU1 (HCoV-HKU1), human coronavirus OC43 (HCoV-OC43), severe acute respiratory syndrome coronavirus (SARS-CoV), Middle East respiratory syndrome coronavirus (MERS-CoV), and SARS-CoV-2, which are betacoronaviruses, and human coronavirus HCoV-229E (HCoV-229E) and human coronavirus NL63 (HCoV-NL63), which belong to the alphacoronaviruses genus. Symptoms caused by these seven coronaviruses can range from mild upper respiratory tract infections, such as rhinorrhea and sore throat, to severe lower respiratory complications, including pneumonia and acute respiratory distress syndrome (ARDS), particularly in the cases of SARS-CoV, MERS-CoV, and SARS-CoV-2[29–32]

SARS-CoV, also known as SARS-CoV-1, was identified as the causative agent of the severe acute respiratory syndrome (SARS) epidemic, the first severe and highly contagious new disease of the 21st century, with an estimated case-fatality ratio (CFR) of around 10%[33,34]. The first case of SARS was detected in China in late 2002, and the epidemic officially lasted until 2004[35]. MERS-CoV was responsible for the second severe respiratory syndrome outbreak of this century, Middle East respiratory syndrome (MERS), beginning in Saudi Arabia in 2012[31]. MERS has a CFR of approximately 40%[31,36] and remains a zoonotic concern with sporadic human cases linked to dromedary camels, primarily in the Arabian Peninsula[36,37]. However, the infection rate is low, with 12 cases reported from January to August 2025, all of them in Saudi Arabia[36].

In December 2019, the world faced a new threat as the novel coronavirus, SARS-CoV-2, was identified in Wuhan, China.[38] This event initiated the COVID-19 pandemic. The origin of SARS-CoV-2 remains under scientific investigation, but the widely supported explanation in the scientific community is that a zoonotic spillover from animals to humans occurred through direct or indirect contact with live wild

animals, such as in the wildlife trade at wet markets like Huanan in Wuhan[39,40]. This hypothesis is consistent with the origins of SARS-CoV (from bats to humans via civets)[41] and MERS-CoV (from bats to humans via dromedary camels)[42]. Bats are identified as the likely natural reservoir of SARS-CoV-2,[28] and the virus shares 96.1% genomic identity with RaTG13, a virus found in Chinese horseshoe bats (Rhinolophus affinis) and 96.9% identity with BANAL-20-52 from Malayan horseshoe bats (Rhinolophus malayanus), further supporting its bat origin[40,43]. Similar to SARS-CoV and MERS, an intermediate host, such as pangolins or raccoon dogs, is believed to have helped the transfer of the virus from bats to humans, allowing for recombination or adaptation events[44,45].

2.1.1 Viral structure

The SARS-CoV-2 virion consists of four main structural proteins: spike (S), membrane (M), envelope (E), and nucleocapsid (N), and it is enveloped in a lipid bilayer derived from the host cell upon exit (Figure 1). It has one of the largest genomes among RNA viruses: a single-stranded, positive-sense RNA molecule of 30 kb. The S, M, and E proteins are embedded in the lipid layer, whereas the N protein encapsidates the genomic RNA.[46]

The structural M protein is the most abundant structural protein in the virion and plays a critical role in virus assembly and RNA packaging, making it essential for virus particle formation.[47] It has potent cytotoxicity, leading to apoptosis, particularly in endothelial cells. Additionally, it possesses viroporin-like functions, resulting in disruption of the cellular homeostasis and inflammasome activation. Similar to the M protein, the E protein functions as a viroporin and induces apoptosis, particularly in neurons and epithelial cells. Together, the M and E proteins are indispensable for the integrity of the virion envelope and for the coordinated assembly and release of the virion.[48,49]

The club-shaped S proteins protrude from the virus envelope, giving the virus its distinctive appearance – reminiscent of the Sun's corona – under an electron microscope. The S protein forms a trimer, with each monomer consisting of two subunits, S1 and S2. The S1 subunit contains the N-terminal domain (NTD) and the receptor-binding domain (RBD), which is essential for recognizing the receptor protein, angiotensin-converting enzyme 2 (ACE2), on host cells. Following RBD engagement with ACE2, the S protein undergoes conformational changes that expose cleavage sites, enabling proteolytic activation. The S1/S2 junction is first cleaved by furin during viral maturation.[50] Then, the S2′ site is cleaved at the cell surface, typically by a cell-surface protease called transmembrane protease serine 2 (TMPRSS2), which is expressed in cells of the nasal area, lungs, and gut in humans.[51,52] The S2 subunit drives membrane fusion between the virus particle and

the host cell, and together with the S1 subunit, it mediates virus entry into a cell, a crucial step for initiating replication.[53] Alternatively, viral entry can occur via the endosomal route, where cathepsin L proteolytically cleaves and activates the S protein in the acidic endosomal environment, facilitating membrane fusion.[54]

By encapsulating the viral RNA, the N protein organizes, packs, and protects the genome. Like the S protein, it also has crucial functions in the virus life cycle: upon entry into the host cell, it dissociates from the viral genome and inhibits antiviral innate immune defense mechanisms (e.g., interferon signaling), while supporting the formation of the viral replication complex.[55,56] In addition, the N protein is highly immunogenic and is expressed in high levels during SARS-CoV-2 infection.[57]

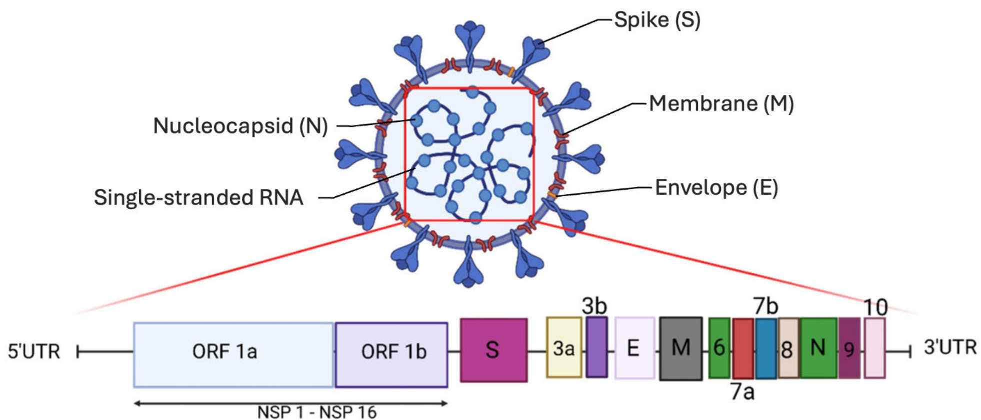

Figure 1. The genomic structure and structural proteins of the SARS-CoV-2 virion. Modified from Torbati et al. 2021.[58]

In addition to the structural proteins, the SARS-CoV-2 genome encodes 16 non-structural proteins (NSP1–16) from the ORF1a/b polyproteins and nine accessory proteins (ORFs 3a, 3b, 6, 7a, 7b, 8, 9a, 9b, and 10) from subgenomic RNAs, which support replication and immune evasion.[59–62] Specifically, NSP1 suppresses host protein translation and promotes host mRNA degradation, thereby favoring viral gene expression.[22] NSPs 2–11 contribute to replication organelle formation and host environment modification, such as RNA-binding (NSP9) and membrane remodeling, to enable efficient viral RNA synthesis.[61,63] NSPs 12–16 provide essential enzymatic activities for RNA replication and transcription, including the RNA-dependent RNA polymerase (RdRp) (NSP12) and proofreading exonuclease (NSP14).[60,63] Collectively, NSPs 1–16 assemble with the N protein into the replication-transcription complex (RTC), which remodels host membranes into protective organelles for viral RNA production while evading host antiviral responses.[59,61]

2.1.2 Antigenic evolution and the emergence of variants of concern

SARS-CoV-2 undergoes continuous antigenic evolution primarily through mutations in the S glycoprotein gene, including point mutations, deletions, and insertions to better adapt to its hosts. Although most mutations are harmful to the virus and hinder replication, leading to their elimination through natural selection, the high occurrence of mutations occasionally results in beneficial outcomes, such as enhanced transmissibility[15]. This process, termed antigenic drift, has driven the sequential emergence of new SARS-CoV-2 variants with improved fitness in immune populations by infecting the host cell more efficiently, replicating faster, or evading detection by the host's antibodies produced after previous infections or vaccinations better than the earlier strains.[15,64] As a consequence, an evolutionary arms race ensues, as the host immune system develops new antibodies to recognize the mutations in key antigenic sites of the S protein.[4] In addition to antigenic drift, recombination contributes to SARS-CoV-2 evolution by generating more abrupt genetic changes. Recombination occurs when two distinct lineages co-infect a cell, and the viral RdRp switches templates during replication, resulting in a new hybrid virus that contains genetic information from both parent strains.[65,66]

Mutation rate is a key determinant of how fast viruses evolve, and this, in turn, depends on the replication fidelity of the virus's polymerase. Coronaviruses possess 3' exonuclease proofreading capability in their replication machinery, leading to a somewhat slower mutation rate compared with most other RNA viruses, such as hepatitis C, human immunodeficiency virus (HIV), or influenza, which lack this ability.[60,67,68] The mutation rate for SARS-CoV-2 is estimated to be $1–4\times10^{-6}$ mutations per nucleotide per replication cycle in vitro, which is 24–167-fold lower than that of influenza A virus ($9\times10^{-5}–2.5\times10^{-4}$ substitutions per nucleotide per replication cycle)[69–71]

All seven significant human coronaviruses show evidence of S protein gene recombination in their evolutionary history.[72] The primary mutations involved in the emergence of new SARS-CoV-2 variants are also found in the S-protein, as it mediates the virus's ability to bind to the host cell receptor and the fusion of the virus and host cellular membranes.[4,69] The first new SARS-CoV-2 variant with a mutated S-protein, characterized by the amino acid substitution of an aspartic acid to glycine (D614G, compared with the Wuhan reference strain), was detected in March 2020. This mutation was quickly associated with increased infectivity, and during the spring, its frequency increased rapidly, resulting in this genotype becoming the dominant variant.[73,74] This new variant, simply referred to by its genotype D614G, simultaneously emerged independently in different parts of the world, starting in Europe and then in North America, Oceania, and Asia.[74]

Since its emergence, SARS-CoV-2 has continued to evolve, leading to the emergence of several lineages and variants, some of which have been classified by the

WHO as variants of concern (VOC), variants of interest (VOI), or variants under monitoring (VUM).[75] According to WHO, an SARS-CoV-2 isolate is considered a VOI if it shows phenotypic differences from a reference isolate or possesses mutations that result in amino acid changes linked to known or suspected phenotypic effects. It must also be identified as causing community transmission (multiple COVID-19 cases or clusters) or detected across multiple countries. A VOI is classified as a VOC if, based on a comparative assessment, it is linked to increased transmissibility or adverse change in COVID-19 epidemiology, higher virulence, altered clinical disease presentation, or a reduction in the effectiveness of public health measures or available diagnostics, vaccines, and therapeutic measures. Alternatively, WHO may classify an isolate as a VOI or VOC after consultation with the WHO SARS-CoV-2 Virus Evolution Working Group. In conclusion, compared to other types of variants, VOCs have gained more efficient transmission and immune-evasion capabilities.

By September 2021, there were four SARS-CoV-2 lineages designated as VOC: Alpha (B.1.1.7), which appeared in November 2020, Beta (B.1.351) in December 2020, Gamma (P.1) and Delta (B.1.617.2) in January 2021 and October 2020, respectively[15] (Figure 2). The variants have been assigned their scientific names by GISAID, Nextrain, and Pango, and the WHO has given them more colloquial names with Greek letters.[7]

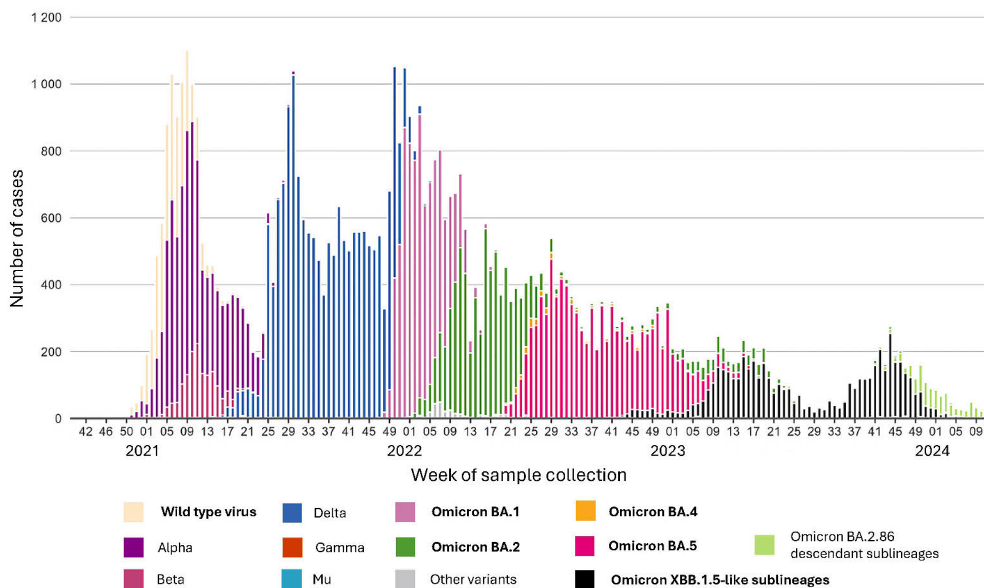

Figure 2. The weekly count of VOCs recorded in the Finnish national infectious diseases register from December 2020, when SARS-CoV-2 genomic surveillance began in Finland, until the beginning of 2024. Modified from THL, 2025.[76]

This pattern of stepwise antigenic evolution reached a new level in late 2021 with the emergence of the Omicron lineage, which introduced the most extensive set of S protein mutations observed to date.[77–79]

2.1.3 Omicron variants

Despite its relatively slow evolution for an RNA virus, SARS-CoV-2 has undergone significant changes in the nearly six years since its emergence. In November 2021, the first Omicron variant, BA.1 (Pango lineage B.1.1.529), was detected in South Africa, replacing Delta as the dominant variant, and marking a pivotal phase in the COVID-19 pandemic. In November 2021, the variant was classified as a VOC and named Omicron by the WHO.[80] This new variant was characterized by over 30 mutations in the S protein, especially in the RBD, which increased its affinity for the host receptor, increasing the likelihood of reinfection compared with other VOCs at that time.[11,79,81] The BA.1 variant was approximately 2.8 times more infectious than the Delta variant, representing a 13-fold increase in infectivity.[79] In addition, studies in cell lines suggest that Omicron sublineages exhibit a shift toward TMPRSS2-independent endosomal entry via cathepsin activation, unlike earlier variants, which mainly utilized TMPRSS2-dependent entry at the plasma membrane.[82] This change may have reduced lung cell infection but increased virus spread in the upper airway, which may partly explain higher transmissibility and milder disease compared to the Delta variant.[12,13,83] However, conflicting evidence from human airway organoids indicates that Omicron entry remains dependent on TMPRSS2.[84–86] Another probable factor for the lesser severity was infection- and vaccine-induced population immunity, which had become more widely established by that time than at the emergence of the Delta variant.[14,16] Although Omicron was less severe than Delta, and cases of severe illness and fatalities were on average lower than in previous waves of infection, the emergence of the Omicron variant caused a significant peak in infections and a high number of deaths globally[16] (Figure 3).

Two additional sublineages, BA.2 and BA.3, emerged nearly concurrently with BA.1 in early November 2021.[87] The BA.3 sublineage was rapidly outcompeted by BA.2, which further evolved into BA.4 and its sister sublineage, BA.5. These sublineages were first identified in South Africa in December 2021 (BA.4) and January 2022 (BA.5).[88] BA.4 was rapidly replaced by BA.5 due to greater fitness of BA.5, leading to its widespread prevalence worldwide.[89]

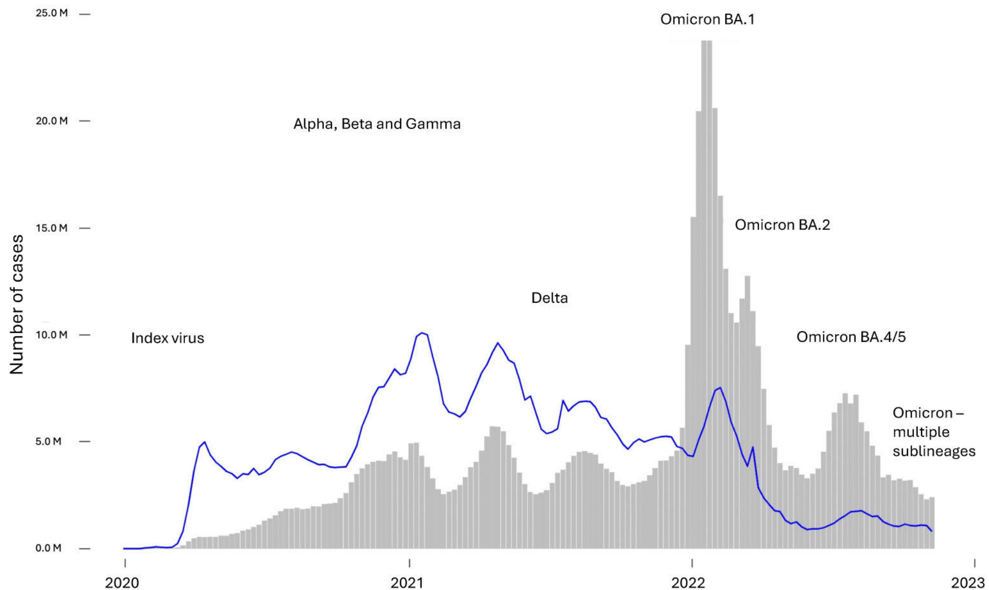

Figure 3. Global COVID-19 cases reported to the WHO are shown in gray, illustrating Omicron's impact upon its emergence in late 2021. The number of deaths is shown in blue. Modified from WHO, 2022.[16]

By mid-2022, Omicron descendant lineages had become the only circulating VOCs, with Alpha, Beta, Gamma, and Delta classified as previously circulating VOCs.[90] The Omicron variant XBB.1.5, a BA.2 sublineage, emerged in October 2022. Compared with earlier Omicron sublineages, XBB.1.5 exhibited even greater immune evasion, primarily through the acquisition of the F486P mutation in the RBD.[91] This enhanced binding affinity to the ACE2 receptor, promoted greater transmissibility, and further reduced neutralization by antibodies elicited by prior infections or vaccines targeting ancestral strains or early Omicrons.[92,93]

By late 2025, Omicron remains the only significant lineage in global circulation. The main Omicron sub-variants currently in circulation worldwide are NB.1.8.1 and XEC (classified as VUMs), together with lower prevalence of XFG, JN.1.18 (also classified as VUMs), and KP.3.1.1 and LP.8.1 (classified as VOIs).[94,95] All trace their ancestry to the JN.1 lineage, which emerged in mid-2023 as a highly divergent descendant of early Omicron BA.2 and achieved global dominance by early 2024.[78,96–98] In contrast to earlier Omicron sublineages (e.g., BA.1, BA.2, BA.4/BA.5), which arose as parallel clades from the initial B.1.1.529 ancestor, or the recombinant XBB.1.5 lineage, current subvariants represent sequential diversification within the JN.1 clade.[99]

2.2 COVID-19

COVID-19 manifests with a variety of symptoms, which vary from mild to severe illness, influenced by factors like the virus variant in question, host immunity, and comorbidities. Clinical symptoms typically appear 2–14 days after exposure to SARS-CoV-2, with an average incubation period of five days. The symptoms range from mild to severe illness, and they can also start as mild and progress to more severe symptoms.[100] Common symptoms include, but are not limited to: fever or chills, cough, sore throat, congestion or runny nose, fatigue, muscle ot body aches, headache, nausea or vomiting, diarrhea, and, characteristic of COVID-19, loss of taste or smell. SARS-CoV-2 exhibits neurotropism, defined as its ability to infect and replicate in nervous system cells, which may contribute to neurological complications alongside other factors such as immune-mediated damage.[101] Besides impacting taste or smell, it can also cause a wide range of other neurological complications in the central and peripheral nervous systems, ranging from headaches and dizziness to more severe conditions such as encephalopathy, peripheral neuropathy, or Guillain-Barré Syndrome.[102]

In milder cases, the symptoms resemble those of a common cold or flu, while more severe forms of illness may involve shortness of breath or difficulties in breathing, chest pain, or confusion, potentially progressing to pneumonia or ARDS. Most individuals experience mild or moderate illness, but older adults and those with underlying conditions have elevated risks for complications.[103]

Sometimes the symptoms do not resolve in a reasonable time, but stay persistent. Post COVID-19 condition, commonly known as long COVID, is by the National Academies of Sciences, Engineering and Medicine (NASEM) defined as a chronic condition that develops following SARS-CoV-2 infection and persists for a minimum of three months, is continuous with relapses and remissions, or has a progressive course, and impacts one or more organ systems.[104] Long COVID can impact anyone, no matter their age or how severe their initial symptoms were. According to the WHO, approximately 10–20% of individuals infected with SARS-CoV-2 may experience symptoms classified as long COVID, affecting their everyday functioning.[105]

SARS-CoV-2 can spread through direct or indirect contact with discharges from an infected individual, such as saliva or respiratory secretions. The primary mode of transmission is airborne, via infectious aerosols or respiratory droplets released when the infected person talks, coughs, sneezes, or sings. Other possible routes include contaminated surfaces and inanimate objects, as well as fecal, bloodborne, mother-to-child, and animal-to-human transmission.[106] The reproductive number, R0, measures the virus's transmission efficiency and depends on the variant; for Omicron, the estimated average reproduction number is 9.5, ranging from 5.5 to 24. In comparison, influenza's R0 has typically been estimated to be between 1 and 1.5,

and the higher R0 for SARS-CoV-2 indicates higher transmissibility.[107] Compared to the Delta variant, the reproduction number of the Omicron variant shows 2.5 to 3.8 times higher transmissibility.[108]

COVID-19 is diagnosed using tests such as reverse transcription polymerase chain reaction (RT-PCR) and rapid lateral flow antigen tests. Both PCR and antigen tests are typically performed on respiratory specimens collected by nasal or nasopharyngeal swab, but differ in their targets: PCR detects SARS-CoV-2 RNA, whereas antigen tests detect viral proteins, most commonly the N protein.[109] Real-time RT-PCR is the gold standard for confirming SARS-CoV-2 infection due to its high sensitivity and specificity, and it can detect viral RNA several weeks after infection, even at somewhat low viral loads. Antigen tests are suitable for the detection of an acute infection, especially within seven days of symptom onset. The ability of antigen tests to detect an antigen depends on the patient's viral load, but they can often detect viral antigens for up to eight to ten days.[110]

In addition to PCR and antigen tests, serological assays measure antibodies in blood. Still, they cannot diagnose active infection, as they detect only the host antibody response to prior infection or vaccination rather than the virus itself. The serological antibody tests measure either immunoglobulin M (IgM) or immunoglobulin G (IgG) class antibodies, or both. IgM antibodies are so-called early antibodies, detected early in infection or post-vaccination, and can be found in the blood for several weeks after infection or vaccination, while IgG antibodies can be reliably detected starting about two weeks post-infection or vaccination and can remain in circulation for years after immunization.[111–113] Since antibodies specific to viral proteins might not have developed in the time between infection and symptom onset, these serology tests were designed not to detect active SARS-CoV-2 infections. Instead, they aim to identify antibodies from previous infections or vaccination, aiding in diagnosis, public health surveillance, and vaccine development.[114]

2.2.1 Globally

The high infectivity and pathogenicity of SARS-CoV-2 allowed it to spread rapidly worldwide. WHO declared the COVID-19 pandemic in late January 2020, and it officially lasted until early May 2023.[115] COVID-19 global surveillance has been done in collaboration with organizations such as the WHO, the European Centre for Disease Prevention and Control (ECDC), and the U.S. Centers for Disease Control and Prevention (CDC), and it depends on countries collaborating to share data and insights about the SARS-CoV-2 spread and characteristics.[116,117] Surveillance helps assess the risks related to COVID-19, including factors like disease severity and the potential emergence of new variants. It is conducted by tracking changes in the virus

through genomic sequencing and monitoring significant changes in virus traits, such as mutations that could impact transmissibility or immune evasion, and by monitoring population immunity – influenced by both SARS-CoV-2 infections and COVID-19 vaccinations – through test positivity rates or other data sources.[117,118]

The impact of COVID-19 has dramatically affected all areas of people's lives worldwide: health, social interactions, the economy, food systems, and food security. In addition, it has caused the death of millions of people directly or indirectly.[119,120] In addition, it caused the most severe decline in the global economy since the Great Depression, with worldwide gross domestic product (GDP) falling by 3.0% in 2020, compared with 2.8% growth in 2019.[1] During the pandemic, many measures were put in place to try to limit the spread of the disease. Mitigation strategies included staying home or keeping distance from others and wearing a mask when out if feeling ill, testing for COVID-19 if infection is suspected, practicing good hand hygiene, staying up to date with COVID-19 vaccines, and seeking treatment if at risk of developing a severe form of the illness.[121,122] Vaccination decreases the number of vulnerable individuals, while non-pharmaceutical measures reduce transmission by limiting opportunities for the virus to spread. Together, these strategies have protected both individuals and society from SARS-CoV-2 infection and severe health outcomes. Despite the pandemic officially having ended, SARS-CoV-2 has not disappeared. In November 2025, COVID-19 remains a global issue, dominated by descendant Omicron subvariants.[94,95]

2.2.2 In Finland

In Finland, responsibility for pandemic preparedness is shared among various actors at national, regional, and local levels and is governed by a law on infectious diseases.[123] It is a joint effort between the Ministry of Social Affairs and Health (STM), the Finnish Institute of Health and Welfare (THL), and hospital districts, regional administrative agencies, and municipalities.[124] Generally, organizations in Finland had pre-COVID-19 preparations and plans for various threats, some including pandemics. However, most of the plans were partially outdated or otherwise not ideal for fighting COVID-19 because they were based on pandemics like the 2009 swine flu (caused by the H1N1 influenza virus) or on regional or short-term, sudden threats. After the pandemic reached Finland, this became evident in practice, for example, with insufficient protective equipment and challenges in information exchange and coordination between administrative authorities.[125,126]

The first detected COVID-19 case in Finland was a Chinese tourist in Lapland at the end of January 2020. In February, COVID-19 was added to the list of generally hazardous communicable diseases, and the spread of the disease in Finland began to accelerate in early March, boosted by Finnish tourists traveling during the winter

holidays in February.[127] The epidemic in Finland is considered to have begun in mid-March, shortly after the WHO declared COVID-19 a pandemic. The Finnish Government announced a state of emergency and implemented various physical distancing measures to slow the spread of the virus and to protect risk groups.[128]

Since July 2023, COVID-19 has not been classified as a dangerous disease but remains under observation. Finland's COVID-19 surveillance focuses on tracking circulating lineages and mutations through whole-genome sequencing of SARS-CoV-2, with diagnostic lab samples sent weekly to THL for analysis. This data is used to identify variants, monitor their prevalence, and is combined with global datasets for a broader picture. The genomic data is reported to the National Infectious Diseases Register with a two-week delay, and viruses are selected for further laboratory testing to evaluate immune evasion.[76]

2.3 COVID-19 vaccines

Since the first properly recorded vaccination in history by an English physician, Edward Jenner, who in the late 1700s found out that inoculating people with pus from cowpox blisters protected against smallpox, vaccination has been one of the most effective methods for preventing and controlling infectious diseases, significantly reducing morbidity and mortality from communicable diseases. Since the early days of vaccination, virus vaccine technology has evolved from live vaccines using whole viruses to live-attenuated and inactivated whole-virus vaccines, then to subunit vaccines containing purified protein components, and most recently to next-generation platforms including DNA and mRNA vaccines, replication-deficient viral vectors, protein nanoparticle-based subunit vaccines, and virus-like particles (VLPs).[129]

After the COVID-19 pandemic started, the importance of slowing virus transmission, reducing disease severity, and lowering death rates was quickly understood. In response, biopharmaceutical companies accelerated vaccine development, compressing a process that typically takes 10–15 years into under one year after the virus was identified.[129,130] Even so, foundational research on mRNA technology had been ongoing for decades, culminating in the key discovery of immunogenicity-reducing base modifications by Karikó and Weissman, which was recognized by the 2023 Nobel Prize in Physiology or Medicine.[131] In December 2020, initial vaccine distributions began in select countries, prioritizing healthcare workers and long-term care residents, with a mRNA vaccine BNT162b2 from BioNTech/Pfizer, the first vaccine authorized by European Medicines Agency (EMA), U.S. Food and Drug Administration (FDA), and CDC.[132–134] By early 2021, four vaccines had been authorized by the EMA: two mRNA vaccines, BNT162b2 (Comirnaty from BioNTech/Pfizer) and mRNA-1273 (Spikevax from Moderna), and

two adenovirus-vector vaccines, ChAdOx1 (Vaxzevria from AstraZeneca/Oxford) and Ad26.COV2.S (Jcovden from Janssen). All except Ad26.COV2.S, which used a single-dose regimen, required two doses spaced three to four weeks apart. In August 2021, the FDA approved BNT162b2 as the first COVID-19 vaccine for wider use and marketing for individuals 16 years of age and older.

In short, all COVID-19 vaccines function by encoding or presenting the SARS-CoV-2 S protein to prime the adaptive immune system (Figure 4). This process stimulates the production of memory $CD4^+$ and $CD8^+$ T cells, B cells, and neutralizing antibodies for future protection against the virus.[135,136] The vaccines induce long-lasting protection against severe outcomes, likely due to Th1-biased responses, and the priming of cytotoxic $CD8^+$ T cells.[23,137,138]

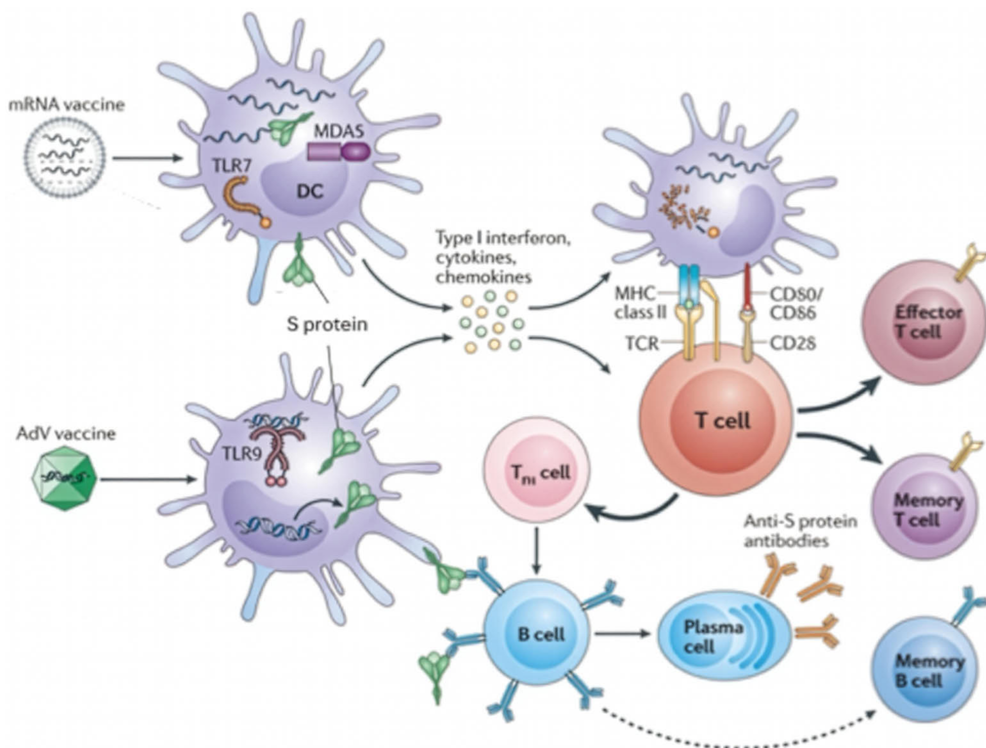

Figure 4. Mechanisms of adaptive immune activation induced by mRNA and adenovirus vector SARS-CoV-2 vaccines, illustrating antigen presentation by APCs and subsequent priming of T- and B-cell responses, leading to immunological memory. Modified from Teijaro et al.[25]

Vaccine formulations have been repeatedly updated to better target evolving variants through improved cross-neutralization. As of August 2025, five vaccines had marketing authorization in the EU through EMA: BNT162b2/Comirnaty,

mRNA-1273/Spikevax, Nuvaxovid (a protein subunit vaccine from Novavax), Bimervax (a protein subunit vaccine from Hipra), and Kostaive (a mRNA vaccine from Arcturus Therapeutics).[26] The early adenovirus-vector vaccines ChAdOx1/Vaxzevria and Jcovden had been linked to life-threatening blood clots and low blood platelet counts, leading to countries, including Finland, limiting or halting the administration of Vaxzevria in spring 2021.[139,140] The FDA and CDC forced a pause on Jcovden in April 2021 for further safety assessment, but the restrictions on its use were later lifted.[141] The last doses of Vaxzevria in Finland were given in November 2021 and Jcovden in March 2023.[3,142] Both vaccines were discontinued in 2023–2024 due to commercial reasons.[143,144] The composition of COVID-19 vaccines has been modified four times since their initial approval in 2020, and for the 2025–2026 season, EMA recommends monovalent updates targeting the LP.8.1 lineage (a JN.1 descendant), with approvals granted in August 2025 for Comirnaty and Spikevax.[145–147]

2.3.1 Vaccination strategy in Finland

In Finland, the national COVID-19 vaccination campaign began at the end of December 2020, with two doses of the mRNA vaccine BNT162b2 (from BioNTech-Pfizer) given with a three-week interval. Because of global and national vaccine shortages at the time, the initial doses were allocated to high-risk groups, such as healthcare workers treating SARS-CoV-2 patients, long-term care residents, the elderly, those with chronic conditions, and other vulnerable populations.[3] Another mRNA vaccine, mRNA-1273 (from Moderna), was authorized for use in January 2021. In early February 2021, the National Advisory Committee on Vaccines (KRAR) and the THL recommended extending this interval to 12 weeks for all authorized vaccines, coinciding with the introduction of the adenovirus vector vaccine ChAdOx1 (from AstraZeneca).[3,148,149] During 2021, vaccinations were gradually expanded to cover the whole population, with healthy adults offered vaccinations in age groups, from the oldest to the youngest. Due to safety concerns linked to coagulation disorders, the use of ChAdOx1 was discontinued for individuals under 65 years in March 2021, and entirely halted in November 2021.[140,142] By March 2022, two more vaccines had been approved for conditional marketing authorization and taken into use in Finland: an adenovirus vaccine Ad26.COV2.S (from Janssen) and a protein-subunit vaccine NVX-CoV2373 (from Nuvaxovid).[3] The majority of COVID-19 vaccines in use in Finland have been the mRNA vaccines BNT162b2 and mRNA-1273. In addition to these, Ad26.COV2.S or NVX-CoV2373 have been given to specific risk groups or in some other exceptions to regular regimens.[26]

Currently, for the season 2025–2026, Finland administers BioNTech-Pfizer's LP.8.1-tailored monovalent mRNA vaccine for all ages with age-adjusted concentrations (3, 10, or 30 µg/dose), and Hipran's LP.8.1-tailored monovalent protein subunit vaccine for those who, due to medical reasons, cannot receive the equivalent mRNA vaccine version.[150] In addition to LP.8.1-tailored vaccines, the monovalent JN.1 and KP.2-variant tailored vaccines are given if the LP.8.1 vaccine is not available. Finland's COVID-19 vaccination strategy has shifted to an annual seasonal approach: vaccinations are administered from September to November, often alongside seasonal influenza vaccines. It targets primarily risk groups, including people aged 75 and above, all severely immunocompromised individuals regardless of age, residents of nursing homes, those receiving regular organised home-care services, and individuals under 75 with two or more serious underlying conditions that increase their risk for severe disease form.[151]

2.3.2 mRNA vaccines

The concept of mRNA vaccines was first proposed in the early 1970s, and the first experimental study was conducted in 1993, demonstrating their effectiveness against influenza.[152] The primary platform for COVID-19 immunization has been, and remains, mRNA vaccines, and Moderna's Spikevax (mRNA-1273) and Comirnaty (BNT162b) from BioNTech/Pfizer are still the most widely distributed mRNA vaccines on the market, both in Europe and the US.[145,153]

Unlike traditional vaccines that contain whole virus or purified structural components, mRNA vaccines instead deliver instructions for target cells to produce the antigen themselves, which in COVID-19 vaccines, is the SARS-CoV-2 S protein. More precisely, these vaccines consist of nucleoside-modified mRNA encapsulated in lipid nanoparticles. After cellular uptake, the mRNA is translated in the cytosol into prefusion-stabilized S protein, which is then displayed on the cell surface of an antigen-presenting cell (APC). This enables efficient antigen presentation via MHC class I and class II pathways, priming $CD4^+$ and $CD8^+$ T cells while directly activating B cells to produce neutralising antibodies.[132,154] In addition to fast-acting antibodies, the process also generates long-lived memory T- and B-lymphocytes capable of mounting a rapid response upon subsequent viral exposure.[154,155]

Compared with other types of vaccines, mRNA vaccines offer significant advantages, including rapid production, scalability, and platform flexibility for rapid virus variant adaptation, at relatively affordable manufacturing costs. However, cold storage requirements and high initial research and development costs pose logistic challenges, especially in low-resource settings. In addition, reactogenicity (many vaccinees develop symptoms such as fever or fatigue) may affect vaccine acceptability.[152] Since mRNA technology does not require pathogen cultivation and

is more scalable than traditional vaccines, it has tackled long-standing limitations of older vaccine methods, such as production delays during previous outbreaks like SARS.[156]

2.3.3 Adenovirus vector vaccines

Viral vector vaccines use a harmless, modified virus that serves as a delivery vehicle, a vector, to bring genetic instructions to target cells. Upon entering the target cell, the virus vector partially uncoats in the endosome, escapes into the cytosol, and the remaining capsid traffics to the nuclear pore complex, where the viral genome is imported into the nucleus. The cells then produce the antigen protein, such as the SARS-CoV-2 S protein, which is presented to the immune system. Adenoviruses, adeno-associated viruses, retroviruses, lentiviruses, and herpes simplex viruses have typically been used as virus vectors.[157,158] Adenoviruses are attractive vaccine vectors because they effectively stimulate both innate and adaptive immune responses in mammalian hosts.[159] Adenovirus vector vaccines originated in the 1980s as gene therapy tools, but pre-existing immunity to adenovirus limited their efficacy.[160,161] In the 90s, to evade anti-vector antibodies, replication-deficient platforms were developed. Early applications for the adenovirus vector vaccines targeted HIV, malaria, and Ebola.[159]

COVID-19 adenovirus-vector-based vaccines include AstraZeneca's Vaxzevria (ChAdOx1) and Janssen's Jcovden (Ad26.COV2.S). In addition, Sputnik V (Gam-COVID-Vac) adenovirus vector vaccine developed by Gamaleya Research Institute of Epidemiology and Microbiology was distributed in Russia and other countries starting in December 2020.[162] In addition, Convidecia (Ad5-nCoV) by a Chinese company, CanSino Biologics Inc., has been approved for emergency use by the WHO and has been administered globally.[163] All the aforementioned adenovirus vector vaccines are based on chimpanzee (ChAd) or human adenovirus (Ad5 and Ad26) backbones.[157]

To create a replication-incompetent vector, the early region 1 (E1) of the adenovirus genome, which is essential for replication, is deleted and replaced with the transgene of interest, such as the SARS-CoV-2 S protein. In many designs, the non-essential early region 3 (E3) and/or parts of the early region 4 (E4) are also deleted or modified.[164] Recombinant viral vectors can be engineered to encode one or more transgenes, thereby expressing single or multiple target antigens. This simultaneously gives them intrinsic adjuvant properties via diverse pathogen-associated molecular patterns (PAMPs) that activate innate immunity and enhance adaptive immune responses, enabling the vectors to induce immune responses without external adjuvants.[157,165] An additional advantage of certain adenovirus-vectored vaccines is the possibility of intranasal administration, which targets

mucosal epithelia and induces local IgA and tissue-resident memory T cell responses. This amplifies both mucosal and systemic immunity, while offering targeted delivery and avoiding injection-site reactions.[166]

However, despite advances of the past decades, the major limitation of pre-existing anti-vector immunity with adenovirus vector vaccines remains. The immune responses neutralize the vaccine vector upon booster administration, thereby reducing successful infection of target cells, lowering transgene expression (SARS-CoV-2 S protein production), and impairing effective boosting with the same platform.[25,167] In addition, unlike mRNA vaccines, they depend on propagation in dedicated producer cell lines, which delays both manufacturing and redesign for new variants.[168] Additionally, the rare but serious risk of vaccine-induced immune thrombotic thrombocytopenia has limited their widespread use, particularly for repeated annual boosting.[139,141,142,169] On the other hand, adenovirus vector vaccines have several advantages over, for example, mRNA vaccines, including lower cost and better thermostability.[170]

2.3.4 Other types of vaccines

Among other vaccine types, inactivated vaccines and live-attenuated vaccines represent the oldest vaccine technologies, which, in contrast to mRNA and virus vector vaccines, use a whole-virus approach. The inactivated whole-virus vaccines were developed in the 1920s to combat, e.g., polio (Salk polio vaccine), and in these vaccines, the viruses are typically inactivated by heat, irradiation, or chemical treatment such as formalin.[171] Modern examples of inactivated whole-virus vaccines include the COVID-19 vaccines CoronaVac by Sinovac and Covaxin by Bharat Biotech, which were developed for use especially in low- and middle-income countries.[172,173] They are not authorized for use by the EMA or the FDA.[146,147] Like inactivated vaccines, live-attenuated vaccines have been used for decades to combat, e.g., polio (Sabin polio vaccine). Due to safety concerns, no COVID-19 vaccines are authorized on this platform.

Recombinant protein subunit vaccines are based on technology developed in the 1980s, and for decades, these vaccines have been successfully used to protect against diseases such as hepatitis B.[174] They are considered very safe because they contain no live virus components and use only purified virus proteins or their fragments. Unlike vectored vaccines or mRNA vaccines, where the antigenic component is induced to be manufactured within the individual's body, protein subunit vaccines use a recombinant approach in which the encoding gene is produced in a heterologous host organism cultivated under controlled laboratory conditions (e.g., yeast, bacteria, insect, or mammalian cells), followed by purification and collection for vaccine formulation.[156] One example of a COVID-19 protein subunit vaccine is

Novavax's Nuvaxovid (NVX-CoV2373), which uses a fragment of the SARS-CoV-2 virus S protein.[175] It has been authorised in both Finland and the U.S. as a non-mRNA alternative for individuals with contraindications to mRNA vaccines.[3,147]

Finally, VLP vaccines represent a highly advanced non-nucleic acid vaccine platform, combining the safety of subunit approaches with virus-like structural mimicry that enhances immunogenicity through repetitive epitope presentation and efficient antigen delivery to immune cells. VLP vaccines targeting human papillomavirus (HPV) were commercialized in 2006 (e.g., Gardasil),[176] but VLP vaccines for SARS-CoV-2 are still in development. The VLP vaccines consist of molecules, such as self-assembling viral proteins, that mimic native virion architecture but lack genetic material and are thus not infectious.[177,178] However, the complexity of multi-protein assembly and their high production costs restrict the scalability of VLP vaccines[179], likely contributing to the delayed approval of COVID-19 VLPs.

2.4 Immune responses to SARS-CoV-2 infection and COVID-19 vaccinations

Effective immunity against SARS-CoV-2 requires coordinated activation of both innate and adaptive immune responses. The innate immune response provides essential early signals by engaging pattern-recognition receptors (PRRs), producing type I interferons, and activating dendritic cells and macrophages, all of which are required to initiate and shape the subsequent adaptive immune response. In SARS-CoV-2 infection, this innate activation is triggered by viral RNA and other PAMPs.[180] In COVID-19 vaccination, the same innate pathways are stimulated by vaccine components, which then drive antigen presentation and prime S-specific adaptive immune responses.[181] As a result, the adaptive immune system forms immunological memory, which includes specialized memory cells that develop during the initial infection or vaccination. Immunological memory is mediated by SARS-CoV-2-specific memory B cells, long-lived plasma cells, and memory CD4^{+} and CD8^{+} T cells. Upon reencountering the virus, these memory cells enable the adaptive immune system to mount a rapid and robust response that confers durable protection against severe disease.[182]

2.4.1 B cell-mediated and humoral immune responses

Humoral immunity against SARS-CoV-2 relies on B cell activation to produce antibodies.[183] There are two main pathways of B cell activation: T-cell-independent and T-cell-dependent activation.[184] The T-cell-dependent pathway is particularly relevant to immunity acquired through SARS-CoV-2 infection or COVID-19

vaccinations.[185,186] It is initiated when B cells encounter protein antigens, such as the SARS-CoV-2 S protein, bind to them with their B cell receptors (BCRs), and internalize them.[187] The protein antigens are then processed into smaller peptides and presented on major histocompatibility complex class II (MHC II) molecules in the germinal centers (GCs) of lymph nodes to follicular helper T (Tfh) cells, which provide signals for full B cell activation.[188]

This pathway also leads to somatic hypermutation, which fine-tunes B cells in the GCs to produce high-affinity anti-S-binding immunoglobulin G (IgG) and neutralizing antibodies, thereby preventing virus entry, often by blocking its interaction with the host cell ACE2 receptor, although conformational effects on the prefusion spike can also contribute to neutralization for certain antibodies.[189] The pathway also leads to antibody class switching, primarily to IgG subclasses, and B cells to rapidly undergo clonal expansion, creating a large number of identical daughter cells, which then differentiate into long-lived S-specific antibody-secreting plasma cells along with memory B cells.[190] After primary COVID-19 vaccination, an initial rapid peak in anti-S antibody levels is driven by short-lived plasmablasts that proliferate vigorously and secrete large amounts of antibodies, but disappear within two weeks. These antibodies then decline with a half-life of approximately 28–34 days, reflecting the short-lived component of the response. However, after the first few months, antibody levels stabilise at a lower plateau, which is sustained by long-lived plasma cells that have migrated to the bone marrow and continuously produce antibodies.[18] This leads to a strong, S-specific antibody response that can wane over time but can be restored with booster vaccine doses or by reinfection.[186,191,192] Although nearly all SARS-CoV-2 neutralizing antibodies are of the IgG isotype and target the RBD, only a minor fraction of anti-RBD or anti-S IgG molecules possess potent neutralizing activity.[193,194] Therefore, analyzing binding IgG serves as a measure of the overall antibody response to the SARS-CoV-2 S protein, while neutralizing antibodies correlate with protection against severe COVID-19.[21,23]

2.4.2 T cell-mediated immune responses and cytokines

After being infected by SARS-CoV-2, infected cells present viral peptides on MHC class I molecules on their surface, which $CD8^+$ cytotoxic T cells recognize. They destroy infected cells by releasing cytotoxic proteins, perforin and granzymes, helping control viral spread early in infection.[195] $CD4^+$ helper T cells are activated when their T cell receptor (TCR) recognizes a virus peptide on MHC class II molecules, which are primarily found on the surface of APCs. The helper T cells then coordinate the immune response by signaling B cells to switch antibody class and to become memory B cells, as well as signaling $CD8^+$ T cells, promoting their survival, expansion, and

cytotoxic function.[190,195] After SARS-CoV-2 infection or COVID-19 vaccination, S-specific $CD4^+$ and $CD8^+$ T cells form memory populations that can persist for months to years and react quickly to reinfection.[196–198]

Memory T cells form the backbone of long-term protection against SARS-CoV-2. Upon encountering the S protein or other viral peptides for the first time, primed naïve T cells begin to divide and differentiate into effector T cells.[182,199] $CD4^+$ effector T cells become Tfh cells in the GCs, or circulating Tfh (cTfh) cells, circulating in the blood and aiding B cells to differentiate into plasmablasts.[182,196,199] After the virus is cleared and the peak of the immune response has passed, most effector T cells die.[200] Those that remain differentiate into memory T cell subsets: central memory T cells (TCM), which reside mainly in the lymph nodes, or into effector memory T cells (TEM), which patrol primarily in the peripheral tissues, or terminally differentiated effector memory (TEMRA) cells, which are mostly $CD8^+$ cells with high cytotoxic activity.[200,201]

Activated T cells release effector cytokines such as interferon-gamma (IFN-γ), and tumor necrosis factor-alpha (TNF-α), secreted by both $CD4^+$ and $CD8^+$ cells, and interleukin-2 (IL-2), secreted mainly by $CD4^+$ cells. These cytokines enhance antiviral defenses by recruiting other immune cells and promoting T cell survival. IFN-γ, IL-2, and TNF-α are T helper 1 (Th1) -type cytokines, which are essential to the immune response against SARS-CoV-2.[202,203]

2.4.3 Hybrid immunity

Hybrid immunity refers to the immunity acquired after SARS-CoV-2 infection that occurs after one or more doses of COVID-19 vaccines, also known as a breakthrough infection, or vice versa. In cases where vaccinated individuals experience a breakthrough infection, the SARS-CoV-2-infection-naïve immune system is initially primed by vaccination to recognize the S protein.[204] This process generates S-specific T and B cell responses, including memory cells, along with high-affinity binding and neutralizing IgG antibodies, primarily targeting the RBD and the NTD of the S1 subunit of the S protein.[74,190,205] When the vaccinated individual is later infected by SARS-CoV-2, the immune system encounters the entire virus, including non-S antigens (N, M, E, and NSPs) in their natural form as well as the S protein in its native trimeric conformation.[206,207] Compared to vaccination alone, a more diverse array of antigens then triggers a broader boosting effect, leading to wider target recognition. This reactivates and diversifies memory cells, promoting B cell affinity maturation (leading to high-affinity antibodies), epitope spreading for recognizing new targets (non-RBD antibodies), and memory amplification in the form of larger pools of long-lived memory cells, such as plasma cells, leading to better sustained antibodies.[208] As a result, hybrid immunity also leads to long-lasting humoral

memory responses, and to higher neutralizing antibody titers and broader cross-neutralization against virus variants of concern, such as Omicron, than vaccination or infection alone.[18,209–213]

On the other hand, the effect of the immune imprinting from vaccinations containing the ancestral Wuhan-Hu-1 strain can impair the de novo response to a breakthrough infection, and thus also the ability to adapt to highly mutated variants like Omicron. Omicron's numerous spike mutations have repeatedly enabled the virus to evade both vaccine- and infection-induced neutralizing antibodies. The acquired evasion ability significantly increases the risk of reinfection, even among people with acquired hybrid immunity.[214–216] In individuals primed by the Wuhan-Hu-1-specific monovalent vaccines, the secondary exposure to a divergent Omicron variant preferentially recalls pre-existing memory B cells specific for the original S immunogen. This can result in a biased antibody output dominated by lower-affinity, RBD-directed antibodies that exhibit reduced neutralization of the new variant.[217,218] This recall dominance can suppress the generation of novel B cell clones against drifted epitopes, limit epitope spreading to conserved non-S antigens, and restrict the breadth of the hybrid immune response.[208] Consequently, repeatedly boosting with ancestral-sequence vaccines may reinforce this imprinting, resulting in increasingly limited protection despite high antibody titers.[217]

Fortunately, like breakthrough infection by antigenically different variants, also boosting immunity through variant-adapted vaccines or multitope vaccines can partially counteract immune imprinting by promoting affinity maturation toward new epitopes.[219–221] In addition, both hybrid immune responses and variant-adapted vaccines improve cross-reactive T cell responses with increased S-stimulated $CD4^+$ and $CD8^+$ T-cell functionality compared to infection-acquired immunity alone.[222] In addition, breakthrough infections in vaccinated individuals are often mild due to pre-existing vaccine-induced immunity, while humoral responses are still strengthened with slower antibody decline and high antibody levels maintained over a long time.[18]

3 Aims

This doctoral study was conducted in 2021–2024. It aimed to characterize the durability and breadth of humoral and cell-mediated adaptive immune responses, particularly in the context of Omicron variants, induced by COVID-19 vaccines and SARS-CoV-2 breakthrough infections in Finnish healthcare workers, with longitudinal follow-up from primary vaccination through multiple booster doses and hybrid immunity.

The specific research objectives were:

1. To evaluate the long-term humoral immunity induced by COVID-19 vaccinations, as well as the antibody decline kinetics, and to continue the long-term follow-up from the first vaccination doses up to the fourth dose.
2. To assess the neutralizing ability of antibodies generated by COVID-19 vaccines and SARS-CoV-2 breakthrough infections against emerging Omicron variants.
3. To analyze the vaccine- and breakthrough infection-induced T cell-mediated immunity and cross-recognizing ability against Omicron variants, including $CD4^+$ and $CD8^+$ T cells and the memory cell subsets.
4. To compare the effectiveness of the different vaccine regimens administered in early 2020, as well as the differences between the mRNA booster doses administered in the following three years.

4 Materials and Methods

4.1 Ethics

The studies were conducted with ethical approval from the Southwest Finland and the Helsinki-Uusimaa health districts. The decision numbers were ETMK 19/1801/2020 (EudraCT 2021-004419-14) for the HCWs at Turku University Hospital (Studies I–III), and HUS/1238/2020 (EudraCT 2021-004016-26) for the HCWs at Helsinki University Hospital (Study I). All participants provided written informed consent before the collection of the first study sample.

4.2 Study participant characteristics and timeline of the vaccinations and samplings

The study participants were recruited among the HCWs of Turku University Hospital (TYKS, Turku, Finland) (Studies I–III) and Helsinki University Hospital (HUS, Helsinki, Finland) (Study I) before receiving COVID-19 vaccination as part of hospital occupational health care in between December 2020 and January 2021 (vaccinees in the short interval regimen) or June 2021 (vaccinees in the long interval regimen). During the follow-up period, September 2020 to April 2023, the HCWs received either an mRNA-based COVID-19 vaccine (BNT162b2 or mRNA-1273) or an adenovirus vector vaccine (ChAdOx1) as the first dose, followed by up to three additional booster doses of the mRNA vaccines. The first and second vaccinations were administered with a three-week (short) or a 12-week (long) dose interval (Figure 5). Serum samples were collected before COVID-19 vaccinations (Pre), three, six and twelve weeks post the first COVID-19 vaccine dose (1D3wk, 1D6wk, 1D12wk), three weeks and three, six and eight months post the second vaccine dose (2D3wk, 2D3mo, 2D8mo), three weeks and three, six and twelve months post the third vaccine dose (3D3wk, 3D3mo, 3D6mo, 3D12mo), and three weeks and three and six months post the fourth vaccine dose (4D3wk, 4D3mo, 4D6mo). Peripheral blood mononuclear cells (PBMCs) were collected at the same time as the serum samples at all time points except the first two (Pre and 1D3wk) in vaccinees with a short interval regimen. Breakthrough infections were identified through self-reported positive SARS-CoV-2 antigen or RT-qPCR test

results, and/or an increase exceeding the cut-off value in anti-S1 or anti-N IgG antibody levels.

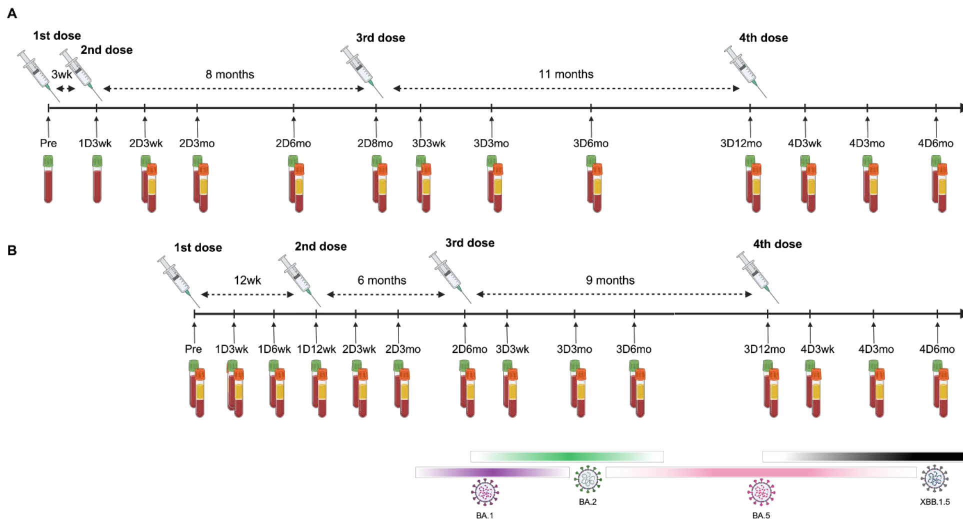

Figure 5. Timeline of the vaccinations and samplings. Serum samples were collected before COVID-19 vaccination and at multiple timepoints after the first, second, third, and fourth doses **A.** in the short three-week interval group and **B.** in the long 12-week interval group. PBMC samples were collected at the same time points as sera, except in the short interval group, where PBMCs were not collected before vaccination or after the first dose. The intervals between the samplings are presented as the average intervals. Created by Milja Belik with BioRender.com

In Study I, the cohort consisted of 328 HCWs, who had received two doses of the COVID-19 vaccine, either with a short interval (n=120) or a long interval (n=208). All participants vaccinated with a short interval received two sequential doses of BNT162b2, followed by a third dose of BNT162b2 (n=47) or mRNA-1273 (n=73). Participants vaccinated with a long dose interval received two sequential doses of BNT162b2 (n=62) or mRNA-1273 (n=72), or a combination of ChAdOx1 and BNT162b2 (n=52) or ChAdOx1 and mRNA-1273 (n=22) vaccines. Participants who received three doses of the vaccine were monitored for up to three weeks after the third dose, while participants in the long vaccine dose group were observed for up to six months following the second dose. A total of 12 participants had a PCR-confirmed SARS-CoV-2 infection prior to the first vaccination, and two participants experienced a PCR-confirmed or EIA-detected breakthrough infection post-second dose.

In Study II, the cohort comprised 100 HCWs, randomly selected from a larger group vaccinated with two initial doses of BNT162b2 mRNA vaccine at a short interval, followed by a third dose of either BNT162b2 (n=46) or mRNA-1273 (n=54). Of these 100 HCWs, two had a previous PCR-confirmed SARS-CoV-2

infection before their first vaccination, and one participant experienced a PCR-confirmed breakthrough infection between the second and third doses. The cohort was followed for up to three months after the third dose, during which four additional PCR-confirmed breakthrough infections occurred between three weeks and three months post third dose.

In Study III, the study included 111 HCWs, who had received two vaccine doses with either a short or long interval, followed by a third dose three to nine months later (n=74). HCWs in high-risk groups for severe COVID-19 (excluding those with severe or moderate immune disorders) also received a fourth dose four to twenty-three months after the third dose (n=37). The first dose consisted of ChAdOx1, BNT162b2, or mRNA-1273, while the second and third doses were BNT162b2 or mRNA-1273; the fourth dose was either of the original monovalent mRNA vaccines (BNT162b2 n=15 and mRNA-1273 n=3) or a bivalent vaccine targeting BA.1 (n=5) or BA.4/5 (n=11). Three participants had no information available about the type of their fourth vaccine dose. Among these 111 healthcare workers, 71% (79/111) had SARS-CoV-2 infections confirmed by PCR or antigen tests, and 9% (10/111) experienced two consecutive infections, totaling 89 infection events by the end of the follow-up period. Of the infected vaccinees, 81% (64/79) had received three vaccine doses, while 19% (15/79) had received four doses. The cohort was followed for up to eight months after the third dose for those vaccinated three times and up to six months after the fourth dose for those with four vaccinations.

4.3 Enzyme immunoassay

An in-house enzyme immunoassay (EIA) was used to measure SARS-CoV-2-specific IgG antibodies in serum samples from the study participants. Recombinant S1 and N proteins of the ancestral SARS-CoV-2 strain were used to evaluate humoral immunity following vaccination and breakthrough infections. These proteins were kindly provided by Dr. Olli Ritvos, University of Helsinki. Ninety-six-well plates (Nunc Maxisorp, Thermo Fisher Scientific) were coated with purified S1 (3.5 μg/ml, 175 ng/well) and N (2.0 μg/ml, 100 ng/well) proteins in phosphate-buffered saline (PBS) and incubated overnight at 4°C. Coating concentrations were based on prior research[223]. Plates were then blocked with assay buffer (5% swine serum [EuroClone] in PBS with 0.1% Tween20) to minimize non-specific binding. Serum samples were diluted 1:1000 for S1-specific assays and 1:300 for N-specific assays in assay buffer, then incubated in antigen-coated wells for 1 hour at room temperature. After washing to remove unbound antibodies, horseradish peroxidase (HRP)-conjugated anti-human IgG (Dako, 1:8000) was added and incubated for 45 minutes. Following additional washes, a tetramethylbenzidine (TMB, Kementec Solutions) substrate was applied, and after 20 minutes, the reaction was stopped with

sulfuric acid. Absorbance was measured at 450 nm using a microplate reader. IgG levels were quantified by converting optical density (OD) values to EIA units through linear interpolation between a positive control serum (100 EIA units) and a negative control serum (0 EIA units). Seropositivity thresholds were set as the mean OD of 20 negative samples plus three standard deviations for S1-based EIA or six standard deviations for N-based EIA. Calibrator sera with known IgG concentrations were included in each assay run to ensure reproducibility.

4.4 SARS-CoV-2 variants

The SARS-CoV-2 isolates used in these studies were isolated from SARS-CoV-2 PCR-positive nasopharyngeal samples at the THL in a biosafety level 3 (BSL-3) facility. Samples were incubated with African green monkey kidney cells clone E6 (VeroE6) (for FIN25-20 and FIN1-20) or VeroE6-TMPRSS2-H10 cells (for FIN37-21, FIN55-21, FIN58-22, FIN61-22 and FIN69-22) and further passaged in VeroE6-TMPRSS2-H10 cells in DMEM (FIN25-20, FIN37-21, FIN55-21, FIN58-22, FIN61-22 [Study III], and FIN69-22) or in VeroE6 cells in EMEM (FIN1-20 and FIN61-22 [Study II]) supplemented with 2% FBS, 2 mM L-glutamine, and penicillin-streptomycin. The supernatants were collected, cell debris was discarded, and the virus stock aliquots were stored at -80°C. To determine virus stock titers, a 50% tissue culture infectious dose (TCID50) assay was performed, in which virus stocks were end-point diluted starting at a 1:10 ratio in infection medium and added to 96-well tissue culture plates (Sarstedt) containing 50.000 cells per well. The dilutions were continued until $1{:}10^{-7}$ or $1{:}10^{-11}$, depending on the isolate. The plates were incubated for three days at 37°C and 5% CO_2, after which the cells were fixed with 4% formaldehyde, stained with crystal violet, and the $TCID_{50}$ was calculated using the Reed-Muench method.[224]

Table 1. SARS-CoV-2 isolates used in microneutralization tests.

Isolate	Pango lineage / WHO label	Sample collected	GenBank ID	GISAID	Study
FIN25-20	B.1 / D614G	2/2020	MW717675.1	EPI_ISL_412971	I, II, III
FIN37-21	B.1.617.2 / Delta	5/2021	MZ945494	EPI_ISL_2557176	I, II
FIN55-21	B.1.1.529 / Omicron BA.1	12/2021	ON532087.1	EPI_ISL_8768822.2	I, II
FIN1-20	B	1/2020	MZ934691	EPI_ISL_407079	II
FIN58-22	B.1.1.529.2 / Omicron BA.2	1/2022	OP199045	EPI_ISL_9695067	II, III
FIN61-22	B.1.1.529.5 / Omicron BA.5	5/2022	OP199047	EPI_ISL_13118918	II, III
FIN69-22	Omicron XBB.1.5	12/2022	OQ509907	EPI_ISL_16526646	III

4.5 Microneutralization test

The microneutralization test (MNT) was conducted in a BSL-3 laboratory to evaluate the in vitro neutralization potential of the serum samples against SARS-CoV-2 variants. In Study I, the MNT was performed in cooperation between THL and UTU, whereas Studies II and III were carried out solely in UTU. For each serum sample, serial two-fold dilutions were prepared in duplicate on 96-well plates, beginning at 1:10 for Studies I and II or 1:5 for Study III, and extending to a maximum of 1:40960 for the D614G strain or 1:2560 for Omicron variants. These dilutions were made in 50 µl of medium (DMEM for UTU and EMEM for THL) supplemented with 2% fetal bovine serum (FBS), 2 mM L-glutamine, and penicillin-streptomycin. A predetermined viral dose was combined with the serum dilutions: 50 $TCID_{50}$ (UTU) or 100 $TCID_{50}$ (THL), yielding a final serum dilution of 1:20. This mixture was incubated for 1 hour at 37°C to enable neutralization. Following this, 50,000 cells per well were introduced into the virus-serum mixture (VeroE6-TMPRSS2-H10 cells in UTU and VeroE6 cells in THL), resulting in a total volume of 150 µl per well. The plates were maintained at 37°C with 5% CO_2 for four days.

After incubation, the cells were fixed with 4% formaldehyde (UTU) or 30% formalin (THL), stained with crystal violet, and assessed for cytopathic effects. The neutralization titer was calculated as the reciprocal of the serum dilution that prevented 50% of cell death (ID_{50}). A sample was deemed positive for neutralizing antibodies if it achieved an ID_{50} at a dilution of 1:20 (Study I and II, and III for D614G) or 1:10 (Study III for Omicron variants) or above. Each plate included a positive control (serum with a known neutralizing titer) and negative controls (cells alone and virus without serum) to validate the assay's consistency.

4.6 PBMC isolation and stimulation

Peripheral blood was collected in lithium-heparin tubes and processed to isolate PBMCs using Ficoll-Paque PLUS (GE Healthcare) density gradient centrifugation, following the manufacturer's protocol. Isolated PBMCs were counted, and viability was assessed using trypan blue dye (BioRad) with a TC20 automated cell counter (BioRad). The cells were then suspended at 5–15 $x10^6$ cells/ml in a freezing medium containing 10% dimethyl sulfoxide (DMSO) and 10% human AB serum (Sigma-Aldrich) and gradually frozen to -135°C for storage until analysis.

Cryopreserved PBMCs were thawed, washed, and resuspended in RPMI-1640 medium (Lonza) supplemented with 10% heat-inactivated human AB serum (Sigma-Aldrich), 2 mM L-glutamine, and penicillin-streptomycin. Cell viability was verified using a TC20 automated cell counter (BioRad), and $1x10^6$ viable cells per well were seeded in 96-well round-bottom plates (Thermo Fisher Scientific).

For stimulation, PBMCs were incubated with SARS-CoV-2 S and N protein peptide pools. In Study II, cells were stimulated with peptide pools covering the full-length S protein of the ancestral SARS-CoV-2 (Wuhan-Hu-1, referred to as wild-type) and Omicron BA.1 and BA.2 variants (Table 2) at a peptide concentration of 0.5 µg/ml. In Study III, stimulation involved overlapping S peptide pools for the wild-type strain and Omicron BA.5 and XBB.1.5 variants at 0.5 µg/ml, along with a wild-type N peptide pool at 1 µg/ml. The S peptide pools consisted of two sub-pools, each containing 157–158 15-mer peptides (with some 13–17-mers) overlapping by 11 amino acids. The Omicron BA.1 and BA.2 pools incorporated 38 and 31 amino acid changes, respectively, with 21 mutations shared between them. Tetanus toxoid (10 µg/ml, AJ Vaccines) served as the positive control, and an equimolar concentration of DMSO served as the negative control. Cells were cultured for 48 hours at 37°C in a 5% CO2 atmosphere.

Table 2. Peptide pools (PepMixTM from JPT Peptide) used in PMBC stimulation.

Source	Product code	Number of aminoacid changes*	Study
SARS-CoV-2 Spike glycoprotein	PM-WCPV-S-1	-	II, III
B.1.1.529 / BA.1 Omicron	M-SARS2-SMUT08-1	38	II
B.1.1.529 / BA.2 Omicron	M-SARS2-SMUT09-1	31	II
B.1.1.529 / BA.4 and BA.5 Omicron	M-SARS2-SMUT10-1	34	III
XBB.1.5 Omicron	M-SARS2-SMUT15-1	42	III
SARS-CoV-2 Nucleoprotein	PM-WCPV-NCAP-1	-	III

*Compared to the S protein of the original Wuhan-Hu-1 strain

4.7 Activation-induced marker assay and flow cytometry

Following stimulation, PBMCs were washed with PBS containing 0.01% sodium azide (NaN_3) and stained with Zombie Green viability dye (BioLegend, 1:1000) for 15 minutes at room temperature in the dark to distinguish live cells. After washing with staining buffer (PBS containing 0.01% NaN_3 and 2% fetal bovine serum [FBS]), cells were incubated at 4°C for 30 minutes in the dark with a panel of fluorochrome-conjugated anti-human antibodies specific for CD45, CD3, CD4, CD8, CD69, CD134, CD137, CD45RA, CCR7, and CXCR5, as detailed in Table 3. After staining, cells were washed with staining buffer and resuspended in either 200 µl (Study II) or 100 µl (Study III) of PBS with 0.01% NaN_3 for flow cytometry analysis.

Table 3. Fluorochrome-conjugated anti-human antibodies used in the AIM assays.

Anti-human antibody	Fluorochrome	Clone	Manufacturer	Catalog no.
CD45	APC-eFluor780	HI30	Invitrogen	47-0459-42
CD3	eFluor506	UCHT1	Invitrogen	69-0038-42
CD4	eFluor450	RPA-T4	Invitrogen	48-0049-42
CD8a	PerCP-eFluor710	SK1	Invitrogen	46-0087-42
CD69	PE	FN50	BD Biosciences	555531
CD134	PE/Cyanine7	Ber-ACT35	BioLegend	350012
CD137	APC	4B4-1	BioLegend	309810
CD45RA	Brilliant Violet 785	HI100	BioLegend	304140
CD197 (CCR7)	PE/Dazzle 594	G043H7	BioLegend	353236
CD185 (CXCR5)	Brilliant Violet 605	J252D4	BioLegend	356930

T cell responses were evaluated using a NovoCyte Quanteon Flow Cytometer (Agilent Technologies Inc.), with data analyzed using NovoExpress v1.5.0 software (Agilent Technologies Inc.). Cell populations were manually gated based on the DMSO-stimulated negative control samples, and this gating was consistently applied to tetanus toxoid- and SARS-CoV-2 peptide pool-stimulated samples. Samples were excluded from analysis if they showed an aberrant $CD4^+$ T-cell response to the tetanus toxoid positive control, had fewer than 10.000 $CD3^+$ T cells, or, for cTfh analyses, had fewer than 500 cTfh $CD4^+$ T cells. A stimulation index (SI) was calculated to quantify SARS-CoV-2-specific T cell responses. The SI was determined by dividing the frequency of activated cells ($CD134^+CD69^+$ for $CD4^+$ T cells or $CD137^+CD69^+$ for $CD8^+$ T cells) after SARS-CoV-2 peptide pool stimulation by the frequency after DMSO stimulation. When the frequency of activated cells post-stimulation was zero, the SI was assigned the lowest observed value for that participant. S-specific T cell responses were considered positive if the SI exceeded 2.5 for $CD4^+$ T cells or 1.25 for $CD8^+$ T cells, as established in a prior study[225].

4.8 Luminex

Cytokine levels in the supernatants of stimulated PBMCs were quantified in THL using a Luminex-based multiplex assay. In Study II, the assay measured secreted interferon-gamma (IFN-γ), interleukin-2 (IL-2), and interleukin-4 (IL-4), while in Study III, only IFN-γ was assessed. The MILLIPLEX kit HCD8MAG-15K-03 (Millipore) was used, with fluorescence detection performed on a Luminex MAGPIX magnetic bead analyzer (Luminex Corporation). Samples were analyzed in singlicates. Cytokine concentrations were determined by comparing sample

fluorescence to a standard curve generated from seven diluted standards, using a five-parameter logistic regression model. Only samples within the linear range of the standard curve were quantified. For statistical analysis, samples below the lowest standard in the linear range were assigned half the value of the lowest standard: 5pg/ml for IFN-γ, 2pg/ml for IL-2, and 10pg/ml for IL-4 in Study II, and 0.5pg/ml for IFN-γ in Study III. Samples exceeding the highest standard were assigned the maximum standard value: 5000 pg/ml for IFN-γ, 7500 pg/ml for IL-2, and 10,000 pg/ml for IL-4 in Study II, and 5000 pg/ml for IFN-γ in Study III. Standard samples were run in duplicate, with a standard deviation of less than 15% required for assay validity. Samples with fewer than 35 beads per well, as specified by the manufacturer, were deemed unreliable and excluded from analysis.

4.9 ELISpot

To identify circulating memory B cells capable of maturing into antibody-secreting cells (ASCs), PBMCs were cultured and stimulated for five days with 1 mg/ml R848 (a TLR7/8 agonist; InvivoGen) and 0.01 mg/ml recombinant IL-2 (R&D Systems) in culture medium containing 10% FBS in AIM-V medium (Thermo Scientific). ELISpot plates (multiscreen filtration plate, Millipore) were coated and incubated overnight at +4°C with solutions of his-tagged SARS-CoV-2 proteins, kindly provided by Dr Olli Ritvos, University of Helsinki: N (8 mg/ml), S1 (3.5mg/ml), and RBD (4 mg/ml). The plates also included mouse myostatin (8mg/ml) as a control for nonspecific binding, along with tetanus toxoid (Td; 10mg/ml; GlaxoSmithKline) and unlabeled anti-human IgG (10mg/ml; MP Biomedicals). The plates were blocked for one hour with culture medium and washed with PBS-Tween-20. Subsequently, antigen-specific amount of stimulated PBMCs in the culture medium was added to the plates: 500,000 cells for SARS-CoV-N and mouse myostatin, 100,000 cells for tetanus toxoid and RBD, 50,000 cells for S1, and 10,000 cells for anti-IgG-coated wells, all in duplicates. After 24 hours of incubation, the wells were washed with PBS-Tween-20, and the cells were lysed using distilled water. The cell debris was then washed away with PBS-Tween-20, and alkaline phosphatase-conjugated goat anti-human IgG (Sigma-Aldrich) was added for one hour at 37°C. The plates were washed with PBS-Tween-20, dried, and then washed three times with PBS before adding 1-Step NBT/BCIP substrate (Pierce) for 5 minutes. After washing and drying, the plates were imaged and analyzed using the ImmunoScan C.T.L ELISpot reader. Nonspecific spots (mouse myostatin spots) were subtracted from the antigen-stimulated spots for each sample. Samples lacking specific spots for both anti-IgG and tetanus toxoid were excluded from the analysis.

4.10 Statistical analysis

Data were collected using Excel 2016 (Microsoft 365) and analyzed and visualized with Prism 8 and 10 (GraphPad Software). Changes in SARS-CoV-2 S structure (PDB: 6VXX) were visualized with UCSF Chimera v1.15 (RBVI, University of California). Shapiro-Wilk and Kolmogorov-Smirnov tests were used to assess data normality. The Wilcoxon signed-rank test or Friedman test, followed by Dunn's multiple comparisons, was used for paired samples when applicable, while the Mann-Whitney U-test or Kruskal-Wallis test, followed by Dunn's multiple comparisons, was used for unpaired comparisons or when participants lacked samples at certain time points. All tests were two-sided, with p-values <0.05 considered statistically significant and displayed in figure legends. Correlations were analyzed using Spearman's correlation test. All statistical tests used are mentioned in the respective figure legends.

5 Results

5.1 Humoral immune responses in COVID-19 vaccinees

5.1.1 Effect of the vaccination intervals and booster vaccine types on antibody responses

In Study I, to assess variation in humoral immune responses elicited by five combinations of three COVID-19 vaccines: two mRNA vaccines (mRNA-1273, BNT162b2), and one adenovirus vector vaccine (ChAdOx1), SARS-CoV-2 S-specific antibody levels were analyzed in sera collected from HCWs (Figure 6). In the case of two doses of BNT162b2, the group was further divided into those with a short dose interval of three weeks and those with a long interval of twelve weeks between the doses. The serum samples were collected before vaccination, and three weeks and three months after the first and second vaccine doses, and analyzed by EIA and MNT.

As shown in Figure 6a, the production of S1-specific IgG antibodies was significantly higher at three weeks after the first dose when BNT162b2 or mRNA-1273 was administered as the first dose, compared to ChAdOx-1. At three months after the first dose, a significant difference in antibody levels persisted between those vaccinated with mRNA-1273 or ChAdOx-1 as their initial dose.

Two doses of any vaccine combination (Figure 6b) elicited a robust response, with GM titers ranging from 89 (ChAdOx+BNT162b2) to 158 (2x mRNA-1273) EIA units three weeks post-second dose. The decline in IgG levels from three weeks to three months post-second dose was similar across all groups (a 1.6–2.0-fold decrease). Within the long interval vaccine regimens, those administered mRNA-1273 as the second dose yielded the highest GM antibody levels (GM of 153–158 and 80–96 EIA units at three weeks and three months post second dose, respectively), while regimens with BNT162b2 as the second dose showed lower levels (GM of 89–127 and 44–67 EIA units).

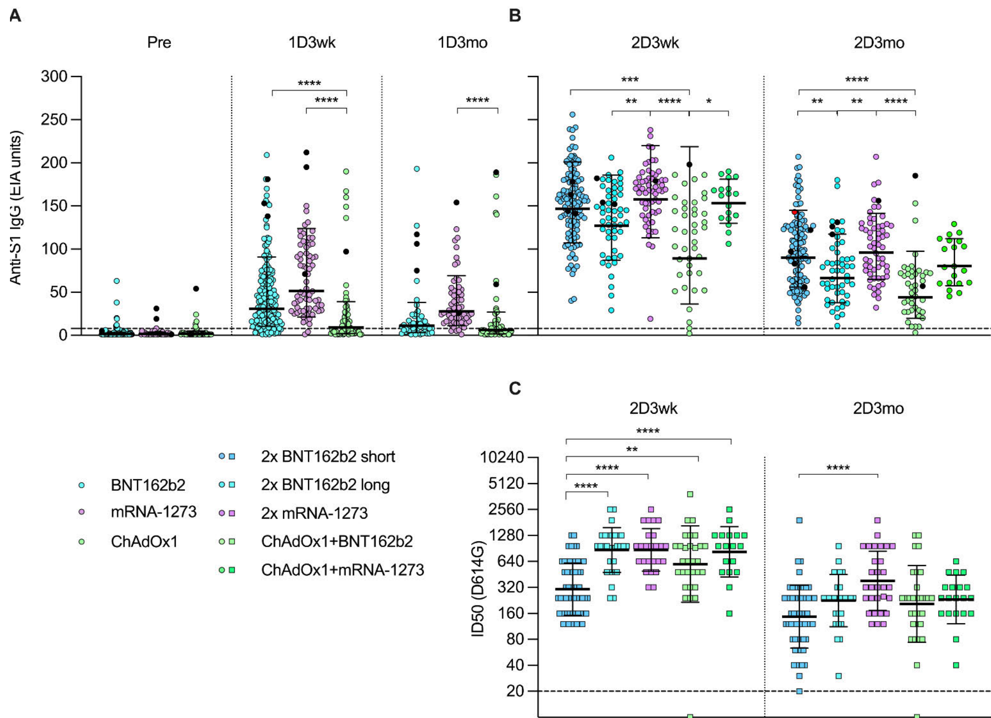

Figure 6. Binding and neutralizing antibody responses of the HCWs after receiving five different vaccine regimens. Vaccinees with SARS-CoV-2 infection contracted before the first vaccination (n=8) are marked with black dots, and those with a post-vaccination breakthrough infection (n=1) with a red dot. **A.** IgG antibody levels in the sera before vaccination and after the first vaccination. **B.** IgG antibody levels in the sera and **C.** neutralizing antibody titers against the D614G variant in uninfected vaccinees after the second vaccine dose. Cut-off values are indicated with dashed lines. Geometric means (GMs) with geometric standard deviations (SDs) are shown. P-values <0.05 were considered statistically significant (*P<0.05; **P<0.01; ***P<0.001; ****P<0.0001). Modified from the Original publication I.

All vaccine regimens induced robust neutralizing antibody responses against the D614G variant (Figure 6c). The levels did not differ significantly at three weeks after the second dose, regardless of whether the second vaccine was mRNA-1273 or BNT162b2. However, long-interval regimens generally resulted in higher titers than the short-interval BNT162b2 group. The decline in neutralizing antibody levels from three weeks to three months after the second dose was 2.1-fold in the short-interval BNT162b2 group and 2.3-fold in the homologous mRNA-1273 vaccine group, whereas in the other groups it ranged from 2.9 to 3.9-fold. As a result, neutralizing antibody levels became more uniform across groups at three months than at three weeks post-second dose.

To evaluate differences in humoral responses after the third vaccination with either mRNA-1273 or BNT162b2, S1-specific binding antibody levels (Study I) and neutralizing antibody levels (Study II) were measured three weeks and three months after the third vaccine dose (Figure 7).

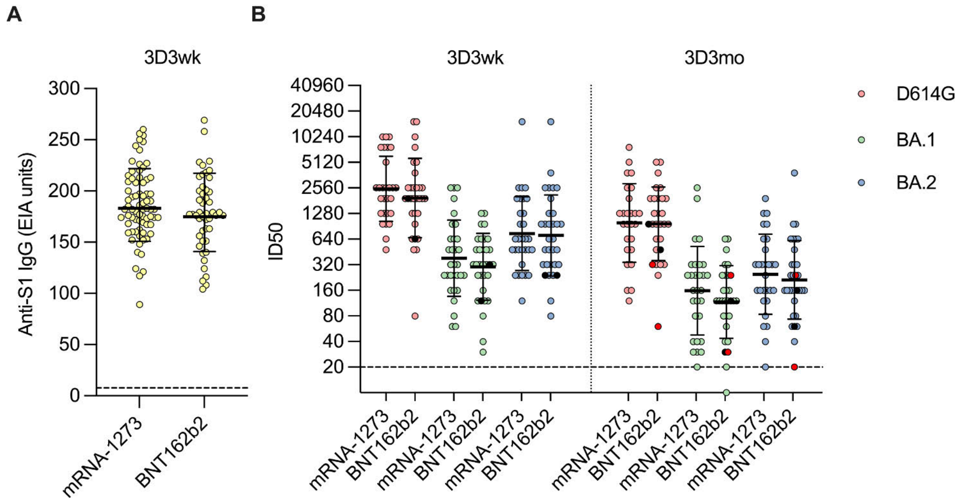

Figure 7. SARS-CoV-2 S1-specific IgG and neutralizing antibody responses of the HCWs after receiving either mRNA-1273 or BNT162b2 as the third vaccine dose. **A.** S1-specific IgG antibody levels and **B.** neutralizing antibody levels in the two vaccination groups compared after the third dose. Vaccinees with SARS-CoV-2 infection contracted before the first vaccination (N=2) are marked with black dots, and those with post-vaccination breakthrough infections (N=2) with red dots. GMs with geometric SDs are shown. The dashed line indicates the cut-off value. P-values <0.05 were considered statistically significant. Modified from the Original publications I and II.

No significant differences were observed in IgG antibody levels after the third dose of either mRNA vaccine, as at three weeks post-third dose, both vaccine types restored binding antibodies to similarly high GM levels of 183 (mRNA-1273) and 175 (BNT162b2) EIA units (Figure 7a). Furthermore, both vaccines elicited equally high neutralizing antibody levels against D614G and the Omicron variants BA.1 and BA.2 at three weeks and three months after the third dose (Figure 7b). The decline in neutralizing antibody levels against all these variants from three weeks to three months after the third dose was similar, with a 2.4–3.0-fold decrease following the mRNA-1273 vaccine and a 2.0–3.3-fold decrease after the BNT162b2 vaccine.

In Study III, 33% (37/111) of the study cohort had received a fourth dose of either a monovalent (mRNA-1273 or BNT-1273, both containing the ancestral strain Wuhan-Hu-1) or bivalent mRNA vaccine (BNT162b2, containing ancestral Wuhan-Hu-1 combined with BA.1 or BA.4-5), and the ability of these vaccines to induce S1-specific binding antibodies and neutralizing antibodies was assessed (Figure 8).

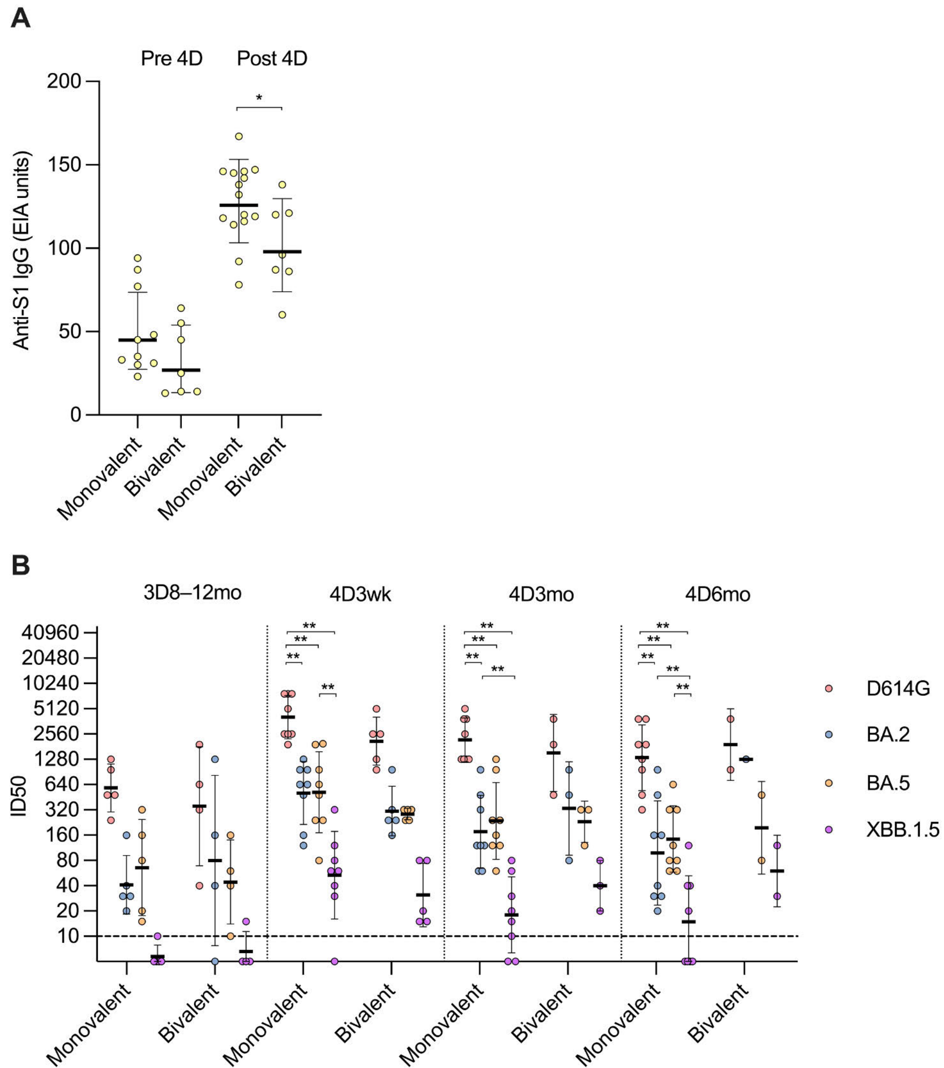

Figure 8. Binding and neutralizing antibody responses of the uninfected HCWs after receiving either monovalent or bivalent mRNA vaccine as the fourth vaccine dose. **A.** SARS-CoV-2 S1-specific IgG antibody levels in the monovalent and bivalent vaccination groups before and after the fourth vaccine dose. **B.** Neutralizing antibody levels in the monovalent and bivalent vaccination groups after the third and fourth doses. GMs with geometric SDs are shown. P-values <0.05 were considered statistically significant (*P<0.05; **P<0.01). Modified from the Original publication III.

Before the fourth vaccine dose, there was no significant difference in IgG antibody levels between the vaccine groups (Figure 8a). However, after the fourth dose, participants who received a monovalent vaccine showed significantly higher IgG antibody levels than those who received a bivalent vaccine.

Serum samples from vaccinees, whether given the monovalent or bivalent vaccine, effectively neutralized D614G and the Omicron variants BA.2 and BA.5, throughout the follow-up period post fourth dose (Figure 8b); all vaccinees neutralized all three variants three weeks, three months, and six months after the fourth dose. However, against XBB.1.5, neutralizing efficacy was lower than against other variants, both among those who received the monovalent and those who received the bivalent vaccine.

In summary, after the first dose, only modest S1-specific IgG antibody responses were induced. However, the second dose elicited a strong response with any vaccine type, although the mRNA vaccines were more immunogenic than the adenovirus vector vaccine. Regarding the second dose, the interval between the first two administrations was a more significant factor than the specific type of the second dose vaccine; long-interval regimens generally lead to higher neutralizing titers than short-interval regimens. As for the third dose, both mRNA vaccines mRNA-1273 and BNT162b2 were equally effective at eliciting S1-specific IgG antibody responses and neutralizing all tested virus variants, including Omicron BA.1 and BA.2. The neutralization efficacy against all tested variants was also fairly similar between the monovalent and bivalent mRNA vaccines given as the fourth dose; both neutralized Omicron BA.2 and BA.5, but were less effective against XBB.1.5. However, in total IgG antibodies, the monovalent vaccine elicited a higher response after the fourth dose compared to the bivalent vaccine.

5.1.2 Binding antibody kinetics in vaccinees with and without SARS-CoV-2 breakthrough infection

In Studies II and III, the research was expanded to include the impact of SARS-CoV-2 breakthrough infections on humoral immune responses following COVID-19 vaccination. To illustrate antibody kinetics over the entire follow-up period from prevaccination to six months after the fourth dose, S1-specific IgG levels were measured in both uninfected and infected HCWs (Figure 9).

In Study II (Figure 9a), IgG antibody levels showed a modest increase three weeks post-first dose (geometric mean of 24 EIA units), followed by an increase to 105 units three weeks post-second dose, and gradual waning to 68 at three months, 27 at six months, and 16 units at eight months post-second dose. However, vaccinees who had contracted SARS-CoV-2 infection pre-vaccination maintained higher antibody levels, and an additional increase in antibody levels was observed following vaccination in these individuals. The third dose elicited a sharp increase in all vaccinees, peaking at 131 units three weeks post-third dose, with gradual waning to 96 units at three months post-third dose.

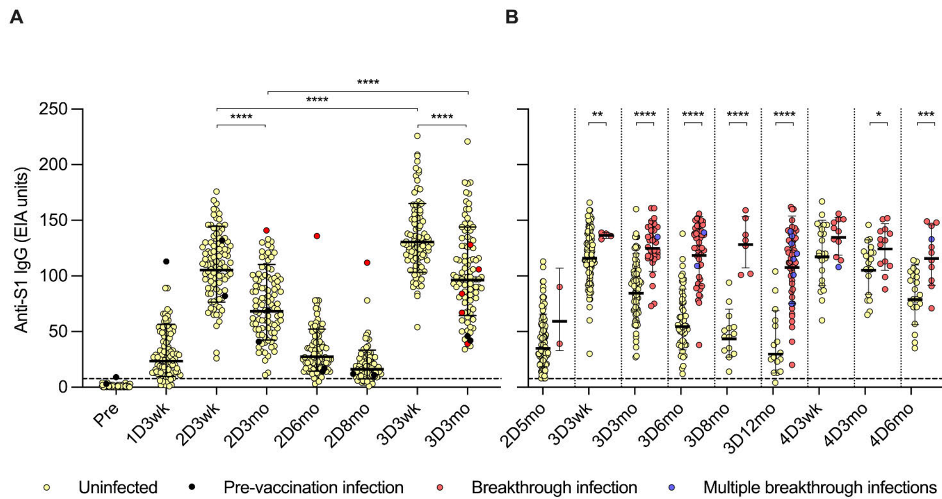

Figure 9. SARS-CoV-2 S1-specific IgG antibody kinetics in HCWs over the entire follow-up period. **A.** Before vaccination and after the first, second, and third vaccine doses, and **B.** after the second, third, and fourth vaccine doses. Cut-off values are indicated with dashed lines. GMs with geometric SDs are shown. P-values <0.05 were considered statistically significant (*P<0.05; **P<0.01; ***P<0.001; ****P<0.0001). Modified from Original publications II and III.

In Study III (Figure 9b), similar to Study II, the three-week post-third-dose time point showed an increase in antibody levels after the third dose, followed by a gradual decline in antibody levels in noninfected vaccinees: the geometric mean titer decreased from 116 units to 85 units at three months, then to 55 at six months, and to 43 at eight and finally to 30 units at 12 months after the third dose. In vaccinees who had contracted one or more breakthrough infections, antibody levels remained at higher levels, declining from 137 units at three weeks post-third dose to 125, 118, 128, and 108 units at three, six, eight, and 12 months after the third dose, respectively. The fourth dose led to a robust increase in antibody levels, with levels rising to 117 EIA units in uninfected vaccinees and 135 units in infected vaccinees at three weeks post-fourth dose. This was followed by a decline to 105 or 124 EIA units at three months, and to 79 or 116 units at six months after the fourth dose in uninfected and infected vaccinees, respectively.

In summary, the S1-specific IgG antibody levels steadily declined in uninfected vaccinees after vaccination, with half-lives of approximately three months after the second dose, six months after the third dose, and over six months after the fourth dose. In vaccinees with breakthrough infections, the half-life could not be determined as breakthrough infections acted as additional boosters, maintaining high antibody levels in the sera. In both groups, each booster vaccine dose raised antibody

levels, but especially among uninfected vaccinees, bringing them back to or above those observed following previous vaccinations.

5.1.3 Immune escape of Omicron variants from vaccine- and infection-induced humoral immunity

In Studies II and III, a microneutralization test was conducted to evaluate the neutralizing capacity of the HCW sera against the D614G and Omicron variants BA.1, BA.2, BA.5, and XBB.1.5 after two, three, and four doses of COVID-19 vaccines and with or without breakthrough SARS-CoV-2 infection (Figure 10).

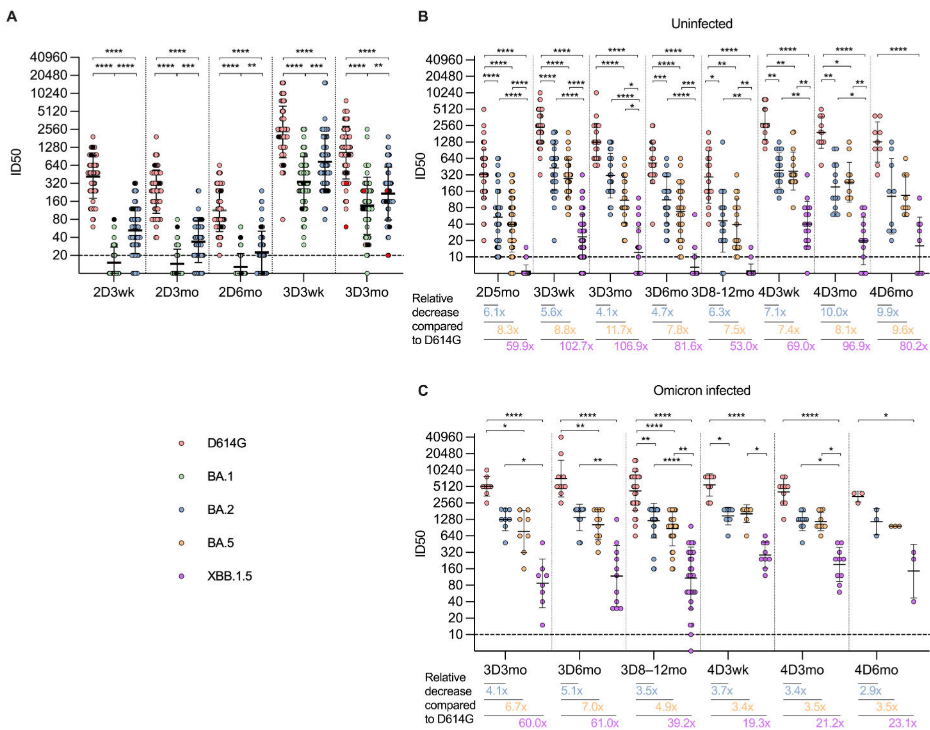

Figure 10. Neutralizing antibody titers against D614G and four Omicron variants, BA.1, BA.2, BA.5, and XBB.1.5, in HCWs after two, three, and four vaccine doses and with or without Omicron breakthrough infection. **A.** Comparison of the neutralizing antibody levels after two and three vaccine doses. Black dots represent HCWs with a PCR-confirmed SARS-CoV-2 infection before the first vaccination, and red dots represent HCWs with a breakthrough infection after the second or third vaccine dose. **B.** Comparison of neutralization levels after two, three, and four vaccine doses in HCWs without breakthrough infection, and **C.** in those with one or more Omicron breakthrough infections. GMs with geometric SDs are shown. P-values <0.05 were considered statistically significant (*P<0.05; **P<0.01; ***P<0.001; ****P<0.0001). Modified from Original publications II and III.

Three weeks after the second vaccine dose (Figure 10a), 100% of the serum samples neutralized D614G, while 37% and 92% of samples neutralized Omicron subtypes BA.1 and BA.2, respectively. The levels of neutralizing antibodies gradually diminished; however, they continued to neutralize the D614G variant at all time points in the following six months post-second dose. The Omicron variants were neutralized by 33% and 83% of the serum samples at three months, and by 23% and 62% of the samples at six months after the second dose for BA.1 and BA.2, respectively. The Omicron variants BA.5 and XBB.1.5 were neutralized by 91% and 13% of the serum samples, respectively, at five months after the second dose (Figure 10b).

The third dose boosted neutralizing ability in uninfected vaccinees against all the variants, with 100% of serum samples neutralizing the Omicron variants BA.2 and BA.5 throughout the follow-up period after the third dose. However, the proportion of samples capable of neutralizing XBB.1.5 declined over time, to 89%, 52%, 23%, and 11% at three weeks and three, six, and eight to 12 months after the third dose, respectively. Throughout the 12-month follow-up period after the third dose, the serum samples showed neutralizing antibody levels 4.1–10.0 times lower against Omicron subtypes BA.2 or BA.5 compared to D614G, whereas against XBB.1.5, the levels were 53.0–106.9 times lower than D614G. The differences in neutralizing capacity were not significant at any time point between BA.1 and BA.2, and between BA.2 and BA.5, significant only at three months post third dose. However, against XBB.1.5, neutralizing titers were significantly lower than against BA.2 and BA.5 at all time points through six months post-fourth dose (Figure 10a, b). The fourth dose again increased neutralizing antibody levels against all variants at three weeks post fourth dose back to levels similar to those three weeks after the third dose. The rate of decline in levels over the next six months was similar after the fourth dose as it was in the six months following the third dose.

Among vaccinees with one or more breakthrough infections, 100% of serum samples neutralized all tested variants at three months after the third dose (Figure 10c). The neutralization efficacy remained at 100% throughout the entire follow-up period against all variants, including Omicron BA.2 and BA.5, except against XBB.1.5, for which 96% of the serum samples retained neutralization ability eight to twelve months after the third dose. Over the 12 months following the third dose, serum samples showed 2.9 to 61.0 times lower neutralizing antibody levels against all tested Omicron subtypes compared to D614G. The fourth vaccine dose showed the strongest effect on neutralizing capacity against XBB.1.5, with antibody levels being 2.6 times higher three weeks after the fourth dose compared to eight to twelve months after the third dose. At the same time points, antibody levels against BA.2 and BA.5 increased by 1.2- and 1.9-fold, respectively. No substantial decline in antibody levels was detected after the third or fourth vaccine dose.

In summary, the D614G variant was well neutralized after the second, third, and fourth vaccine doses, and neutralizing antibody levels remained high throughout the follow-up period. After the second dose, BA.2 and BA.5 Omicron variants were neutralized significantly better than BA.1 or XBB.1.5, and after the third and fourth doses, XBB.1.5 remained significantly less neutralized than all the other variants in both uninfected vaccinees and those with breakthrough infections. The decrease in neutralizing antibody levels over the 12 months after the third dose was less pronounced in vaccinees with breakthrough infections than in those without. Overall, neutralizing responses in the infected vaccinees were higher and less varied than in uninfected vaccinees; the fold differences between variants were smaller, and the fourth dose did not significantly increase neutralizing antibody levels above those seen after the third dose.

5.2 Cell-mediated immune responses in COVID-19 vaccinees

5.2.1 Cross-reactive and durable T cell immunity

HCWs were evaluated for T cell-mediated vaccine responses by analyzing PBMCs for activation-induced markers by flow cytometry after two and three (Study II), and three and four (Study III) doses of COVID-19 vaccines (Figure 11). The T cell-mediated responses were analyzed against SARS-CoV-2 wild-type (Study II and III) and Omicron variants BA.1 and BA.2 (Study II), and BA.5 and XBB.1.5 (Study III) S protein peptides. In addition, in Study III, the wild-type nucleoprotein-specific T cell responses were measured in vaccinees with and without one or more SARS-CoV-2 breakthrough infections.

The two COVID-19 vaccination doses effectively primed S-specific $CD4^+$ T cells against the S protein peptide pools of the wild-type SARS-CoV-2, and the Omicron variants BA.1 and BA.2, as 93–100% of vaccinees showed a positive response across the variants at three weeks after the second dose (Figure 11a). The stimulation levels remained relatively consistent for six to eight months after the second dose, and the third dose did not significantly increase them. The levels remained robust and equal against wild-type and the Omicron variants BA.1 and BA.2, as well as BA.5 and XBB.1.5, until three months after the third dose, and they remained stable against BA.5 and XBB.1.5 until eight to twelve months after the third dose. However, at six months after the third dose, responses against the wild-type variant were significantly higher than against those of XBB.1.5 (Figure 11b). After the fourth dose, $CD4^+$ responses remained relatively stable against wild-type, Omicron BA.5, and XBB.1.5.

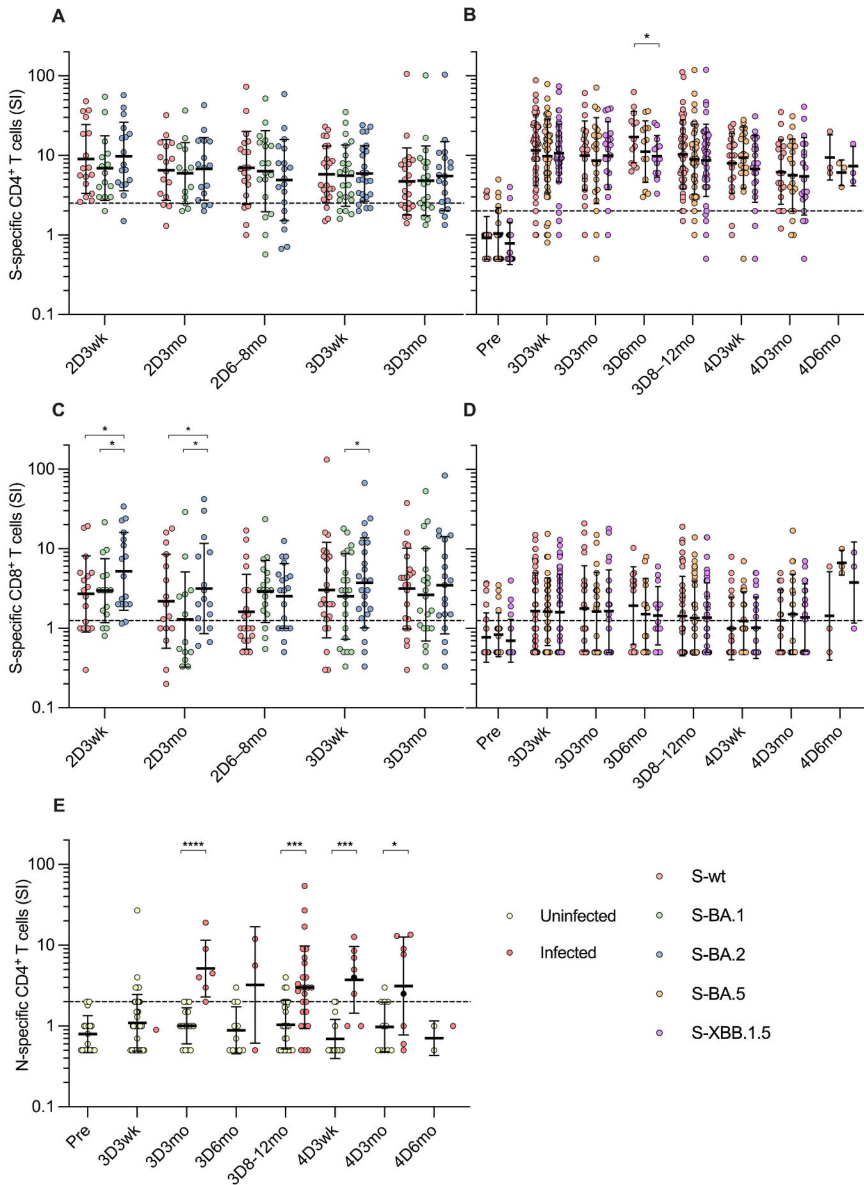

Figure 11. CD4⁺ and CD8⁺ T cell responses to SARS-CoV-2 wild-type (wt) and Omicron BA.1, BA.2, BA.5, and XBB.1.5 S protein peptides following vaccinations and to wild-type N protein peptides in the HCWs with and without breakthrough infection. **A.** S-specific CD4⁺ T cell responses to wild-type, BA.1 and BA.2, and **B.** to wild-type, BA.5 and XBB.1.5 S protein peptides **C.** S-specific CD8⁺ T cell responses to wild-type, BA.1 and BA.2, and **D.** to wild-type, BA.5 and XBB.1.5 S protein peptides. **E.** CD4⁺ T cell responses to wild-type N protein peptides after the third and fourth vaccine doses in uninfected and infected vaccinees. Dashed lines indicate the cut-off values. GMs with geometric SDs are shown. P values <0.05 were considered statistically significant (*P<0.05; ***P<0.001; ****P<0.0001). Modified from Original publications II and III.

The measured S-specific $CD8^+$ T cell responses were generally lower than those of $CD4^+$ T cells (Figure c, d). Three weeks after the second vaccine dose, 59–60% of participants showed a positive response across the variants (Figure 11c). Additionally, stimulation levels against the wild-type variant decreased to some degree over the eight-month observation period after the second dose. The third vaccine dose restored $CD8^+$ wild-type S-specific responses to a level higher than after two doses, and at three weeks and three months following the third dose, 71% and 77% of participants, respectively, exhibited positive $CD8^+$ responses against the wild-type variant. Additionally, $CD8^+$ T cells effectively cross-recognized Omicron BA.1 and BA.2 S protein peptides during the follow-up period after the second and third doses, although there were significant differences in responses between the two Omicron variants at specific time points. The Omicron variants BA.5 and XBB.1.5 were also cross-recognized, although the SIs observed were relatively low against all tested variants, including responses to the wild-type variant: 48% of the vaccinees showed a positive response at three weeks, 33% at three months, and 55% at six months after the third dose (Figure 11d). At eight to twelve months after the third dose, only 25% of these vaccinees displayed a positive $CD8^+$ T cell response to wild-type peptide pool stimulation. In addition, no apparent increase in wild-type-specific $CD8^+$ responses was observed after the fourth dose.

The formation of N-specific $CD4^+$ T cells was triggered by breakthrough infections, and while at three weeks after the third dose, N-specific responses were observed in only 2% of the vaccinees, at three months post-vaccination, they were seen in 29% of the vaccinees (Figure 11e). The stimulation indices remained at relatively similar levels throughout the follow-up period, with 27% at six months after the third dose, 21% at eight to twelve months after the third dose, 36% three weeks after the fourth dose, and 42% of the vaccinees three months after the fourth dose showing positive responses for wild-type N-specific $CD4^+$ cells. During follow-up, responses to N peptides were observed in some uninfected vaccinees, but their activation levels were significantly lower than those seen in vaccinees with breakthrough infections.

In summary, the S-specific $CD4^+$ T cells were effectively primed, and they successfully recognized all of the included variants – wild-type, and Omicron BA.1, BA.2, BA.5, and XBB.1.5 – after vaccinees received the second, third, and fourth vaccine doses. The stimulation levels stayed relatively stable throughout the observation period. In general, the measured S-specific $CD8^+$ T cell responses against all variants were lower than the $CD4^+$ responses, and stimulation levels were less consistent. Nonetheless, like $CD4^+$ T cells, the $CD8^+$ T cells also cross-recognized the Omicron variants. Breakthrough infections efficiently triggered the formation of N-specific $CD4^+$ T cells, and the stimulation levels remained consistent throughout the follow-up period.

5.2.2 Memory T cells

In addition to $CD4^+$ and $CD8^+$ T cell responses, the activation levels of $CD4^+$ circulating follicular T helper cells were analyzed, along with the division of both $CD4^+$ and $CD8^+$ T cells into memory cell subsets of Tcm ($CD45RA^-CCR7^-$), Tem ($CD45RA^-CCR7^+$), Temra ($CD45RA^+CCR7^-$), and Naïve-like cells (Naïve; $CD45RA^+CCR7^+$) (Figure 12). In Study II, the cTfh cells were analyzed for their activation after stimulation with S protein peptide pools from the wild-type variant and the Omicron variants BA.1 and BA.2. In Study III, in addition to the wild-type S protein peptides, cTfh responses were analyzed following stimulation with Omicron BA.5 and XBB.1.5 S protein peptides.

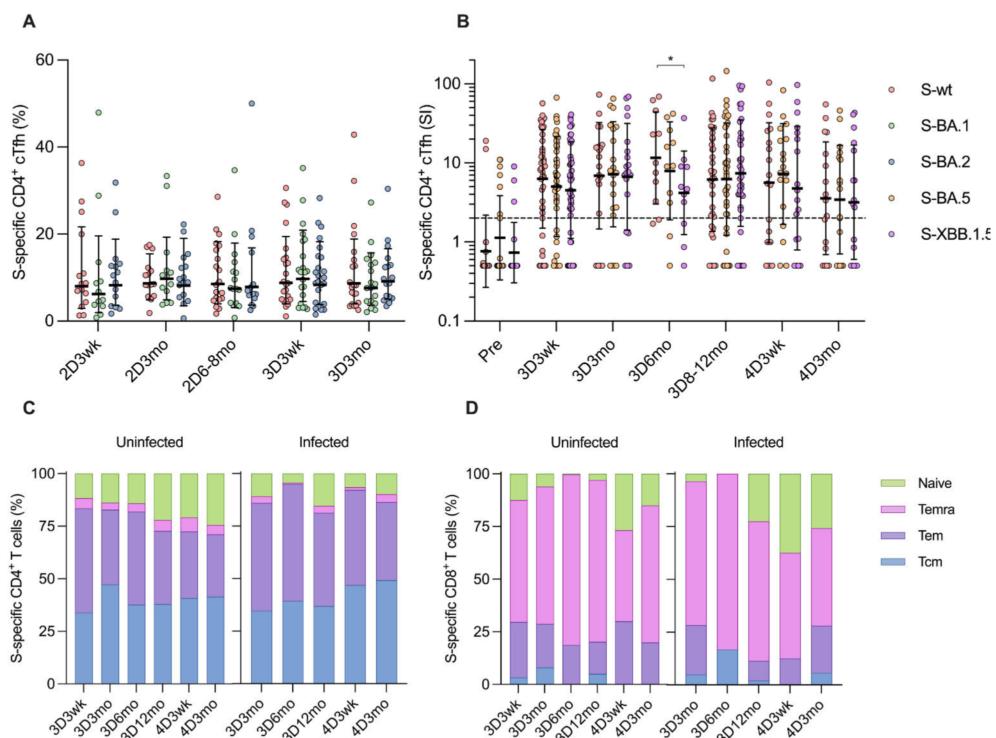

Figure 12. SARS-CoV-2 S-specific $CD4^+$ circulating follicular T helper cell (cTfh) responses and the distribution of S-specific $CD4^+$ and $CD8^+$ T cells into memory cell subsets after the second, third, and fourth vaccine doses. cTfh responses are shown upon stimulation with **A**. S protein peptide pool from wild-type, BA.1, and BA.2, as proportions (%) within S-specific $CD4^+$ T cells, and upon stimulation with **B.** S-protein peptide pools from wild-type, BA.5, and XBB.1.5 variants, as SI. **C**. The distribution of $CD4^+$ and **D.** $CD8^+$ T cells into memory cell subsets: Tcm, Tem, Naïve, and Temra. Dashed lines indicate the cut-off values. GMs with geometric SDs are shown. P values of <0.05 were considered statistically significant (*P<0.05). Modified from Original publications II and III.

Three weeks after the second vaccine dose, wild-type S-specific cTfh cells were detected in 88% of the vaccinees, and at three weeks after the third dose, in 96% of the vaccinees (Figure 12a). The proportions of detected S-specific cTfh remained stable over the follow-up period, with no decline in the following six to eight months post-second dose or in the three months post-third dose, and the proportions were similar between all analyzed variant peptide pools, including Omicron BA.1 and BA.2. In addition, most participants showed stable levels of S-protein-specific cTfh cells against Omicron BA.5 and XBB.1.5 variants and these cells were detected in 77% of vaccinees at three weeks and in 81% at eight to 12 months after the third vaccine dose (Figure 12b). Notably, neither the fourth vaccine dose nor a breakthrough infection significantly increased the frequency of S-protein-specific cTfh cells.

After the third vaccine dose, the wild-type S-protein-specific $CD4^+$ memory cell subsets in both uninfected and infected vaccinees consisted mainly of Tcm and Tem cells, with fewer Naïve and Temra cells (Figure 12c). The distribution of memory cells remained similar after the fourth vaccine dose, and no clear differences between uninfected and infected vaccinees were observed during the follow-up. However, a modest increase in the proportion of S-protein-specific Naïve memory cells and a corresponding decrease in Tem cells were observed among uninfected vaccinees over the follow-up period. In wild-type S-protein-specific $CD8^+$ T cells, the predominant memory cell subset was Temra cells, with smaller proportions of Tem, Tcm, and Naïve cells (Figure 12d). These proportions were similar between uninfected and infected vaccinees and stayed relatively stable. However, at three weeks after the fourth dose, both groups showed a small increase in the proportion of Naïve cells and a modest decrease in Temra cells.

In summary, S-protein-specific cTfh responses against all tested variants, including Omicron subtypes, were detected in most vaccinees after the second, third, and fourth vaccine doses. The levels and proportions remained stable throughout the follow-up period, with no significant decrease in the subsequent months after the vaccine doses, and neither a booster dose nor a breakthrough infection significantly increased their frequency. Similarly, the distributions of $CD4^+$ and $CD8^+$ memory cells remained consistent throughout the follow-up period, with no evident differences between uninfected and infected vaccinees.

5.2.3 Functional memory B cell response

In Study III, to better characterize humoral immune responses after the third and fourth COVID-19 vaccinations, as well as following a SARS-CoV-2 breakthrough infection, the ability of circulating memory B cells to differentiate into functional ASCs specific to the S1 subunit of the SARS-CoV-2 S protein, the RBD protein, and the N-protein was evaluated using an enzyme-linked immunosorbent spot assay (ELISpot) (Figure 13).

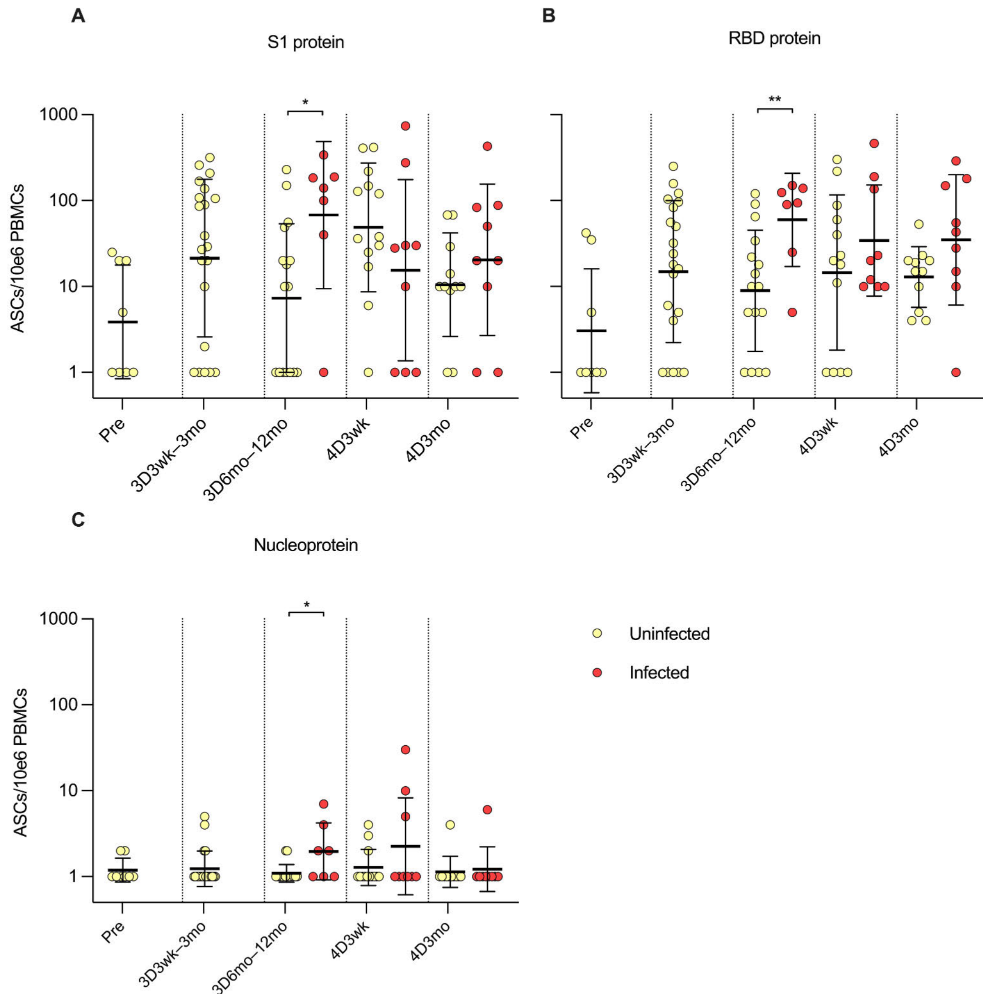

Figure 13. SARS-CoV-2 S1, RBD, and N protein-specific memory B cell responses in HCWs with and without SARS-CoV-2 breakthrough infection after three and four COVID-19 vaccination doses. **A.** S1 protein, **B.** RBD protein, and **C.** N protein-specific memory B cells capable of transforming into ASCs at various time points before vaccination and after the third and fourth vaccine doses. Uninfected vaccinees are represented by yellow dots, and vaccinees with a breakthrough infection by red dots. GMs with geometric SDs are shown. P values of <0.05 were considered statistically significant (*P<0.05; **P<0.01). Modified from the Original publication III.

Before the initial COVID-19 vaccination (Pre), 50% of vaccinees showed detectable S1-specific ASC responses (Figure 13a). Following three doses, the percentage of S1-specific ASCs rose to 76% at the time point of three weeks to three months post-third dose. At six to twelve months after the third dose, the proportion of S1-specific ASCs decreased to 56%, but breakthrough SARS-CoV-2 infections or the fourth vaccine dose increased it to 92%. A similar pattern was observed with

RBD-specific ASCs compared to S1-specific ASCs (Figure 13b). Throughout the follow-up, the number of ASCs was similar between uninfected and infected vaccinees, except at six to twelve months post-third dose, where infected vaccinees showed a significantly higher number of S1, RBD, and nucleoprotein-specific ASCs (Figure 13a–c). However, nucleoprotein-specific ASCs were not detected in all infected vaccinees.

In summary, the third and fourth COVID-19 vaccine doses or a SARS-CoV-2 breakthrough infection led only to a modest increase in the number of S1- and RBD-specific ASCs, and these were similar between uninfected and infected vaccinees throughout the entire observation period. In addition, not all infected vaccinees showed detectable N protein-specific ASCs.

6 Discussion

6.1 The effect of the timing and the combinations of vaccines on humoral immune responses

Little was known beforehand about the durability of immune responses induced by mRNA vaccines or the impact of extending the interval between vaccine doses. However, evidence from studies, particularly those on BNT162b2 conducted early in the pandemic, indicated that a longer interval improved vaccine immunogenicity, resulting in higher antibody responses and potentially increased vaccine effectiveness against infection and hospitalization after the second dose.[226–232] It has been proposed that the better vaccine immunogenicity could be attributed to the fact that an extended vaccination interval allows more time for mRNA vaccine-induced GC formation, leading to the maturation of the immune response, especially of memory B cells, after the first priming dose. This enhances the effect of additional doses and may also extend the duration of immunity[231]. In addition, extended interval promotes enriched virus-specific $CD4^+$ T cells producing IL-2, which supports B cell help and GC activity[230]. This evidence supported the delay of the second dose, while also giving the possibility to cover more people with first-dose protection in the meantime. Along with differences in interval length, it was recognized early on that mRNA-based COVID-19 vaccines elicited a somewhat stronger humoral immune response than adenovirus vector vaccines.[25,227,228,233]

This fast-paced, vital research was contributed to by our Study I at the appropriate time: in fall 2021, vaccination regimens for the first two doses were compared, involving two types (and three brands) of vaccines: one adenovirus vector vaccine, ChAdOx1, and two mRNA vaccines, mRNA-1273 and BNT162b2. In addition, the homologous doses of BNT162b2 were administered with two different intervals: a short three-week interval and a long twelve-week interval. In line with studies published at the same time as ours[230,231,234], we demonstrated that two doses of BNT162b2, mRNA-1273, or ChAdOx1+mRNA with a long interval induced equally high anti-S IgG and neutralizing antibodies against the D614G and Delta variants post-second dose, whereas BNT162b2 with a three-week interval yielded two to three-fold lower neutralizing antibody titers. In other words, a longer interval between doses generally resulted in higher neutralizing antibody levels than a shorter

interval. The decrease in binding antibody levels post-second dose was consistent between all the groups, however, the decline in neutralizing antibody levels in the long-interval group was slower than in the short-interval group. In conclusion, when it comes to neutralizing antibodies, the longer gap between the first two vaccine doses was a more significant factor than the type of vaccine administered. These findings were reassuring because, early in the pandemic, widespread public concern and speculation arose over whether vaccines from different manufacturers might offer unequal protection against COVID-19.

For this thesis, the data were partially reorganized to include a comparison of the three different vaccines administered as the first dose, and consequently, to better understand the overall picture starting from the initial dose. The two mRNA vaccines (mRNA-1273 or BNT162b2) induced a significantly more robust S1-specific IgG antibody response in comparison to the adenovirus vector vaccine ChAdOx1 as the first vaccine dose, but equally good compared to one another. The kinetics of antibody decline after the first dose were similar, regardless of whether the vaccine was an mRNA or an adenovirus vector vaccine, although the difference between mRNA-1273 and ChAdOx1 remained significant three months after vaccination. The superiority of the mRNA COVID-19 vaccines over adenovirus vector vaccines has also been demonstrated in other studies conducted at the same time or after our research. [232,235–237] The reason mRNA vaccines produce a stronger S1-specific IgG response after the initial dose in naïve individuals may be due to the highly efficient and rapid delivery of large quantities of S protein encoding mRNA directly into the cytoplasm by lipid nanoparticles, resulting in very high-level antigen production.[238] In addition, simultaneously with encoding the viral protein, the viral mRNA also acts as an adjuvant because of RNA's natural ability to stimulate the immune system.[25,238] When it enters cells, single-stranded RNA (ssRNA) and double-stranded RNA (dsRNA) are recognized by distinct endosomal and cytosolic sensors, which trigger type I interferon production and dendritic-cell activation.[154,238] This process results in sustained antigen production over several days to weeks and promotes potent GC reactions.[17,238] In contrast, adenovirus vector vaccines deliver DNA that requires nuclear entry and subsequent nuclear transcription for antigen expression, which delays antigen production compared to cytosolic mRNA translation.[239] Additionally, some people may have immunity to the vector used in adenovirus-based vaccines, which can decrease successful infection of target cells and, as a result, lower SARS-CoV-2 S protein production.[25,154,167]

Regarding differences in antibody-inducing capacity between the two mRNA vaccines, our studies show that the second dose of mRNA-1273 produced higher levels of S protein-specific binding and neutralizing antibodies than BNT162b2 in both homologous and heterologous regimens. In line with this, other studies have shown that mRNA-1273 outperforms BNT162b2 as a second dose by inducing

higher binding[240] and neutralizing antibodies against wild-type SARS-CoV-2 as well as Delta variant in both homologous and heterologous vaccine combinations,[241] and in overall VE.[242,243] This is likely partly due to dose-dependent boosting: mRNA-1273 can contain over three times more mRNA than BNT162b2 (50–100 µg vs 30 µg, respectively).[244,245]

6.2 Third and fourth vaccine doses in restoring humoral immune responses against Omicron variants

To further analyze differences in induced humoral antibody responses between mRNA-1273 and BNT162b2, their effectiveness as a third vaccine dose was compared in Studies I and II, and to create a better overall picture, those results were combined in this thesis. This demonstrated that in both study groups, the mRNA vaccines given as a third dose produced equally high levels of binding antibodies, significantly increasing their levels compared to the levels after the second dose. Thus, although mRNA-1273 has demonstrated stronger responses during the initial vaccination series[228,240–243], our Studies II and III showed that responses between the two mRNA vaccines became comparable with the second booster doses (third vaccine doses). This likely occurs because booster doses target established memory B cells from the primary vaccination, where dose differences are mitigated by recall efficiency rather than by new priming.[246] On the other hand, our results somewhat contrast with a study that, by comparing nearly 200,000 individuals with no prior SARS-CoV-2 infection and various primary vaccine series backgrounds, shows the superiority of mRNA-1273 in its VE as the third dose[244]. In addition, mRNA-1273 has been shown to induce more sustained humoral response against Omicron variants BA.1, BA.5, and XBB.15.[247] Additionally, another study found mRNA-1273 superior compared to BNT162b2 as the fourth dose in its ability to induce anti-RBD IgG and IgA responses, as well as neutralizing antibody responses against Omicron BA.1 and BA.2, and overall VE against infection and symptomatic disease.[248]

Previous studies have shown that although two doses of the COVID-19 vaccines are enough to elicit a strong humoral response, they do not provide strong long-term protection against the Omicron BA.1 or BA.2 variants, as six months after the second mRNA vaccine dose, only a small percentage of recipients remain protected against symptomatic disease.[10,249,250] Our data from Studies I and II clearly support these findings, showing insufficient neutralizing antibody titers against BA.1 from three weeks and up to six months after the second dose. Interestingly, Study II showed that most vaccinees had neutralizing antibodies against BA.2 up to six months after the second dose, and the difference in titers between BA.1 and BA.2 was significant. Neutralizing antibodies have been shown to be substantially evaded by both

subvariants (20–40-fold reduction compared to the ancestral Wuhan strain or D614G), and VE has generally been comparable against BA.1 and BA.2.[249–252] However, in some cohorts, vaccine-induced neutralizing antibodies have been shown to neutralize BA.2 somewhat better than BA.1, with no major clinical impact on protection against severe disease.[249,251,253]

Nevertheless, as shown in Studies I–III, a third dose of an mRNA vaccine restores high levels of cross-neutralizing antibodies against both Omicron BA.1 and BA.2. Both mRNA-1273 and BNT162b2 elicited equal neutralizing antibodies against these two Omicron variants, and the decline in neutralizing antibodies was also comparable after vaccination with either vaccine. The cohorts in Studies I–III received their third doses (either mRNA-1273 or BNT162b2) between the end of September 2021 and early February 2022, simultaneously with BA.1 replacing Delta as the dominant circulating variant in Finland.[76] BA.1 dominated from December 2021 to January 2022, but BA.2 quickly took over in February-March 2022, due to slight fitness advantages.[8,254] At the time our Studies I and II were conducted, it could be concluded that because the third dose significantly boosts antibody levels and neutralization ability, especially against Omicron BA.1 and BA.2, current vaccines, when administered in at least three doses, were able to provide sufficient protection against Omicron variants.

Next, the impact of the fourth vaccine dose on humoral immune responses was analyzed in Study III. After antibody levels declined again in the months following the third vaccine dose, the fourth dose restored them to high levels, although they did not increase beyond levels seen with the third vaccination. In fact, the peak of S1-specific IgG antibody levels was observed after the third vaccine dose, and subsequent fourth doses or breakthrough infections did not lead to distinctly higher levels. This phenomenon, known as the ceiling effect, has been observed already before the appearance of SARS-CoV-2, for example with influenza, and in more recent studies also with SARS-CoV-2, now that nearly five years have passed since the vaccinations began, and sufficient follow-up has been possible.[18,255] Nevertheless, the decline of both binding and neutralizing antibodies was slower after the fourth dose than after the second or third doses, indicating that repeated vaccination prolongs the presence of S1-specific antibodies.

The emergence of the Omicron subvariant BA.5 in early 2022, which became dominant in Finland by summer 2022, was followed by the appearance of XBB.1.5 in late 2022, necessitating a re-evaluation of humoral immunity against circulating Omicron lineages. In Study III, neutralizing antibody titers were compared in uninfected vaccinees following their third and fourth doses; titers were similar against BA.2 and BA.5 after each dose. However, notably lower antibody titers were observed at all time points for XBB.1.5, indicating immune evasion of the neutralizing antibody response elicited by the three or four original Wuhan-Hu-1-

type monovalent vaccinations this cohort had received, as well as by the fourth bivalent BA.1 or BA.4/5 vaccine version. In other words, these bivalent vaccines did not show increased specificity for neutralizing antibodies against XBB.1.5, a finding also observed by others[247,256], confirmed in our more recent study[257], and confirmed by the FDA.[258] However, it could be observed that, based on the neutralizing antibodies, the fourth vaccine dose overall did provide marginally better protection against the XBB.1.5 variant than the previous three doses.

To investigate this further, in Study III, the ability of monovalent and bivalent mRNA vaccines, administered as a fourth dose, to induce S1-specific binding and neutralizing antibodies was evaluated. Most participants in our cohort who received the monovalent vaccine as their fourth dose received BNT162b2, and those who received the bivalent vaccine likely received BNT162b2 Original/BA.4-5 or Original/BA.1 bivalent version. The estimate of the type of vaccine administered is based on self-reports, and the vaccination dates falling between February and August 2022, or between the end of August 2022 and January 2023, and the vaccinations given in Finland during those periods.[259] Nonetheless, due to the reliance on self-reported data, absolute certainty cannot be assured, which represents a limitation of this study. Another limitation is the small number of participants who received a fourth dose, which reduces the reliability of the statistical tests and comparisons. Before the fourth vaccine dose, there was no notable difference in S1-specific IgG levels between the monovalent and bivalent vaccine groups. However, after the fourth dose, participants who received a monovalent vaccine showed moderately higher IgG antibody levels compared to those who received a bivalent vaccine. However, the neutralizing effectiveness against all tested variants was relatively similar between the monovalent and bivalent vaccine groups. This aligns with findings from other research indicating that the Omicron-specific BA.4/5 booster vaccine generates cross-reactive antibodies against newer variants but does not elicit a stronger antibody response than the original monovalent vaccine.[260] On the other hand, data also show that neutralizing activities were more widespread after bivalent BA.1 or BA.4/5 boosters compared to monovalent boosters.[260–262]

The reason why bivalent vaccination offers little or no advantage over monovalent vaccination as a booster in inducing neutralizing antibodies, and why it does not cross-neutralize newer Omicron variants as effectively as the ancestral strain or the Omicron variants BA.1 or BA.2, is hypothesized to be due to a phenomenon called immune imprinting.[9] This means that the immune systems of previously vaccinated people who then receive a booster of the bivalent vaccine were primed by the first vaccinations to respond to the older variants SARS-CoV-2, and therefore likely responded to epitopes shared by the ancestral Wuhan strain and BA.4 and BA.5, instead of the new epitopes on BA.4 and BA.5 (in the case of monovalent versus bivalent BA.4-5) or on XBB.1.5.[256,263] Overall, the Original/BA.1 and

Original/BA.4–5 bivalent vaccines have provided less protection against BA.5 and XBB.1.5 than initially expected.[20,264,265] Luckily, research on the XBB.1.5 vaccine shows that a monovalent, XBB.1.5-variant-specific booster generates robust immune responses against later Omicron strains variants.[266] However, this does not fully resolve the issue of vaccine-induced immune imprinting.

6.3 Persistent and cross-reactive T cell responses

Both Studies II and III demonstrated that the SARS-CoV-2 S protein-specific $CD4^+$ and $CD8^+$ T cells are comparatively stable and capable of effectively cross-recognizing S protein peptide pools from all the analyzed Omicron variants: BA.1, BA.2, BA.5, and XBB.1.5, which is in line with other studies.[205,251,262] This cross-reactivity likely stems from a broad array of conserved T cell epitopes across variants, that are targeted especially by $CD8^+$ cells, but also from their ability to cross-recognize mutated epitopes.[205,267–269] In contrast, neutralizing antibodies predominantly target mutation-prone regions of the S protein, such as the RBD, which are heavily mutated in Omicron sublineages BA.1, BA.2, BA.4/5, and XBB1.5.[77,270] Thus, cross-reactivity enables existing T cells to recognize mutated strains even when antibodies fail to do so. It is already established that SARS-CoV-2-specific T cells persist longer after humoral immunity wanes and continue to protect against reinfections even in the absence of antibodies, and that the severe disease form is likely prevented even if antibody levels are low or absent.[24,271,272]

Our findings from Study III show that S protein-specific T cell responses elicited by COVID-19 vaccines do not increase after repeated vaccinations or infections, nor do they decrease, as they persist for at least 12 months post-vaccination. Furthermore, these responses remain stable over time, regardless of booster doses. Generally, this persistence differs between $CD4^+$ and $CD8^+$ T cell subsets: $CD8^+$ T cells often form long-lived central memory populations post-vaccination, while $CD4^+$ T cells provide essential helper functions that sustain the overall durability of the immune response.[21,192] Other studies have measured T cell dynamics showing half-lives of only roughly three to five months for SARS-CoV-2-specific $CD4^+$ and $CD8^+$ T cell responses in infected individuals[273] and in uninfected vaccinees[274], while others line with our studies, suggesting longer periods of over six months[275,276] or more than 12 months[277], simultaneously highlighting the variability influenced by factors like vaccine type and individual immune status.

In addition, the activation of SARS-CoV-2 S protein-specific circulating T follicular helper (cTfh) cells – considered essential for the production of effective neutralizing antibodies – was measured. The frequency of S protein-specific cTfh cells remained stable for months after the second and third vaccine doses, consistent with one study[155] but inconsistent with another.[249] Even though a correlation between

the number of activated cTfh cells and S1-specific binding or neutralizing antibodies was not found, a strong link between an early cTfh response and subsequent antibody response has been demonstrated by others.[185] Additionally, although the correlation between cTfh cells and antibody responses against SARS-CoV-2 remains still somewhat poorly established, evidence for this association exists for other viruses, such as influenza and HPV.[176,278–280] In addition, cTfhs have been shown to correlate with antibody levels during acute SARS-CoV-2 infection and disease severity.[23] Our result may be explained by our vaccinees having received multiple vaccinations, leading their antibody responses to be primarily memory B cell-driven, which is less dependent on new Tfh help due to pre-existing high-affinity clones and GC re-entry. The data conducted by us and others suggest that cTfh responses are more important in primary antibody responses elicited by the first vaccine dose.[17,23]

T cell functionality in our studies was assessed by measuring IFN-γ production, a marker of Th1 cytokine activity. Th1 cells are known to stimulate $CD8^+$ T cell responses, which aligns with findings in Study II showing that IFN-γ secretion was associated with S protein-specific $CD8^+$ T cell activity. A significant correlation was observed between the activation of $CD4^+$ and $CD8^+$ T cells in Study II, and CD4+ T cells in Study III, with IFN-γ levels. Throughout all time points, stimulation with wild-type, Omicron BA.1, BA.2, or XBB.1.5 S protein peptide pools led to a notable increase in IFN-γ secretion, indicating effective recognition of these variant peptides. Polyfunctional T cells expressing IFN-γ are linked to protection from severe disease by supporting antiviral responses.[281] A weak positive correlation was also noted between wild-type peptide-activated cTfh cells and IFN-γ levels from wild-type-stimulated PBMCs. These findings confirm that SARS-CoV-2-specific T cells remain functional up to one year post-immunization.

6.4 Hybrid immunity

In our Studies I and II, it could be noted that breakthrough infections were rare at the time (only one PCR-confirmed case in Study I and seven in Study II), yet the third dose still significantly increased neutralizing antibodies against Omicron BA.1 and BA.2, establishing a vaccine-induced basis for enhanced hybrid immunity upon future exposure. Study II expanded on this by analyzing T cell-mediated responses after the third mRNA dose, showing persistent $CD4^+$ and $CD8^+$ T cells that cross-recognized Omicron BA.1 and BA.2 S protein peptides, highlighting the durability of vaccine-induced T cell responses.

Study III provided better insights into long-term hybrid immunity, tracking participants for up to twelve months after the third dose and six months after the fourth dose. Almost two-thirds of the cohort experienced breakthrough infections during the follow-up, mainly during the period when Omicron sublineages BA.2,

BA.4, and BA.5 sequentially predominated in circulation (from early to mid-2022). Hybrid immunity led to significantly higher levels of S1-specific IgG and neutralizing antibodies against D614G, Omicron BA.2, BA.5, and XBB.1.5 compared to vaccine-only immunity.

The elevation in S protein-specific IgG levels and improved neutralization against diverse variants, including Omicron sublineages, likely occurs due to the exposure to whole-virus antigens during infection, which diversifies the epitope repertoire beyond vaccine-encoded S protein[9,282] In addition, neutralizing responses induced by breakthrough infections are somewhat more resilient to S protein mutations, with potency and breadth correlating with the number of antigen exposures[212], whereas in unvaccinated individuals, Omicron infection shows limited cross-neutralization.[213] Neutralizing antibodies in infected vaccinees declined with a half-life of three to four months after vaccination, but breakthrough infections slowed down this decline. These findings are in line with for example a study by Hoffmann et al., which showed that breakthrough infections with BA.1, BA.2, or BA.5 in triple-vaccinated individuals led to significantly higher neutralization of Omicron sublineages, including BA.1, BA.2, BA4./5. and BJ.1 (which is parental lineage to the XBB recombinant), than in vaccinees without breakthrough infections.[9] In our study, the fourth dose combined with infection reached peak total antibody levels, and Omicron BA.2/BA.4/BA.5 infection provided better cross-neutralization breadth against XBB.1.5, outperforming monovalent (Wuhan-Hu-1) or bivalent (Wuhan-Hu-1/BA.4/5) vaccines alone. These are interesting results, considering that breakthrough infection with the earlier Omicron variants does not typically induce robust cross-neutralizing antibody levels against XBB.1.5.[209,210,256] In addition, a recent study by Linger et al. has shown that using a homologous bivalent (Wuhan-Hu-1/BA.4/5) mRNA vaccine as the third dose elicited stronger neutralizing antibody and cell-mediated immune responses than an XBB.1.5 breakthrough infection against the XBB.1.5 variant in naïve mouse models.[283] In our study, T cell responses, however, were not further strengthened by additional vaccine doses or breakthrough infections, although the responses did remain well-maintained and cross-reactive against Omicron variants. Importantly, no evidence of T cell exhaustion was detected, consistent with the finding that T cell exhaustion in SARS-CoV-2 is linked to severe or persistent disease rather than to resolved acute infections or vaccinations.[19,284]

An additional noteworthy finding from our Studies I–III is the ceiling effect on antibody responses, previously discussed herein in the context of multiple vaccinations, and now also considering hybrid immunity. In participants with acquired breakthrough infections, baseline antibody levels were already high due to previous exposure to both vaccine and natural antigens, which aligns with data showing hybrid immunity starts from a higher baseline than vaccine-only

immunity.[18,282] Consequently, further boosting added little additional benefit, as the immune system had likely already reached its maximum response capacity.[18] This is particularly evident in Study III, where the fourth dose in infected participants did not significantly increase IgG or neutralizing antibodies beyond the peak levels seen after the third dose or infection, unlike in naïve vaccinees.

These notions suggest that when pre-existing immunity is high, either from vaccination, infection, or both, and particularly if recent, additional boosting may not substantially enhance responses. Simultaneously, the observed T cell stability in Studies II and III, even following breakthrough infections, underscores the robustness of cellular immunity, which appears less sensitive to antigenic drift than humoral immune responses. Nevertheless, in high-risk populations, periodic booster doses updated to match current circulating variants remain clinically justified to combat antigenic drift–induced decreases in neutralizing antibody titers and to further lower the residual risk of severe disease, even in individuals with hybrid immunity.

6.5 Future aspects of vaccine development

Although the S protein has been the primary vaccine target, its mutational hotspots, which are evident in the accumulating changes observed in Omicron variants such as XBB.1.5, JN.1, and its descendants (e.g. JN.1.8.1, LP.8.1.1, NB.1.8.1, and XFG), highlight limitations in eliciting long-lasting immunity against emerging variants.[78,90,209] Given the extensive antigenic drift observed in the S protein[11,79,81,91] and the associated challenges of immune imprinting[9,214,215,219], future vaccine development efforts could expand to include, in addition to the S protein, more conserved non-structural proteins, like N protein.[285–287] While the S protein has accumulated dozens of mutations, the N protein remains notably stable, with only three to seven substitutions across dominant 2024–2025 variants, including NB.1.8.1 and LP.8.1.1.[288] The N protein has been shown to boost T cell memory and interferon responses in SARS-recovered patients,[289] potentially offering broader protection against new variants and providing variant-resistant cellular immunity that complements waning humoral protection.

However, possibly the most promising future COVID-19 vaccines are multitope vaccines that include multiple epitopes. Examples include Vaxxinity's UB-612 protein subunit vaccine, which contains S1-RBD, S2, N, and M epitopes, and which has demonstrated enhanced T cell cross-reactivity in preclinical studies,[220] SK Bioscience's GBP511, a recombinant protein nanoparticle-based pan-sarbecovirus vaccine candidate that recently entered phase 1/2 clinical trials in Australia[290], and Caltech's mosaic-8b, a preclinical RBD nanoparticle displaying eight diverse sarbecovirus RBDs, which elicited cross-reactive antibodies in pre-immunized

animal models and has completed preclinical validation toward phase one human trials in 2025.[291–293]

As of mid-2025, multiple intranasal vaccine candidates have progressed to phase 2/3 testing globally, supporting the potential of mucosal immunization to complement intramuscular vaccination. Nasal and mucosal vaccines induce secretory IgA and tissue-resident memory T and B cells at the primary site of viral entry, thereby limiting upper respiratory viral replication and onward transmission more effectively than systemic S protein-only vaccines alone.[294] Preclinical studies have demonstrated near-sterilizing immunity in the respiratory tract with live attenuated intranasal vaccines (no detectable viral RNA in Syrian hamsters challenged with multiple variants, including Omicron BA.1, BA.2, and BA.5)[295], and with adenoviral-vectored intranasal vaccines expressing full-length S protein or RBD (providing full protection against Beta challenge in mice and HLA-humanized models).[296] Additionally, the Finnish intranasal trimeric sherpabody TriSb92, applied as a "biological mask", neutralized SARS-CoV-2 (including Omicron BF.7, XBB, and BQ.1.1) in vitro and prevented infection in mice even when administered four hours post-exposure.[297] In early human trials, the parainfluenza virus 5-vectored vaccine CVXGA1 confirmed induction of mucosal IgA, serum neutralising antibodies, and $CD8^+$ T cell responses.[298] Lastly, a large systematic review and meta-analysis of 65 mucosal vaccine studies (with over 200,000 participants) concluded that intranasal and inhaled COVID-19 vaccines elicit significantly higher neutralizing antibody titres at mucosal surfaces than intramuscular vaccines and provide measurable protection against symptomatic infection, although efficacy varies by platform and route.[299]

Co-administration of separate COVID-19 and seasonal influenza vaccines is already recommended in Finland to maximise uptake and reduce healthcare burden.[151] Phase three data published in 2025 show that Moderna's single-dose combination vaccine mRNA-1083 induces humoral immune responses that are at least as strong as, and often stronger than, separately administered influenza and COVID-19 vaccines in adults aged 50 years and older, with frequent but mostly mild-to-moderate reactogenicity and no safety concerns.[300] In contrast, the Pfizer/BioNTech combination vaccine candidate did not achieve equivalent antibody levels against influenza B strains in its phase three trial, highlighting an ongoing challenge with influenza B-lineage immunogenicity in mRNA platforms.[301] Preclinical research has since addressed this limitation: a novel multivalent hemagglutinin design significantly enhanced antibody responses against influenza B/Victoria in mice and, when combined with a COVID-19 mRNA component, outperformed commercial high-dose influenza and variant-adapted COVID-19 (Moderna's Spikevax XBB.1.5) vaccines.[302]

6.6 Limitations of the study

This thesis work has limitations. First, the type of the SARS-CoV-2 vaccine and dose timing were based on participants' self-reported information. Self-reported data may be subject to recall bias, particularly for booster doses administered during extended follow-up. In Study III, for instance, the fourth vaccine type remained unknown for three individuals, which may reduce the precision of comparisons between specific regimes. Similarly, breakthrough infections were primarily identified through participant-reported positive rapid antigen tests or RT-qPCR results, with supporting evidence from increases in anti-S1 or anti-N IgG levels. This combined approach carries the risk of both underreporting (especially for mild or asymptomatic cases) and overreporting. These factors could influence the classification of hybrid immunity and introduce bias into the evaluation of infection-induced boosting across all three studies.

Furthermore, although the participant cohorts were adequate in size, in some cases, participant cohort subgroups for specific vaccine sequences were relatively small, such as those comparing homologous mRNA primary series with heterologous primary series, or combinations involving viral vector vaccines followed by mRNA boosters. Limited statistical power in subgroup analyses increases the possibility of failing to detect modest but biologically relevant differences in immune parameters.

Moreover, the study population consisted of HCWs recruited from two Finnish university hospitals. This group was predominantly healthy, working-age adult females with likely elevated occupational exposure to SARS-CoV-2. Consequently, the results may not be readily generalizable to other demographic groups, including older adults, children, individuals with comorbidities, immunocompromised persons, or populations with lower or different exposure patterns and vaccination schedules. A cohort of unvaccinated, infection-only participants was not included in this study, nor were participants with a wide range of underlying health conditions. The lack of such comparators limits the ability to distinguish between vaccine-induced and infection-induced contributions or to account for potential additional factors, such as pre-existing immunity or other host characteristics.

Additional methodological constraints include the use of cryopreserved PBMCs in Studies II and III, as cryopreservation can reduce cell viability. Peptide stimulation employed 15-mer overlapping pools, which may be suboptimal for detecting maximal $CD8^+$ T cell responses, as naturally presented MHC class I ligands and optimal $CD8^+$ T cell epitopes are generally shorter (8–10 amino acids).[303] In addition, cytokine quantification in supernatants from stimulated PBMCs provided the total amount secreted by the cell population but prevented assessment of T cell polyfunctionality or identification of the specific cell types responsible for cytokine production.

Lastly, the immune assays were limited to a selected panel of SARS-CoV-2 variants (ancestral D614G, Delta, and Omicron sublineages up to XBB.1.5), and the cross-reactivity findings do not fully reflect the current or future circulating strains, limiting conclusions about the overall breadth of protection against ongoing virus evolution.

Overall, these limitations emphasize the importance of interpreting the observed patterns of immune durability carefully. They also highlight the need for future research involving larger, more diverse cohorts, real-world clinical outcomes, and an updated selection of variants.

7 Conclusions

The main conclusions of the study were:

1. Waning binding antibodies were restored with concurrent booster doses. The administration of the additional doses activates both humoral and cell-mediated immune responses.
2. Additional booster vaccinations restore neutralizing antibody levels but do not override them, and neutralizing antibodies showed cross-neutralization. The five vaccine combinations used in Finland elicited similar antibody responses.
3. Vaccinating with the bivalent BA.1/BA.4-5 showed no clear advantage over vaccination with a monovalent vaccine. Both heterologous and homologous vaccinations induced strong immune responses.
4. Two vaccine doses already provide strong T cell immunity. T cell responses are not exhausted by repeated vaccinations, but they also do not necessarily expand the antibody response further. Therefore, booster vaccines should be updated to match circulating variants to reduce the impact of multiple SARS-CoV-2 infections.
5. The repeated vaccination delayed the decline of S1-specific antibodies. Omicron breakthrough infection further induced and maintained high antibody levels, acting as an additional booster dose.
6. Repeated Omicron exposure does not override immunity from ancestral SARS-CoV-2 variant imprinting.

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