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IMMUNOLOGICAL INSIGHTS INTO COVID-19

From vaccination to long-term sequelae

Antti Hurme

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SARJA – SER. D OSA – TOM. 1839 | MEDICA – ODONTOLOGICA | TURKU 2024

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To my wife, Riikka, and our beautiful daughter, Saimi.

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ABSTRACT

COVID-19 was a new type of infection caused by a novel coronavirus, SARS-CoV-2, and a challenge for our naïve immune system. With no pre-existing adaptive immunity, SARS-CoV-2 spread rapidly worldwide in the beginning of 2020. The pandemic caused enormous pressure on healthcare systems and medical organizations, leading to global isolation protocols and the development of new vaccines in record-breaking time.

However, serum levels of neutralizing antibodies declined a few months after the immunization, although some protection was seen in epidemiological studies. In addition, many patients reported symptoms lasting even months after the acute SARS-CoV-2 infection. The condition was named "long COVID" or "post-COVID-19 condition" (PCC). However, little is known about the pathophysiological basis of the condition.

In this thesis, I studied cellular immunity generated by COVID-19 vaccination in healthcare workers, immunocompetent COVID-19 patients, and immunocompromised individuals. Moreover, I assessed the role of inflammation, humoral immunity, and hypocortisolism in PCC in a 24-month follow-up of COVID-19 inpatient and outpatient cohorts.

I showed that SARS-CoV-2 vaccination induces long-lasting cellular immunity in healthy individuals and immunocompromised patients, possibly protecting against severe disease. The prevalence of PCC decreased from 51.2% in three months to 18.3% over the 24-month follow-up period. Patients with PCC had elevated levels of anti-SARS-CoV-2 antibodies compared to recovered individuals. Interestingly, the clustering of patients to subgroups revealed that patients with fatigue, myalgia, or ongoing respiratory problems seemed to have elevated serum levels of interleukin six and high-sensitivity C-reactive protein. In contrast, patients with cognitive problems seemed to have lower cortisol levels. Unfortunately, the subgroup sizes were too small for proper statistical analysis.

KEYWORDS: COVID-19, immunology, vaccination, immunodeficiency, long-term sequelae

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TIIVISTELMÄ

Alkuvuonna 2020 Kiinan Wuhanissa havaittu uusi koronavirus SARS-CoV-2 aiheutti maailmanlaajuisen COVID-19 pandemian. Virukselle ei ollut väestössä hankinnaista immuniteettia, minkä vuoksi se kykeni leviämään nopeasti ympäri maailmaa. COVID-19 aiheutti alkuvaiheessa huomattavaa kuolleisuutta, ja pandemian aiheuttama terveydenhuollon kuormitus johti maailmanlaajuisiin eristystoimenpiteisiin. Toisaalta vakavan pandemian vuoksi uusia rokoteteknologioita onnistuttiin kehittämään ennätyksellisen nopeasti.

Levitessään maailmalla viruksesta kehittyi uusia variantteja, jotka onnistuivat väistämään aiempien infektioiden ja rokotusten synnyttämää immuniteettia. Rokotukset suojasivat jonkin verran myös uusilta varianteilta, mutta niiden aikaansaamasta soluvälitteisestä immuniteetista ei juuri ollut tutkittua tietoa. Tieto immuunivasteista on erittäin tärkeää etenkin potilailla, joiden vasta-ainetuotanto on heikentynyt. Moni potilas kärsii akuutin taudin jälkeen pitkäkestoisista ja hankalista oireista, joita kutsutaan akuutin COVID-19-taudin jälkeiseksi oireyhtymäksi (PCC). PCC heikentää merkittävästi elämänlaatua mutta valitettavasti sen taustasyytä ei tarkkaan tiedetä eikä siihen ole tällä hetkellä tehokasta hoitoa.

Tässä väitöstutkimuksessa tutkin COVID-19-rokotusten aikaansaamaa soluvälitteistä immuniteettia terveillä terveydenhuollon työntekijöillä ja vasta-ainevajausta sairastavilla potilailla. Lisäksi tutkin PCC:tä sairastavien potilaiden tulehduksen välittäjäaineiden sekä SARS-CoV-2-vasta-aineiden pitoisuuksia. Tutkimukseni osoitti, että COVID-19-rokotteet saavat aikaan tehokkaan soluvälitteisen immuunivasteen sekä terveillä että immuunipuutteisilla potilailla. Potilaiden pitkäaikaiset oireet helpottivat merkittävällä osalla kahden vuoden seurannan aikana. Pitkäaikaisista oireista kärsivillä oli korkeammat vasta-ainetasot SARS-CoV-2 viruksen pinta- ja nukleoproteiineja kohtaan muihin taudin sairastaneisiin verrattuna. Potilailla, joilla keskeisimmät oireet olivat uupumus sekä lihaskivut tai pitkäaikaiset hengitystieoireet, seerumin interleukiini 6:n ja herkän C-reaktiivisen proteiinin pitoisuudet vaikuttivat korkeammilta. Pääosin kognitiivisesti oireilevien kortisolitasot vaikuttivat matalammilta. Valitettavasti näiden osaryhmien koot jäivät kuitenkin liian pieneksi tilastollisia analyysejä varten.

AVAINSANAT: COVID-19, immunologia, rokotus, immuunipuute, pitkäaikaisoireet

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Abbreviations

6MWT	6-minute walking test
ACE2	angiotensin-converting enzyme 2
APC	antigen-presenting cell
ARDS	acute respiratory distress syndrome
BCR	B cell receptor
BMI	body mass index
CBT	cognitive behavioral therapy
CD	cluster of differentiation
COPD	chronic obstructive pulmonary disease
COVID-19	coronavirus disease 2019
CTL	cytotoxic T lymphocyte
CVID	common variable immunodeficiency
DAMP	damage-associated molecular pattern
DC	dendritic cell
EBV	Epstein-Barr virus
ECDC	European centre for disease prevention and control
EL	Encephalitis lethargica
FEV_1	forced expiratory volume in the first second
FEV_1/FVC	forced expiratory ratio
FVC	forced vital capacity
GET	graded exercise therapy
GPCR	G-protein-coupled receptor
HCoV	human coronavirus
HCW	healthcare worker
HRCT	high-resolution computed tomography
HPA axis	hypothalamic-pituitary-adrenal axis
hs-CRP	high-sensitivity C-reactive protein
ICU	intensive care unit
IFN	interferon
IGRT	immunoglobulin replacement therapy
IL	interleukin

IRF	interferon-regulatory factor
IVIG	intravenous immunoglobulin
MAC	membrane attack complex
ME/CFS	myalgic encephalomyelitis/chronic fatigue syndrome
MHC	major histocompatibility complex
MIS-C	multisystem inflammatory syndrome in children
NF-κB	nuclear factor kappa B
NK cell	natural killer cell
NLR	NOD-like receptor
NRS	numerical rating scale
NSP	nonstructural protein
ONS	Office of National Statistics, the United Kingdom
ORF	open reading frame
PACS	post-acute COVID-19 syndrome
PAD	primary antibody deficiency
PAIS	post-acute infection syndrome
PAMP	pathogen-associated molecular pattern
PANGOLIN	phylogenetic assignment of named global outbreak lineages
PBMC	peripheral blood mononuclear cell
PBS	phosphate-buffered saline
PCC	post-COVID-19 condition
PEM	post-exertional malaise
PID	primary immunodeficiency
PRR	pattern recognition receptor
RBD	receptor binding domain
RdRP	RNA-dependent RNA polymerase
RT-PCR	reverse transcriptase polymerase chain reaction
RT-qPCR	quantitative reverse transcriptase polymerase chain reaction
SAD	specific antibody deficiency
SARS	severe acute respiratory syndrome
SARS-CoV-2	severe acute respiratory syndrome coronavirus 2
SHG	secondary hypogammaglobulinemia
SI	stimulation index
sST2	soluble suppression of tumorigenicity 2
STAT	signal transducer and activation of transcription
T1D	type 1 diabetes
T_{CM}	central memory T cell
TCR	T cell receptor
T_{EM}	effector memory T cell
T_{EMRA}	terminal effector memory T cell

THL	Finnish Institute of Health and Welfare
TLR	Toll-like receptor
TMPRSS	transmembrane serine protease
Tyks	Turku University Central Hospital
VOI	variant of interest
VOC	variant of concern
VUM	variant under monitoring
WHO	World Health Organization
XLA	X-linked agammaglobulinemia

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 Hurme Antti*, Jalkanen Pinja*, Heroum Jemna, Liedes Oona., Vara Saimi, Melin Merit, Teräsjärvi Johanna, He Qiushui, Pöysti Sakari, Hänninen Arno, Oksi Jarmo, Vuorinen Tytti, Kantele Anu, Tähtinen Paula A., Ivaska Lauri, Kakkola Laura, Lempainen Johanna, Julkunen Ilkka: 'Long-Lasting T Cell Responses in BNT162b2 COVID-19 mRNA Vaccinees and COVID-19 Convalescent Patients'. *Front. immunol.* 2022; 13 (April).**

II Hurme Antti, Jalkanen Pinja, Marttila-Vaara Minna, Heroum Jemna, Jokinen Heidi, Vara Saimi, Liedes Oona, Lempainen Johanna, Melin Merit, Julkunen Ilkka, Kainulainen Leena: ' T cell immunity following COVID-19 vaccination in adult patients with primary antibody deficiency – a 22-month follow-up'. *Front. immunol*, 2023; publishing year; 14 (May)

III Hurme Antti, Viinanen Arja, Teräsjärvi Johanna, Jalkanen Pinja, Feuth Thijs, Löyttyniemi Eliisa, Vuorinen Tytti, Kantele Anu, Oksi Jarmo, He Qiushui, Julkunen Ilkka: 'Post-COVID-19 condition in prospective cohorts of inpatients and outpatients – a 24-month follow-up.' Manuscript.

* Authors contributed equally to this publication.
** This original publication has already been included in the thesis of PhD Pinja Jalkanen in 2023

1 Introduction

At the end of 2019, the world changed with the emergence of a novel coronavirus, SARS-CoV-2, causing a severe respiratory and multisystem disease pandemic – Coronavirus disease 2019, or COVID-19. The pandemic led to global isolation procedures and a race to develop vaccines. Different vaccination approaches were developed, but only a few were finally approved for Finnish national vaccination strategies. The vaccinations proved effective against severe COVID-19 and reduced symptoms in milder disease.

However, while spreading throughout the globe, SARS-COV-2 evolved variant lineages, causing breakthrough infections even after vaccination or previous infection. The uncertain efficacy of immunization sparked the research of SARS-CoV-2 antiviral immunology, especially in the kinetics of neutralizing antibodies after infection and vaccination. Studies showed that neutralizing antibodies declined rapidly after immunization, and novel variants of concern (VOCs) could escape and evade humoral immune responses. With the declining levels of neutralizing antibodies, the effectiveness of vaccination was questioned. However, cellular immunity is less sensitive to a few amino acid mutations in the antigenic epitopes than humoral immunity and could protect against the new variants. There were limited studies into cellular responses following SARS-CoV-2 vaccination and infection. In addition, patients with hypogammaglobulinemia cannot produce sufficient levels of antibodies to provide protection and rely on cellular immunity for protection against severe COVID-19. Therefore, studying the kinetics of cellular responses following SARS-CoV-2 vaccination and infection was vital.

As the pandemic progressed and vaccinations became available, the mortality rate of COVID-19 decreased. However, many patients reported symptoms lasting months after the initial infection, including anosmia, ageusia, fatigue, shortness of breath, palpitations, arrhythmias, and cognitive difficulties or ‘brain fog’. At first, the medical professionals were skeptical of the condition, believing it to be mainly a psychosocial phenomenon. However, as more cases began to arise and the patient advocate groups started to lobby vocally, the research into long COVID or post-COVID-19 condition started to take interest globally. The epidemiological studies showed increased cases with specific symptoms after COVID-19, with common risk

factors for developing the condition. A dysregulated immune system causing long-term and low-grade inflammation was suggested as a pathophysiological mechanism for the condition. Later studies showed that SARS-CoV-2 RNA can be found in multiple tissues months after the acute infection. Residual RNA has been linked to tissue-based T-cell activation, potentially leading to chronic inflammation.

This dissertation assesses the role and function of the immune system in acute SARS-CoV-2 infection, COVID-19 vaccination, and post-COVID-19 condition. I showed that COVID vaccination induces strong cellular responses in immunocompetent individuals and patients with hypogammaglobulinemia. The responses are sustained by the generation of memory T cells that recognize multiple epitopes on SARS-CoV-2, possibly leading to protection against many variants. Additionally, I studied humoral immune responses and the role of inflammation and hypocortisolism in cohorts of COVID-19 inpatients and outpatients. Fortunately, during the 24-month follow-up, most patients with post-COVID-19 condition experienced alleviation of symptoms. However, for some patients, the symptoms persisted. Patients with prolonged symptoms had elevated serum antibody levels against the spike and nucleoprotein of SARS-CoV-2. Furthermore, patients experiencing myalgic or respiratory symptoms seemed to have elevated levels of proinflammatory IL-6 and hs-CRP, while patients with neurocognitive problems seemed to have lower cortisol levels. Unfortunately, the subgroup sizes at the end of the follow-up were too small for proper statistical analysis. Therefore, no conclusion can be made about the subgroups. Still, this finding may offer ideas for future studies on PCC or other post-viral syndromes.

2 Review of literature

2.1 Human coronaviruses

2.1.1 Classification

Coronaviruses are positive-sense single-stranded RNA viruses classified under the family *Coronaviridae* and order *Nidovirales,* consisting of four genera – *Alphacoronavirus, Betacoronavirus, Deltacoronavirus* and *Gammacoronavirus*. Alpha- and betacoronaviruses infect only mammals, while delta- and gammacoronaviruses infect birds but can also infect some mammals. In humans, alpha- and betacoronaviruses cause respiratory and gastrointestinal infections[1]. However, many coronaviruses have reservoirs in animals, such as bats, dogs, cats, horses, and camels, that could transmit the disease to humans via zoonotic infection[2].

So far, seven human coronaviruses (HCoVs) have been discovered: Four seasonal endemic viruses – HCoV-229E, HCoV-OC43, HCoV-HKU1, and HCoV-NL63 – cause mild respiratory disease and are responsible for 5–30% of global common cold cases. HCoVs have adapted to multiple hosts during their complex evolution[3].

In 2002, a new HCoV with a significant mortality rate causing acute respiratory failure emerged in southern China. It was named severe acute respiratory syndrome coronavirus or SARS-CoV, having a mortality rate of 10%. It was phylogenetically proven to be a distinct line in group B betacoronaviruses with origins in bats and civet cats[4]. However, the SARS epidemic was controlled with a rapid cooperative response from global medical organizations, and since the 2004 reemergence in China, no new SARS infection has been detected[5]. Ten years after SARS, a new severe betacoronavirus was isolated from a patient with acute pneumonia in Saudi Arabia, named Middle Eastern respiratory syndrome coronavirus or MERS-CoV[6]. The virus caused severe pneumonia that often progressed to acute respiratory distress syndrome (ARDS), leading to a 20–40% mortality rate. Unlike SARS, which disappeared, MERS cases have been periodically detected in endemic areas in the Middle East. Later, MERS-CoV RNA was found in bats and dromedary camels, explaining the endemic epidemiology of MERS[7].

2.1.2 Structure and replication cycle

The coronavirus genome is the largest among RNA viruses – up to 32 kb in size. The virions are round, enveloped, and range from 100–150 nm in diameter. The large surface (S) glycoprotein is a trimeric fusion protein divided into two subunits: The S1 subunit binds the virion to the cellular receptor, and the S2 subunit fuses the virion with the target cell membrane[8]. The S protein gives the virion the characteristic shape – the crown or the corona. Other structural proteins include two membrane-embedded proteins: the membrane (M) protein, needed for the viral assembly, and the envelope (E) protein, which stabilizes the virus. Some betacoronaviruses also have hemagglutinin esterase (HE), a glycoprotein that helps release the virus from infected cells[1,9].

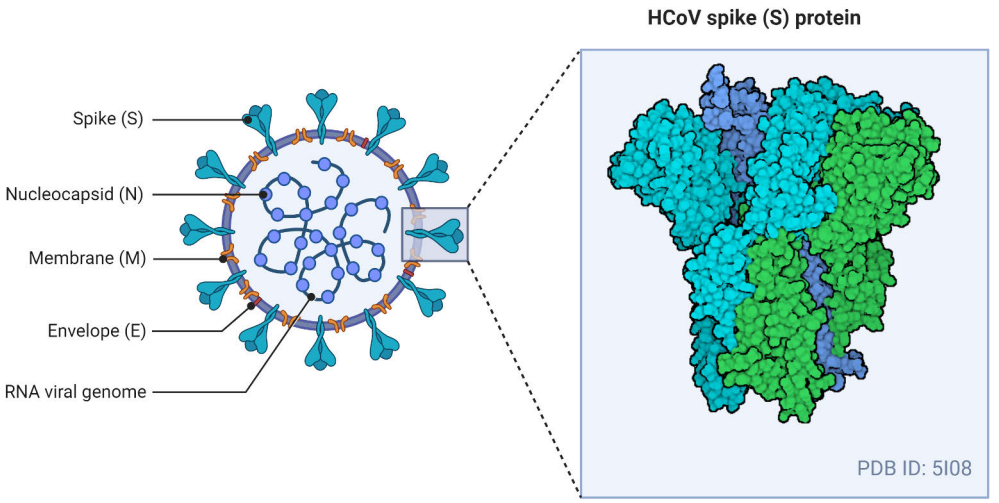

Figure 1. Coronavirus structure. Created by Jerry Gu from BioRender.com.

SARS-CoV-2 binds angiotensin-converting enzyme 2 (ACE2) on the target cell membrane with the S protein to initiate cell entry by fusing the viral and plasma membranes or by receptor-mediated endocytosis[10]. Some coronaviruses use cellular transmembrane serine proteases (TMPRSS) to prime the S protein for viral entry. In the cytoplasm, the viral RNA is translated by host mechanisms. First, a polyprotein, encoded by open reading frame 1a/b (ORF1a/b) of the viral genome, is translated and cleaved by cellular and viral proteases to produce 16 nonstructural proteins (NSPs), including RNA-dependent RNA polymerase (RdRP). The function of RdRP is to produce a negative-sense copy of the viral RNA to serve as a template for mRNA synthesis[11]. Then, the ORFs encoding the structural proteins S, E, M, and N

are assembled by host machinery and assist in budding newly synthesized virions at the Golgi apparatus to finalize the replication cycle by exiting the cell by exocytosis[9,12].

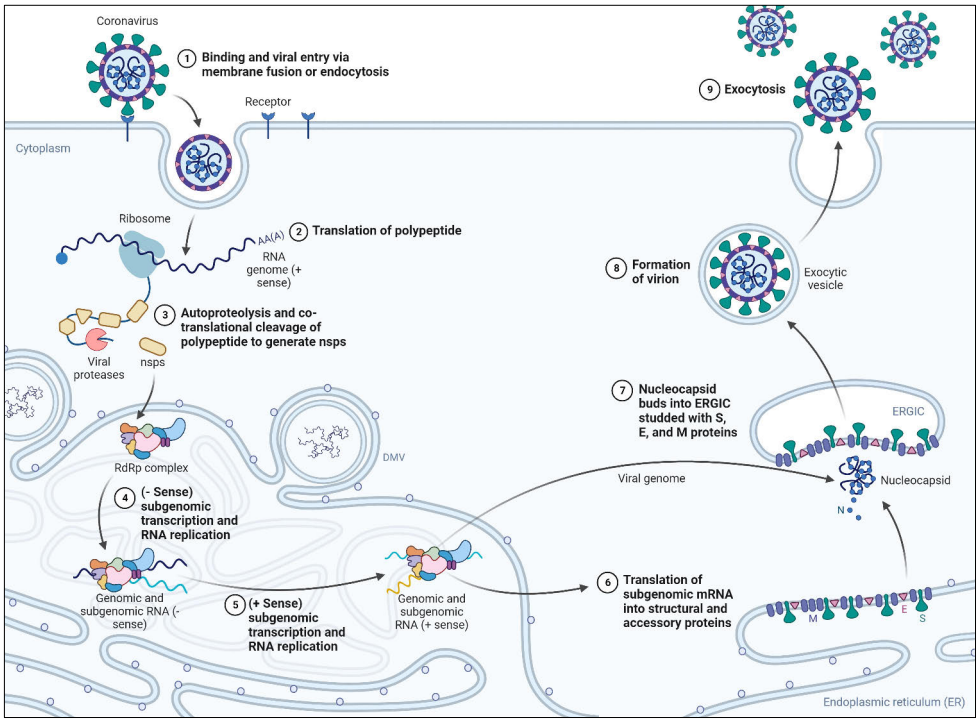

Figure 2. Coronavirus replication cycle. Created by Jessica M Tucker from BioRender.com.

2.2 COVID-19

2.2.1 Epidemiology

2.2.1.1 Worldwide

In late December 2019, a cluster of patients with pneumonia with an unknown causative agent was linked to a seafood and wet animal market in Wuhan, Hubei Province, China[13] On 10 January 2020, another outbreak of unexplained pneumonia was reported. The causative agent was determined to be a novel betacoronavirus, and epidemiological analysis revealed human-to-human transmission and confirmed the local wet market as the origin of the infection[14]. The virus was first named the 2019 novel coronavirus (2019-nCoV). After determining the sequence of the novel virus,

it was found to form a sister clade to SARS-CoVs. Therefore, the International Committee on Taxonomy of Viruses named the virus SARS-CoV-2, and the World Health Organization (WHO) named the disease Coronavirus Disease 2019 or COVID-19[15]. SARS-CoV-2 spread rapidly to all 34 provinces in China by the end of January 2019, and from late February, large clusters of infections were reported from all over the world[16]. Eventually, on 11 March 2020, the WHO declared COVID-19 as a pandemic[17].

2.2.1.2 Variants

Coronaviruses are susceptible to genetic mutations due to their large RNA genome, moderately error-prone RNA polymerase, and discontinuous style of RNA synthesis[18]. However, to stabilize replication from a catastrophic rate of point mutations, coronaviruses encode proofreading nsp14 exoribonuclease, leading to an estimated nucleotide substitution rate of 1:1000 annually[19]. This relatively stable rate of mutations allows for a high rate of recombination, insertions, and deletions, providing a source for antigenic escape and the formation of novel virus variants[20]. The evolutionary pressure for mutations in SARS-CoV-2 is highest for the receptor binding site (RBD) of the S protein – a target for the neutralizing antibodies[21].

Due to the relatively high rate of genetically diverse variants and massive generation of genomic sequence data, a practical variant classification system was generated, with the Wuhan lineage as the root sequence. Classification was based on lineages that contributed most to the spread of the virus rather than individual phylogenetic changes. Major SARS-CoV-2 lineages were denoted as A and B, further descending to numerical sublineages, such as A.1 and B.1, according to geographical areas and phylogenetic similarities with ancestral lineages. The early lineage A shared an identical genome sequence with the most recent common ancestor. However, lineage B.1 became the predominant global lineage, further evolving into multiple sublineages[22]. The rapid production of SARS-CoV-2 genome data required a new dynamic nomenclature system called Phylogenetic Assignment of Named Global Outbreak Lineages (PANGOLIN) to track global SARS-CoV-2 transmission lineages[23]. To better understand and communicate the impact of different variants circulating globally, the WHO and European Centre for Disease Prevention and Control (ECDC) characterized some of these variants as variants under monitoring (VUMs), variants of interest (VOIs), or variants of concern (VOCs) according to transmissibility and clinical significance of the variants[24]. VOCs were named by Greek letters, namely Alpha (B1.1.7), Beta (B.1.351), Delta (B.1.617.2), Gamma (P.1), and Omicron (B.1.1.529)

The Alpha variant, containing 23 amino acid mutations (most notably N501Y, P681H, and D614G), was first detected in the United Kingdom in September 2020,

becoming the main variant in Finland from January 2021 to May 2021[25]. The beta variant with 12 mutations (most notably K417N, D614G, E484K, and N501Y) was first detected in South Africa in December 2020[26]. It spread to Finland in the early spring of 2021 and caused a minor epidemic alongside the alpha variant[27]. The Delta variant, with 17 mutations (most notably K417N, L452R, T478K, E484K, E156del, and F157del), emerged in October 2020 in India[28]. It arrived in Finland in April 2021 and became the main variant in Finland from June to December 2021. It was remarkably more transmissible and caused a more severe form of COVID-19[29]. The Omicron BA.1 variant was first spotted in South Africa in late November 2021 and was first detected in Finland in early December 2021. It had multiple novel mutations in the RBD of the S protein, making it significantly more transmissible and causing an epidemic of breakthrough infections. Fortunately, however, the disease it caused was considerably mild, especially among vaccinated individuals. Omicron has further evolved into sublineages BA.2, BA.3, BA.4, and BA.5, all with multiple variable mutations and disease severity[30].

The mutated virus variants were able to evade previous immunity generated against the ancestral lineages and vaccinations and presented a considerable challenge to healthcare systems[31]. The neutralizing antibodies had reduced affinity to variants of concern, raising concerns about diminished vaccine efficiency with the possible emergence of novel variants[32].

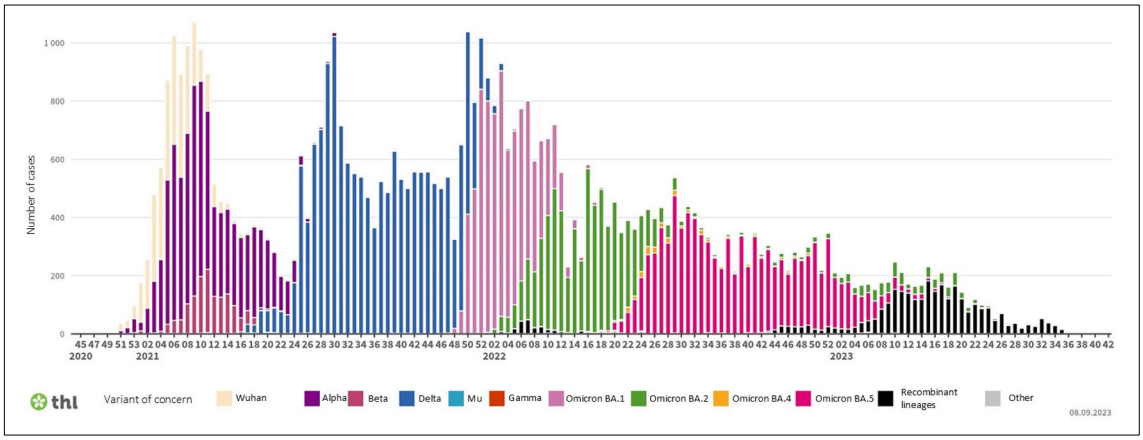

Figure 3. SARS-CoV-2 VOCs in Finland, according to the Finnish National Infectious Diseases Register. Image created by Finnish Institute of Health and Welfare (THL).

2.2.1.3 In Finland

The first reported case of SARS-CoV-2 infection in Finland was a 32-year-old Chinese tourist traveling from Wuhan to Lapland[33]. Sporadic SARS-CoV-2

infections were reported in February 2020, and the Finnish Ministry of Social Affairs and Health declared COVID-19 a generally hazardous communicable disease. In the beginning of March 2020, Finland witnessed the first wave of hospitalizations, reaching its peak on 7 April 2020[34]. Social isolation and testing were rapidly implemented, and the rate of infections started to decline. To better assess the asymptomatic spread of COVID-19 in the Finnish population, the Finnish Institute of Health and Welfare (THL) started to monitor SARS-CoV-2 RNA from untreated wastewater at the beginning of August 2020[35]. In December 2020, infections started accumulating again, and the second wave peaked on 8 December 2020.

Vaccinations in Finland started in 2021 with two mRNA vaccines (BNT162b2, mRNA-1273) and an adenoviral vector vaccine (ChAdOx1-S)[36]. In March 2021, the third wave of infections started, peaking in the beginning of April 2021. During the summer of 2021, the rate of infections stayed relatively low, and most of the population had been vaccinated at least once. In early April 2021, the Delta variant started to spread in Finland and caused a wave of infections and an increased rate of hospitalized patients in late summer 2021. The first Omicron BA.1 lineage case was detected on 2 December 2022[37]. THL started monitoring the wastewater for SARS-CoV-2 RNA, which began accumulating rapidly at the beginning of January 2022, and the fifth wave of hospitalized patients began in March 2022 with Omicron BA.2 as the primary variant[38]. The sixth wave, with Omicron BA.5 as the main variant, peaked between July and August 2022. It quickly became the main variant circulating in the Finnish population[39]. The number of hospitalized patients started declining at the beginning of 2023.

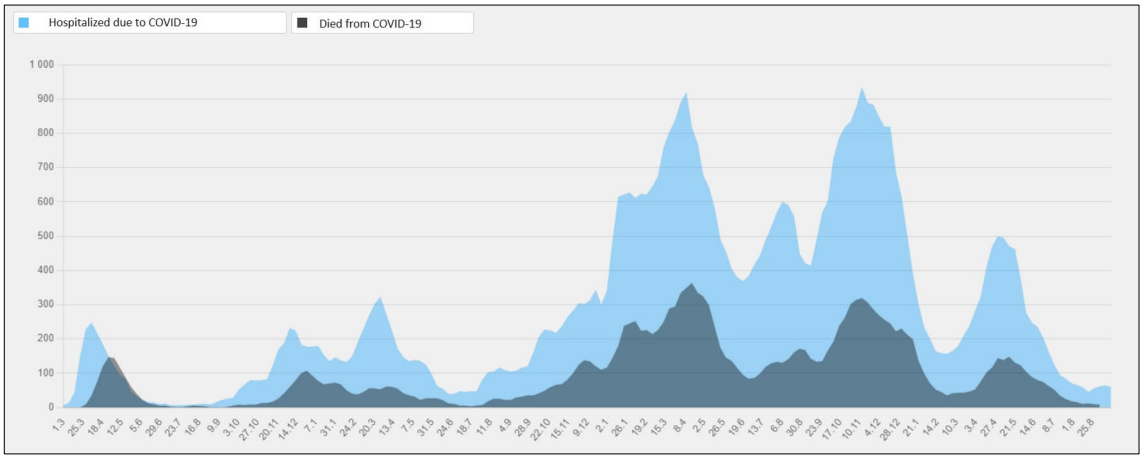

Figure 4. The number of hospitalized patients and deaths due to COVID-19 between 1 March 2020 and 25 August 2023 in Finland. Image created by THL.

2.2.2 Diagnostics

In the early stage of the pandemic, it became essential to distinguish patients with SARS-CoV-2 infection for isolation to prevent spreading of the disease. At first, diagnostic methods relied on typical symptoms, elevated blood inflammatory markers, lymphopenia, elevated serum D-dimer, and typical radiographic findings[40]. After detecting and characterizing the virus, multiple different testing methods were developed[41]. Rapid diagnostics helped prevent spreading within hospitals in the early stages of the pandemic[42]. Three main methods are still used for COVID diagnostics:

After characterizing and publishing the genome of SARS-CoV-2, a reverse transcriptase polymerase chain reaction (RT-PCR) test was created for specific diagnostics. It is a sensitive and specific test, but on the other hand, it is time-consuming, expensive, and needs laboratory space and unique instruments and skills[43]. Another method of diagnosing acute SARS-CoV-2 infection is antigen detection from a nasopharyngeal swab. Antigen tests have good sensitivity and specificity in outpatient populations and quickly became the primary testing method[44]. Immunoglobulin G (IgG) antibody response against SARS-CoV-2 S and N proteins develops after a few weeks. Therefore, enzyme immunoassays (EIAs) are designed to detect serum IgG antibodies to determine exposure or asymptomatic infection in pandemic surveillance[45].

2.2.3 Clinical features

The severity of acute SARS-CoV-2 infection ranges from mild upper respiratory infection to respiratory failure and severe multisystem disease. After a median incubation period of five days (ranging from two to 14 days), the disease presents with fever, cough, fatigue, myalgia, and dyspnea in most patients[46]. For some patients, the disease progresses to severe pneumonia and hyperinflammatory disease with multiorgan failure[47]. The most common radiographic features include bilateral consolidations, ground-glass opacities, and interlobular septal wall thickening[48]. COVID also increases the risk for thromboembolic complications, which greatly contribute to the mortality and morbidity[49].

Vaccination against SARS-CoV-2 has reduced the severe and critical forms of the disease[50]. Also, while the prevailing Omicron variant and its sub-variants are more transmissible, they have reduced disease severity compared to previous variants[51].

2.2.4 Treatment

Two main features of COVID-19 increase mortality significantly – hypoxemia and inflammation. Hypoxemia is treated with supplemental oxygen, and the severity of

hypoxemia determines the mode of oxygen treatment. Early in the pandemic, 5% of patients required intensive care, and 75% of hospitalized patients required some form of supplemental oxygen[52]. Inflammation causes arterial and venous vasculopathy, with subsequent lung thrombosis, acute cardiorenal damage, and an increased risk for secondary infection and sepsis[53]. Hemodynamic support with inotropes and careful fluid therapy is used to treat hypotension in hyperinflammation[54]. Antithrombotic prophylaxis is recommended for hospitalized patients to prevent venous thromboembolisms but has little benefit for the outpatient population[55]. Immunomodulatory medication, especially corticosteroids and anti-cytokine therapies, have been shown to reduce mortality and duration of hospitalization in COVID[56]. In addition, some antiviral drugs, such as remdesivir, nirmatrelvir-ritonavir, and molnupiravir, and monoclonal antibodies, have been accepted for the treatment of patients at risk for severe SARS-CoV-2 infection[57].

2.3 Immune responses to SARS-CoV-2 infection

2.3.1 Innate immunity

Innate immune cells cannot distinguish specific microbes but recognize pathogen-associated molecular patterns (PAMPs) and products of cellular damage (DAMPs) via their pattern recognition receptors (PRRs). Viral RNA is recognized by cytosolic Toll-like receptors (TLRs), NOD-like receptors, and RIG-like receptors (RLRs), resulting in the phosphorylation of interferon-regulatory factors (IRF) 3 and 7 or the translocation of nuclear factor kappa B (NF-κB) to induce the expression of IFNs, cytokines, or interferon-stimulated genes (ISGs)[58]. Type I interferons (IFNs) induce an antiviral state by inhibiting cellular RNA synthesis and promoting RNA degradation, increasing expression of major histocompatibility complex (MHC) class I and II molecules and activating natural killer (NK) cells and phagocytes to kill infected cells[59]. In addition, type I IFNs promote differentiation and migration of dendritic cells, leading to enhanced antigen presentation and better adaptive immune responses[60].

Cytokines are potent activators of innate and adaptive immune responses needed to control the infection. They are produced in multiple different cell types and have both pro- and anti-inflammatory roles[61]. IL-6 is a proinflammatory cytokine with diverse immunological functions. It is produced by alveolar and circulating macrophages in response to infection, ischemic injury, or trauma. In uncontrolled inflammatory responses, overproduction of IL-6 can activate a positive feedback loop through interplay with NF-κB and signal transducer and activator of transcription (STAT) 3, resulting in chronic inflammation, autoimmunity, or cytokine storm associated with severe SARS-CoV-2 infection[62]. Suppression of

Tumorigenicity (ST2) is a member of the IL-1 receptor family expressed in various cell types in exposure to stress and inflammation[63] Its soluble form (sST2) acts as a decoy receptor for IL-33, diminishing the anti-remodeling effect of the IL-33/ST2 ligand (ST2L) complex, resulting in the reduction of anti-inflammatory cytokines and the promotion of proinflammatory cytokines[64].

SARS-CoV-2 first infects respiratory epithelial cells, which detect the cytosolic viral RNA via their TLR, NLR, and RIG-I receptors. This results in the upregulation of type I and III IFNs, proinflammatory cytokines, and chemokines, aiming to control of the infection[65]. Additionally, the release of mitochondrial DNA caused by SARS-CoV-2 infection can result in the activation of the cyclic-GMP-AMP synthase (cGAS) – stimulator of interferon genes (STING) pathway resulting in immunopathology associated with acute SARS-CoV-2[66].

SARS-CoV-2 has evolved ways to evade innate immune responses by inhibiting the production of type I IFNs. This is achieved through increased expression of Orf6 and Orf9b, blocking nuclear translocation of STAT1, STAT2, and interferon regulatory factor 3, leading to inhibition of interferon-stimulated genes[67]. On the other hand, SARS-CoV-2 infection can induce a substantial production of proinflammatory cytokines, leading to a hyperinflammatory state, which, in combination with defective IFN response early in the infection, greatly increases the risk for severe disease[68].

2.3.2 Adaptive immunity

2.3.2.1 Humoral immunity

B cells play an integral part in adaptive immune response to viruses by differentiating into long-lived plasma cells that produce antibodies. The differentiation of B cells into antibody-producing plasma cells starts with recognizing the virus by the membrane-bound B cell receptor (BCR) usually in the secondary lymphoid tissues[69]. Then, B cells present the antigen in their MHC II class molecules to co-specific CD4+ T helper cells that activate the B cells to start producing more specific antibodies – a process called cross-presentation[70]. Antibodies have many different roles in protecting against viral infections, such as prohibition of entry and enhanced detection by other immune cells[71]. Antibodies are also potent activators of the classical pathway of the complement system, resulting in enhanced clearance by phagocytes, inflammation, and disruption of the microbial cell wall by membrane attack complex (MAC)[72].

Before the SARS-CoV-2 pandemic, most individuals had encountered seasonal HCoVs and had virus-specific antibodies in their sera. However, these cross-reactive antibodies did not adequately protect against SARS-CoV-2 infection or

hospitalization[73]. However, SARS-CoV-2 infection activates B cells to differentiate into antibody-producing plasma cells[74]. The formation of high-affinity antibodies in follicular centers is essential for protection against severe disease[75], while delayed production of neutralizing antibodies is associated with increased mortality in COVID-19[76]. Neutralizing antibodies against SARS-CoV-2 bind to the RBD of the S protein, inhibiting the viral entry to the cell and providing protection from symptomatic infection[77]. However, the evolved SARS-CoV-2 variants could escape most existing SARS-CoV-2 neutralizing antibodies[78].

2.3.2.2 Cellular immunity

T cells are needed to eradicate intracellular pathogens and to develop immunological memory against viruses. Briefly, cytotoxic T lymphocytes (CTLs) are needed to kill the infected cells, while T helper cells are needed to activate macrophages and CTLs and to induce B cell class-switching and affinity maturation[79].

Naïve T cells, capable of recognizing various epitopes, are generated in the thymus. They circulate to lymph nodes and other secondary lymphoid organs, where they interact with APCs, such as dendritic cells (DCs), that present foreign antigens they have acquired from the periphery. Activation of naïve T cells through their TCR leads to IL-2 production, proliferation, and differentiation to effector T cells with diverse functions. Some effector T cells survive as memory T cells that can persist and be activated by re-encountering their specific epitopes. Immune cells are categorized phenotypically according to their surface antigens, called clusters of differentiation (CDs) and chemokine receptors (CCRs).

CD69 is a membrane-bound lectin receptor, and a classical early lymphocyte activation marker mainly found in tissue-resident memory T cells[80]. CD134, or OX40, is a T cell co-stimulatory receptor and a tumor necrosis factor (TNF) receptor superfamily member. It is expressed in activated CD4+ and CD8+ lymphocytes 12-24 hours after activation[81]. CD137, or 4-1BB, is also a member of TNF receptor superfamily. It is crucial to T cell proliferation, memory formation, and effector cell functions for cytotoxicity. It is mainly expressed on activated CD8+ cells but also activated CD4+ cells within 24h hours after activation[82]. Upon activation, central memory T cells ($T_{CM;}$ $CD45RA^-$, $CCR7^+$) proliferate rapidly and home to lymphoid organs, while effector memory T cells (T_{EM}; $CD45RA^-$, $CCR7^-$) migrate to inflamed tissues and start their effector functions. Some T_{EM} cells start expressing CD45RA again differentiating into terminal effector memory cells (T_{EMRA}; $CD45RA^+$, $CCR7^-$) in response to antigen load or viral persistance[83].

A robust memory T cell response is generated against multiple epitopes after SARS-CoV-2 infection, and the disease severity correlates with the breadth and magnitude of the response[84]. Moreover, a greater CD4+ T cell response leads to

higher antibody levels. A robust CTL response, stimulated by TCR signaling or external proinflammatory stimuli (bystander activation), is needed for SARS-CoV-2 clearance. CTL response is generated within seven days of SARS-CoV-2 infection, often resulting in milder disease[85]. An early, robust bystander CD8+ activation correlated with a mild infection, while inefficient bystander responses were linked to more significant systemic inflammation and more severe disease[86].

2.3.3 Vaccination strategies against SARS-CoV-2

After determining the genetic sequence of SARS-CoV-2, the global vaccine development started at an unprecedented rate. Many different types of vaccines, such as inactivated virus, adenoviral vector, and nucleic acid-based vaccines, were trialed in a record time and shown to be effective and safe[87–90] In the end, four significant vaccines were approved and selected for immunization programs by ECDC: Two recombinant adenoviral vectors (Jcovden® or Ad26.COV2-S, and Vaxzevria® or ChAdOx1-S) and two mRNA-based vaccines (Comirnaty® or BNT162b2, and Spikevax® or mRNA-1273)[91].

However, as the virus mutated and evolved new variants, the neutralization of the novel variants was markedly reduced, leading to uncertainty of vaccine efficacy against VOCs[92,93].

2.3.3.1 Recombinant adenovirus vector vaccines

Adenoviruses are double-stranded, nonenveloped DNA viruses with a 34–43 kb genome that usually cause minor respiratory and ocular infections. Because of their safety and immunogenicity, adenoviruses have been used in gene delivery for medical and biotechnological purposes[94]. Adenoviruses provide a heat-stable and affordably producible vaccine platform, making it easier to ship and distribute vaccines around the world[95].

University of Oxford and AstraZeneca constructed a ChAdOx1 and Johnson & Johnson Ad26.COV2 adenoviral vectors with trimeric prefusion S protein as a gene of interest. They were shown to be effective and safe in the clinical trials, but unfortunately, in the middle of 2021, reports of unusual thrombotic complications halted their distribution in many countries[96].

2.3.3.2 mRNA vaccines

The common problem with many vaccines is the difficulty of delivering the target antigen for presentation without risk of infection. Nucleic acid-based mRNA vaccines are safe options for immunization since they do not have any mechanisms

to incorporate into the host genome[97]. Moreover, mRNA vaccines are fast to produce and induce strong cellular and humoral responses without adjuvants[98]. However, mRNA is quite vulnerable to heat, making these vaccines harder to store and distribute[95]. The main idea is to deliver *in vitro* transcribed mRNA inside lipid nanoparticles via intracutaneous or intramuscular injection[99]. The lipid nanoparticles containing the mRNA enter APCs by endocytosis and release the mRNA to the cytosol after escaping the endosome[100]. During this process, endosomal TLR signaling activates type 1 IFN signaling, producing a proinflammatory environment that enhances the immune response [98]. Cytosolic mRNA is then translated to protein, which will be either proteolytically degraded and presented to CD8+ T cells by MHC class I molecules or secreted extracellularly. Extracellular antigenic peptide activates B cells via BCR, is also taken in by other APCs, and is presented via MHC class II molecules to CD4+ cells. Activated CD4+ T+ cells produce inflammatory cytokines and help B cells generate maturated and class-switched antibodies against the protein[101].

Pfizer-BioNTech and Moderna developed the first authorized SARS-CoV-2 vaccines, BNT162b2 and mRNA-1273, for commercial use in the US[102]. They encode a membrane-anchored spike in prefusion conformation, which is immunogenic and safe, although some patients had severe allergic reactions[103]. After the adverse immunothrombotic events from the adenovirus-based vaccines, the main interest turned to the safe and effective mRNA vaccines, quickly becoming Europe's most popular type of COVID-19 vaccine. Most of Finnish patients

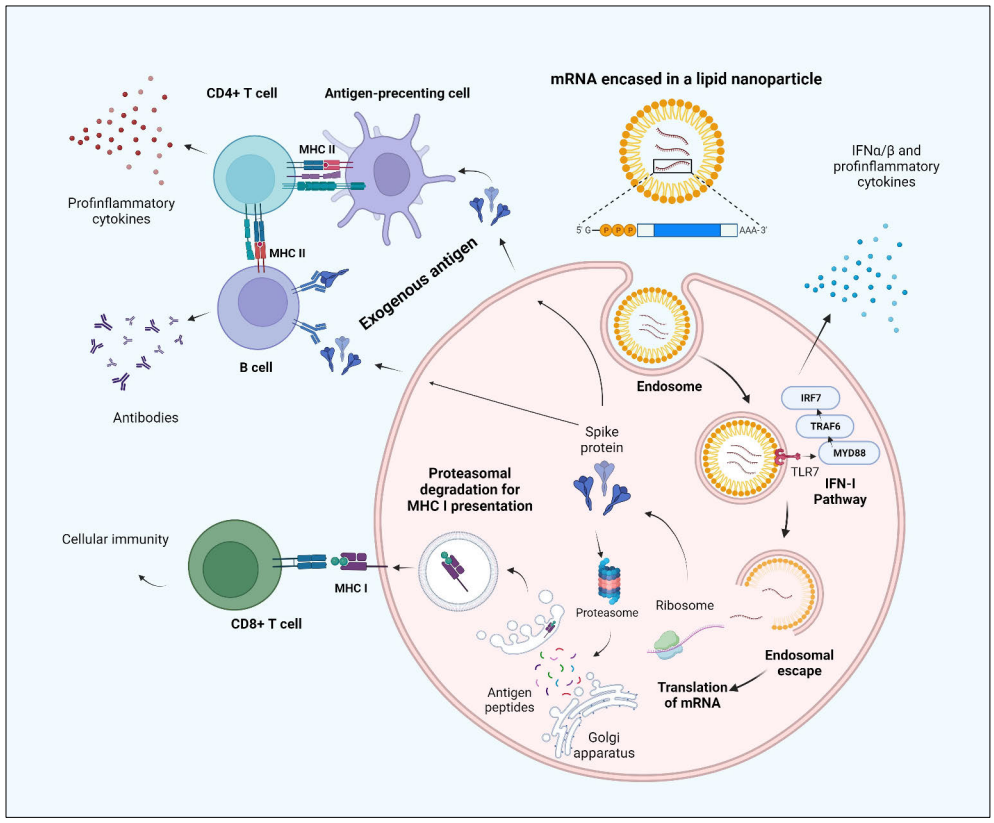

Figure 5. mRNA vaccines, mechanism of action. Created by Antti Hurme with BioRender.com.

2.4 Humoral immune deficiencies

2.4.1 Primary antibody deficiencies

Primary antibody deficiencies (PADs) result from defects in B cell development and activation that result in lower levels of immunoglobulins and reduced or missing antibody production after vaccination or infection[104]. Patients with PAD are at increased risk of invasive bacterial and viral infections, often needing longer antibiotic treatments and antibiotic prophylaxis[105]. To prevent recurrent infections, patients are given lifelong immunoglobulin replacement therapy (IGRT)[106]. Some PADs present with apparent monogenetic mutations, while some are more ambiguous in their genetic background[107]. The influence of chronic infections and defects in regulatory T cell function may lead to reduced peripheral tolerance in patients with primary immune deficiencies (PIDs), which can paradoxically lead to the development of autoimmune diseases[108].

2.4.1.1 Common variable immunodeficiency

Common variable immunodeficiency (CVID) is clinically one of the most important PADs among adolescents and young adults. It is a heterogeneous disease entity with a multifaceted genetic background. The main feature is hypogammaglobulinemia, presenting with low levels of serum IgG, decreased IgM and IgA, and reduced antibody response to vaccination[109]. Clinically, CVID is characterized not only by recurrent infections, especially with pyogenic infections such as *S. pneumoniae*, but also by autoimmune diseases and a higher incidence of malignant tumors[110]. Additionally, dysregulated B cells can increase the risk of developing interstitial lung disease and bronchiectasis with increased morbidity and mortality[111].

2.4.1.2 X-linked agammaglobulinemia

X-linked agammaglobulinemia (XLA) is a heritable hypogammaglobulinemia caused by mutations in a cytoplasmic tyrosine kinase, resulting in arrest in B cell differentiation and failure to develop B lymphocytes and plasma cells[112]. Patients with XLA are at increased risk for severe and persisting COVID, but interestingly, had better outcomes than patients with CVID, suggesting a potential role for dysfunctional B cells in disease severity[113].

2.4.1.3 Specific antibody deficiency

Patients with specific antibody deficiencies (SADs) can produce normal levels of immunoglobulins but fail to generate antibodies against polysaccharide antigens, resulting in recurrent infections with pyogenic bacteria[114]. Patients with SAD usually require recurrent immunization with *S. pneumoniae* vaccines, aggressive management of asthma and allergic rhinitis to prevent infections at sinopulmonary sites, appropriate antibiotic prophylaxis and treatment for diseases, and sometimes IGRT[115].

2.4.2 Secondary antibody deficiencies

While PADs are often a result of inherited or sporadic genetic defects in immune cell development, secondary antibody deficiencies result from external factors. Globally, malnutrition and malaria are among the leading causes of hypogammaglobulinemia. However, hematological malignancies and their treatment, especially CD20-cell depleting rituximab, contribute to most cases of secondary antibody deficiencies in the Western world[116]. Moreover, protein-losing enteropathy and nephropathy, cancer metastases in the bone marrow, and immunosuppressive agents and certain other medications can lead to secondary

hypogammaglobulinemia[117]. Patients with secondary antibody deficiencies benefit from antibiotic prophylaxis and proper antibiotic treatment during infections. However, the benefit of using IGRT for secondary antibody deficiencies is more controversial, requiring a more clinical approach to identify patients that would potentially benefit from exogenous immunoglobulin treatment[118].

2.5 Post-COVID-19 Condition (PCC)

2.5.1 Definition

Acute SARS-CoV-2 infection and its complications have been studied thoroughly since the beginning of the pandemic. Medical treatments and prophylaxis, including effective vaccination, have improved the outcomes of acute COVID-19. However, many patients report long-lasting symptoms after the initial disease, defined as "post-acute COVID-19 syndrome" or PACS[119]. WHO Delphi consensus defines the post-COVID-19 condition (PCC) as "the continuation or development of new symptoms three months after the initial SARS-CoV-2 infection, with these symptoms lasting at least two months with no other explanation. Common symptoms include fatigue, shortness of breath, and cognitive dysfunction, and generally impact everyday functioning." [120]

2.5.2 Epidemiology

In 2021, the UK Office of National Statistics (ONS) estimated the prevalence of patients experiencing at least one persisting symptom at 11.4% five weeks after SARS-CoV-2 infection, declining to 3.0% after 12 weeks. The self-diagnosed "long COVID" prevalence among SARS-CoV-2 positive individuals is 3.3% in the UK[121]. Early meta-analyses estimated the prevalence of PCC at four months to be 43–50.6% (Table 1), and risk factors for developing the condition were female sex, BMI (overweight or obese), pre-existing asthma, and the severity of acute infection[122–125]. On the other hand, a more recent meta-analysis of 54 studies from 22 countries comprising 1.2 million individuals with symptomatic SARS-CoV-2 infection estimated a significantly lower prevalence of 6.2% at three months and 0.9% at 12 months after the acute infection after considering pre-COVID health data[126]. The high variation in the estimated prevalence is likely caused by the heterogeneity of the studies, the unspecific nature of the symptoms, and the lack of standardized criteria for the condition[127]. A Finnish study of 1326 participants found PCC-associated symptoms (except for anosmia and ageusia) as prevalent among SARS-CoV-2 negative as SARS-CoV-2 positive individuals[128].

Table 1. Systematic reviews and meta-analyses on PCC.

Study	Prevalence of symptoms	Follow-up time	Most common symptoms
Montani et al. 2021	NA	3–12 months	Fatigue (40–70%) Dyspnea (5–81%) Cognitive impairment (15–40%)
O'Mahoney et al. 2021	45% overall, 52.6% hospitalized, 34.5% home-treated	1–12 months	Fatigue (17.6–57.2%) Dyspnea (13.9–29.1%) Myalgia (5.0–44.2%)
Surveillance report, ECDC 2022	50.6% overall, 66.5% hospitalized, 50.6% home-treated	3–12 months	Fatigue (21–40.6%) Weakness (28.1–34.6%) Cognitive impairment (14–45.5%)
Chen et al. 2022	43% overall, 54% hospitalized, 34% home-treated	4 months	Fatigue (17–30%), Cognitive impairment (10–19%)
Natarjan et al. 2023	NA	4–8 months	Fatigue (21.6–39.5%), Myalgia (7.5–23.7%)

2.5.3 Post-acute infection syndromes

Infectious diseases are usually resolved after the acute phase. However, some patients fail to recover after exposure to certain microbes (Table 2) and suffer for up to decades from chronic symptoms that are still poorly understood and understudied[129]. However, there is evidence of infectious diseases affecting the immune system, and infections can sometimes induce the development of autoimmune diseases[130,131]. Early childhood wheezing episodes caused by respiratory syncytial virus (RSV) or rhinovirus (RV) increase the risk of later development of asthma[132].

Table 2. Infectious organisms associated with chronic sequelae, modified from[148]

Pathogen	Name of the associated syndrome
Viral pathogens	
SARS-CoV-2	Post-COVID-19 condition (PCC) Post-acute COVID-19 syndrome (PACS) Long COVID
Chikungunya	Post-chikungunya chronic inflammatory rheumatism (pCHIK-CIR)[133] Chronic chikungunya (CHIK)[134]
Dengue	Post-infectious fatigue syndrome in Dengue[135]
Ebola	Post-Ebola syndrome (PES)[136–138]
Polio	Post-polio syndrome (PPS)[139]
SARS-CoV	Post-SARS syndrome (PSS)[140,141]
Tick-Borne encephalitis virus (TBEV)	Post-enchepalitic syndrome (PES)[142]
Coxsackie B	Post-viral syndrome[143]
Epstein-Barr virus (EBV)	ME/CFS[144]
Influenza	ME/CFS[145]
Ross River virus	ME/CFS[146]
West Nile virus	No name[147]
Other pathogens	
Coxiella burnetii	Q-fever fatigue syndrome (QFS)
Borrelia burgdorferi	Post-treatment Lyme disease syndrome (PTLDS)
Giardia lamblia	Irritable bowel syndrome after giardiasis

2.5.3.1 Encephalitis lethargica

The first remarks of unexplained post-infectious syndrome were found during the Russian flu epidemic – possibly caused by a coronavirus[149] – in 1892, as some patients reported suffering from extreme fatigue, insomnia, and mood disturbances after infection – a condition later described as *neurasthenia*[150]. In 1915–1917, French physician Jean-René Cruchet and Austrian neurologist Constantin von Economo described an epidemic of *Encephalitis lethargica* (EL) – a mysterious disease with diverse neuropathological clinical outcomes, including disturbances of alertness and extrapyramidal movement disorders and sometimes high mortality[151] After the Spanish flu pandemic in 1918–1919, caused by a highly virulent H1N1 influenza A virus, there was a cluster of EL cases throughout the world [152]. Epidemiological data, including the geographical, seasonal, and patient age distribution, suggested patient-to-patient transmission, leading to a hypothesis of influenza virus as the causative agent of EL. In addition, later analysis of the genetic structure of the H1N1 virus

suggested adaptation to human hosts before the pandemic, possibly explaining the first cluster of EL during 1915–1917[153].

Still, there is evidence against the viral hypothesis, as there are no records of influenza-like outbreaks preceding the EL pandemic, and the epidemiology of Spanish flu and EL differ in many ways[154]. However, the unreliability of clinical judgment without modern testing could explain why possibly mild infections could go undiagnosed[155]. Viral RNA could not be later extracted from the autopsy samples, and the inflammation of the central nervous system in EL was restricted to the midbrain and brainstem – not usually affected by the H1N1 influenza virus[156] In conclusion, the viral hypothesis of EL is mainly speculative and currently not supported by empirical evidence[157].

2.5.3.2 Myalgic encephalomyelitis/chronic fatigue syndrome

Myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS) is a controversial debilitating illness with unclear etiology. The diagnosis is based on medical history and excludes other distinct causes of fatigue, such as psychotic disorders, depression, dementia, or eating disorders (anorexia or bulimia nervosa). ME/CFS is characterized by chronic fatigue persisting over three months, not alleviated by rest, and accompanied by various post-exertional malaise (PEM) symptoms. Additional diagnostic criteria are unrefreshing sleep and cognitive impairment[158]. ME/CFS impacts the quality of life like many chronic severe diseases[159].

Research into ME/CFS has proposed multiple causes for the disorder, but no single explanation is sufficient. One of the adversities in researching ME/CFS is the vague diagnostic criteria and the resulting heterogeneous populations. Depending on the definition, the prevalence of ME/CFS can be anywhere between 0.006% to 3% of the global population[160]. Most studies raise the need for longitudinal studies of ME/CFS with more stringent inclusion criteria. Pooled ME/CFS populations have some distinct characteristics, however: Majority of the patients are female, unemployed, have comorbidities, and generally have lower quality of life. Infectious diseases are often associated with CFS but are not demonstratively the cause of the disorder[161]. For example, Influenza A increases the risk for chronic fatigue and impacts respiratory function and quality of life for up to 24 months after the hospitalization[145].

Autoimmunity after infections has lately been proposed as one explanation behind the condition[162]. Mitochondrial dysfunction and low ATP production in post-viral ME/CFS patients can inhibit apoptosis and stimulate necrotic cell death, presenting self- and neoepitopes to autoreactive immune cells[163]. Various autoantibodies against neurotransmitters correlate with ME/CFS[164].

2.5.3.3 Post-infectious autoimmune disorders

Microbial infections can cause the development of autoimmune diseases, possibly by activating APCs to produce high levels of costimulators and further activating autoreactive immune cells seen in mouse models[165]. Many viruses are linked to the development of autoimmune diseases. For example, Epstein-Barr virus (EBV) is suspected of reprogramming immune cells in multiple sclerosis[166]. Enteroviruses infecting the pancreas are closely linked to the development of type 1 diabetes (T1D) in children, and many of the risk genes for T1D involve the regulation of antiviral immune responses, suggesting virus-induced autoimmunity[167]. Moreover, the tissue damage in enteroviral myocarditis may also be caused by an autoimmune mechanism[168]. Dysregulated immune response to Lyme disease may also lead to autoimmune responses, and inflammation[169].

Additionally, ordinarily unexposed self-antigens can be released during an antiviral immune response, processed by APCs, and presented to autoreactive T cells[170]. Molecular mimicry is a mechanism in which microbial peptide epitopes resemble self-epitopes, leading to immune-mediated damage to self-tissue[171]. Another suspected mechanism is bystander activation, where an inflammatory environment activates self-reactive immune cells[172]. The destruction of self-tissue can lead to even more release of self-antigens, activating even more autoreactive cells – a process called epitope spreading[173].

2.5.4 Proposed mechanisms of PCC

2.5.4.1 Direct viral toxicity and cellular damage

Various pathophysiological mechanisms have been proposed for PCC. PEM after COVID-19 correlates with increased mitochondrial damage, metabolic dysfunction, and capillary amyloid composition[174]. A significant proportion of inpatients with moderate to severe COVID-19 during the first years of the pandemic had radiological abnormalities and pulmonary impairment six months after discharge[175]. During acute respiratory infection, the mesenchymal and immune cells must balance clearing the virus (resistance) and modulating the inflammatory responses to maintain or repair the vital alveolar structures (tolerance)[176,177]. Uncontrolled inflammation can substantially damage the lung and airway tissues[178]. In most severe cases, the resulting cytokine storm can lead to ARDS[179]. However, persistent symptoms after COVID-19 can be found even without radiological abnormalities or functional defects and patients with mild to moderate disease severity can also develop PCC[180,181].

2.5.4.2 Viral persistence and chronic inflammation

Viral clearance requires a robust adaptive immune response, while delayed clearance of SARS-CoV-2 is associated with severe acute disease, lower levels of naïve CD4+ cells, and higher levels of anti-SARS-CoV-2 antibodies and proinflammatory cytokines[182]. Normal antibody response against SARS-CoV-2 peaks approximately 1–2 months post-infection[183]. However, patients with PCC have persistently elevated serum levels of antibodies against SARS-CoV-2 spike (S) glycoprotein and nucleocapsid (N) protein[184].

Continued viral replication causing chronic inflammation could explain why some patients experience long-lasting symptoms even after resolving the initial infection[185]. Persisting SARS-CoV-2 RNA was found in fecal samples in 12.7% at four months and 3.8% of patients at seven months after infection[186]. Some autopsy studies have found SARS-CoV-2 RNA in multiple tissues regardless of their nasopharyngeal PCR status[187,188]. Patients with PCC have been shown to have elevated levels of intermediate and non-classical monocytes and fragments of spike protein and viral RNA compared to recovered COVID-19 patients[189]. A Chinese study found patients with PCC after mild SARS-CoV-2 infection had a higher amount of persisting viral RNA in multiple tissue samples, and a higher initial viral load was a significant risk factor for developing PCC[190]. Moreover, the persisting viral RNA in patients with PCC has been shown to correlate with tissue-based activation of T cells even two years after the initial SARS-CoV-2 infection[191].

Recently, sST2 has been shown to correlate with the long-term clinical outcome in coronary artery disease[192] and as an inflammatory marker in the severity of acute COVID-19[193]. However, sST2 kinetics have not been studied in patients with PCC. Prolonged, higher levels of sST2 could suggest sustained inflammation after COVID-19.

2.5.4.3 Immune dysregulation

Acute SARS-CoV-2 infection can dysregulate the immune system, especially CD8+ bystander cells, which may lead to inflammation and a more severe form of acute disease or persistent symptoms[194]. COVID-19 patients have been shown to have elevated serum levels of proinflammatory cytokines four months after infection and levels of IFN-β and IFN-λ1 remain elevated at eight months post-infection in patients with PCC[195]. Elevated levels of IL-6, acute phase reactants, and autoantibodies 3–8 months after the initial disease have also been linked to PCC[196,197]. Moreover, relief in PCC symptoms correlates with improved immune dysregulation at 24 months[198]. One study found that patients with PCC had higher levels of circulating non-conventional monocytes, double-negative B lymphocytes ($IgD^-CD27^-CD24^-CD38^-$)

and anti-S1 and anti-RBD antibodies, and lower levels of circulating T_{CM} cells than non-PCC COVID-19 patients or negative controls[184].

2.5.4.4 Autoimmunity

At the beginning of the pandemic, clinicians noticed an increase in autoimmune-like diseases, such as Multisystem inflammatory syndrome in children (MIS-C) in COVID-19 patients[199]. COVID-19 infection also increases the risk of autoinflammatory connective tissue disorders[200]. Moreover, vaccination against SARS-CoV-2 could increase the risk of developing autoimmune diseases[201]. Multiple studies have shown elevated levels of autoantibodies and inflammatory biomarkers, such as IFN-γ, CRP, and IL-6, correlate with the production of autoantibodies three months after COVID-19[196,202].

2.5.4.5 Hypocortisolism

Hypocortisolism shares many symptoms with PCC: postural hypotension, profound fatigue, myalgia, and abdominal pain. Corticosteroids are recommended for patients with severe COVID-19 as they reduce mortality by reducing the effect of inflammation, especially in patients with ARDS[203]. The use of corticosteroids can activate the negative feedback loop to cause iatrogenic hypocortisolism after ending the corticosteroid treatment[204]. Additionally, SARS-CoV-2 can directly infect adrenal and thyroid cells because of the expression of ACE2 and TMPRSS[10]. Thus, adrenal insufficiency could explain long-term symptoms after SARS-CoV-2 infection.

A Japanese study of 186 patients and an American study of 275 patients found lower cortisol levels to be a predictor for developing PCC[184,205]. However, these studies were criticized for not accounting for oscillations in physiological cortisol production, and other studies have not been able to replicate the findings[206].

3 Aims

This thesis aimed to investigate immunological responses to COVID-19 vaccination and infection and the role of immune system in the post-COVID-19 condition.

The aims of individual studies in this thesis were the following:

I. To describe the cellular and humoral responses to vaccination with COVID-19 mRNA vaccine BNT162b2.

II. To investigate SARS-CoV-2 vaccine-induced immune responses in patients with hypogammaglobulinemia compared to healthy vaccinees.

III. To analyze serum inflammatory markers, anti-SARS-CoV-2 antibodies, and cortisol levels in a cohort of inpatients and outpatients with post-COVID-19 condition (PCC).

4 Materials and methods

4.1 Ethics

All studies adhered to the ethical principles of the Declaration of Helsinki and Good clinical practice. All patients provided written informed consent, and the ethics committees of the Southwest Finland Health District and the Helsinki-Uusimaa Health District approved the study protocols. The permission numbers were ETMK 19/1801/2020 and EudraCT 2021-004419-14 for the Turku University Central Hospital (TYKS) HCW vaccinees (I-II), ETMK 89/1800/2021 and EudraCT 2021-004891-33 for immunocompromised patients (II), and HUS/1238/2020 and EudraCT 2021-004016-26 for immunocompetent patients and negative controls (I, III)

4.2 Study participants

4.2.1 Immunocompetent COVID-19 patients (I, III)

In studies I and III, immunocompetent patients with PCR-confirmed SARS-CoV-2 infection were recruited from TYKS and Turku City Health Stations. In the acute phase (up to 14 days after symptom onset), we collected serum samples from the inpatients, and in the convalescent phase (later than 14 days after symptom onset), we collected serum and whole blood samples from inpatients and outpatients.

Study I included 15 inpatients from TYKS, aged 32–78 (mean 53 years; six females and nine males). Study III included 62 inpatients from TYKS, aged 25–79 (mean 55 years; 26 females and 40 males) and 53 outpatients aged 21–63 (mean 34 years; 33 females and 20 males). The patients were recruited from March 21 to December 13, 2021, and enrolled in two one-month periods in March and December.

4.2.2 Immunodeficient COVID-19 patients (II)

In study II, we recruited 31 patients with PAD and secondary hypogammaglobulinemia (SHG) treated and followed up at TYKS. Enrollment for the study started in January 2020. The diagnosis of CVID and XLA was based on

international diagnostic criteria[207]. Almost all patients (30/31) were treated with regular IGRT.

4.2.3 COVID-19 vaccinees (I–II)

In studies I-II, healthcare workers (HCWs) from TYKS vaccinated with the BNT162b2 mRNA vaccine were recruited to the study. Vaccinees received two vaccine doses at a three-week interval through their occupational health care.

Study I included 23 HCWs from a larger cohort of vaccinated HCWs. The participants were aged 26–60 (mean 39 years; 20 females and three males). Study II included 10 HCWs aged 27–58 (mean 42 years; all females).

4.2.4 Uninfected unvaccinated individuals (I)

In study I, whole blood and serum samples were collected from 13 unvaccinated people with no previous SARS-CoV-2 infection for negative control samples.

4.3 Methods

4.3.1 PBMC isolation (I–II)

In studies I and II, peripheral whole blood was collected in lithium-heparin vials. PBMCs were isolated with Ficoll-Paque PLUS (GE Healthcare, USA) density gradient centrifugation according to the manufacturer's instructions. The viable cells were counted using a TC20 automated cell counter with trypan blue dye (BioRad, USA). The isolated cells were gradually frozen to -135°C by suspending them in freezing medium containing 10% dimethyl sulfoxide (DMSO) and 10% human AB serum (Sigma-Aldrich, USA).

4.3.2 Optimization series for stimulation culture (I)

Before analyzing the samples from patients and vaccinees, we optimized the T cell stimulation protocol for cellular concentration, resting time after thawing, and peptide stimulation time. First, we optimized the cellular concentration and resting time after thawing. PBMCs from three HCWs vaccinated 3–6 months prior to sampling were allowed to rest for 4h, 8h, 16h, or 24h after thawing, followed by peptide stimulation at final concentrations of 0.5, 1, 2, 5, or 10×10^6 cells/200µl. Then, we optimized the peptide stimulation time. PBMCs from five HCWs vaccinated 3–9 months prior to sampling were rested for the optimal time of 8h and then incubated with stimulant peptides for 4, 8, 16, 24, 48, or 72h. T cell activation-

induced markers (AIM) were analyzed with flow cytometry, and the expression of intracellular IFN-γ, IL-2, TNF-α, and IL-4 mRNA was measured with quantitative reverse transcriptase polymerase chain reaction (RT-qPCR).

Based on the flow cytometry and RT-qPCR data, we determined the optimal conditions for stimulation culture to be (1) a resting time of 8h, (2) a cellular concentration of 1x106 cells/200µl, and (3) a stimulation time of 48h.

4.3.3 RT-qPCR

Total RNA was isolated from PBMCs using the RNeasy Mini kit (Qiagen) or MagnaPure 96 Cellular RNA Large Volume Kit (Roche) according to the manufacturer's protocols. For amplification and quantitation, 5 µl of purified RNA was used in One Step PrimeScript III RT-qPCR Kit (Takara Bio Inc) with predesigned TaqMan FAM-MGB IFN-γ (Hs00989291_m1), IL-2 (Hs00174114_m1), IL-4 (Hs00174122_m1), TNF-α (Hs00174128_m1) and β-actin (Hs01060665_g1) primer/probe sets (Thermo Fisher Scientific) in Rotor-Gene Q (Qiagen). The conditions for the RT-qPCR thermal cycling were the following: One reverse transcription cycle at 55°C for 10 min and 95°C for 10 sec, followed by 45 amplification cycles at 95°C for 5 sec and 58°C for 30 sec. The relative fold changes of the target genes were obtained with the $2^{-\Delta\Delta Ct}$ method by using β-actin Ct-values for normalization and a DMSO-treated sample as the control.

4.3.4 Cell culture and stimulation

To avoid the toxic effect of DMSO after cryopreservation, PBMCs were quickly thawed and washed with culture media RPMI (Gibco) containing 10% human AB serum, two mM L-glutamine, and penicillin/streptomycin. PBMCs were counted and allowed to rest in a 5×10^6 cells/ml concentration for 8h at 37°C and 5% CO_2. After resting, the cells were pelleted and resuspended to a fresh culture medium.

To activate the SARS-CoV-2-specific T cells, 1×10^6 viable cells in a 100µl culture media were plated into a 96-well U-bottom plate (Thermo Fisher, USA) well. The cells were stimulated with commercially produced peptide pools diluted to 1ml of DMSO containing the whole SARS-CoV-2 spike protein as 15mers with 11mer overlap (PepMix™, JPT Peptide Technologies, Germany, described in Table 3) at a concentration of 0.5 µg/ml in a culture media for 48h at 37°C and 5% CO_2. Purified tetanus toxoid (AJ Vaccines, Denmark) at a concentration of 10 µg/ml was used as a positive control, and 0.4% of DMSO in culture media was used as a negative control. The final volume of media during stimulations was 200 µl per well.

Table 3. SARS-CoV-2 spike peptide pools (PepMix™, JPT Peptide Technologies) used in T cell stimulations.

SARS-CoV-2 pango lineage (WHO label)	Cat#	Number of amino acid changes
B	PM-WCPV-S-1	0
B.1.1.7 (ALPHA)	PM-SARS2-SMUT01-1	10
B1.351 (BETA)	PM-SARS2-SMUT02-1	10
P.1 (GAMMA)	PM-SARS2-SMUT03-1	12
B.1.617.2 (DELTA)	PM-SARS2-SMUT06-1	10

4.3.5 Flow cytometry (I–II)

In studies I and II, stimulated PBMCs were washed with phosphate-buffered saline (PBS), and dead cells were stained with 1:1000 diluted Zombie Green dye (Biolegend, US) in the dark for 15 minutes at room temperature. Afterward, the cells were washed with PBS containing 2% fetal bovine serum (FACS buffer) and stained with fluorochrome-labeled anti-human antibodies recognizing cell surface markers CD45, CD3, CD4, CD8, CD69, CD134, and CD137 (Table 4). PBMCs were incubated with the antibodies for 30 minutes at +4°C in the dark, washed with FACS buffer, and resuspended in 200 µl of PBS.

Table 4. Fluorochrome-labeled antibodies used in FACS.

Antibody	Fluorochrome	Manufacturer	Cat#
Anti-human CD45	APC-eFluor780	Invitrogen/Life technologies	47-0459-42
Anti-human CD3	eFluor506	Invitrogen/Life technologies	69-0038-42
Anti-human CD4	eFluor450	Invitrogen/Life technologies	48-0049-42
Anti-human CD8a	PerCP-eFluor710	Invitrogen/Life technologies	46-0087-42
Anti-human CD69	PE	BD Biosciences	555531
Anti-human CD134	PE/Cyanine7	BioLegend	350012
Anti-human CD137	APC	BioLegend	309810

T cell subtypes were characterized with a NovoCyte Quanteon Flow Cytometer (Agilent Technologies Inc, US) and analyzed with NovoExpress v1.5.9 (Agilent Technologies Inc, USA). Cell gating was done manually from the main population of samples treated with DMSO. Identical gating was used for each respective cell population stimulated with tetanus or peptide pools.

Samples that had cell populations with a lymphocyte percentage of less than 4% in the forward vs side scatter (FSC vs SSC) gating, CD3 cell count lower than 10000, CD4 or CD8 cell count lower than 5000, or total events fewer than 100000 were excluded from the analysis. Missing CD4+ cell response (SI < 4) to tetanus toxoid also excluded the sample from further analysis.

4.3.6 Enzyme immunoassay (I–III)

In all studies, antibodies against SARS-CoV-2 spike protein subunit 1 (S1) and nucleocapsid protein (N) were measured using an in-house enzyme immunoassay (EIA). Antibody dilutions were selected based on previous research[208]. 96-well microtiter plates coated with N or S1 protein were incubated with a serum sample diluted to 1:300 in PBS supplemented with 5% swine serum (Biological Industries, Israel) and 0.01% Tween-20.

In studies I-II, SARS-COV-2 specific IgG antibodies were detected with HRP-conjugated anti-human IgG (1:8000 dilution; Dako A/S, Denmark) and total IgG (1:20000 dilution; Abcam, UK). In study III, SARS-CoV-2 specific IgA, IgM, and IgG antibodies were detected with HRP-conjugated anti-human IgA (1:8000, Dako A/S, Denmark), IgM (1:4000, Dako A/S, Denmark) and IgG (1:8000, Dako A/S, Denmark) antibodies. Absorbance values were measured at 450 nm wavelength using TMB One (Kementec Solutions A/S, Denmark) as a substrate. Optical density values were converted to EIA units with linear interpolation between negative (0 EIA units) and positive (100 EIA units) control samples.

4.3.7 Surveys and clinical data

In studies II and III, relevant clinical data, including medical history, prior medication, age, sex, comorbidities, and treatment during hospitalization, were collected from the electronic health records (EHRs) of TYKS (Uranus™, CGI, Canada). This included the duration of stay in hospital wards and intensive care unit, modality of supplementary oxygen therapy (nasal cannula, high-flow nasal oxygen therapy, non-invasive ventilation, and invasive mechanical ventilation), pharmacological treatment (antiviral therapy, corticosteroids, antibiotics, low-molecular-weight heparin), the severity of the acute disease, and complications during hospitalization.

Based on the symptoms and need for medical attention, patients were divided into four disease severity clusters: (1) Asymptomatic or very mild disease group had positive PCR result found in screening or very mild flu-like symptoms (mild cough, runny nose, sore throat), (2) mildly symptomatic group had fever over 38°C mild dyspnea, severe fatigue, and myalgia with difficult cough, (3) moderately

symptomatic group had dyspnea with native blood oxygen saturation less than 95% on admission requiring hospitalization less than 7 days and supplemental oxygen, but not needing intensive respiratory support, and (4) severely symptomatic group needed intensive respiratory support (nasal high-flow cannula, non-invasive ventilation or respirator), had ARDS or needed intensive care or hospitalization over 7 days.

In study III, no validated questionnaire was available to assess PCC symptoms during the study design. Therefore, we designed a questionnaire based on available evidence and clinical experience to evaluate the symptoms and detailed medical history. Patients were asked about their smoking status, use of alcohol, and initial recovery after the acute SARS-CoV-2 infection.

The patients filled in the online questionnaires three months, six months, one year, and two years after the acute infection. These self-reported symptoms were assessed using a numerical rating scale (NRS) from 0 to 10 on the questions with verbal descriptors. At three months post-infection, patients were also asked retrospectively to score the severity of symptoms before and during COVID-19. For statistical analysis, an increase of at least four points on the NRS compared to the pre-infection situation was defined as a potential PCC symptom to be evaluated by experts.

4.3.8 Physiological testing (III)

In study III, patients performed the 6MWT, spirometry, and orthostatic tests at the clinical research clinic, TYKS, three and six months after the acute disease.

The 6MWT was performed adhering to the European Respiratory Society guidelines[209]. Briefly, patients were asked to walk along a 50-meter-long, flat, straight, and hard surface. The ends of the course were marked visibly for patients to see. A trained study nurse supervised the test and encouraged the patient every 60 seconds. Patients were allowed to rest during the test, but the timer was not stopped, and the study nurse encouraged the patients to continue walking. The test was terminated after six minutes, and walking distance (6MWD) was recorded. Oxygen saturation was measured with a pulse oximeter, and dyspnea was assessed with a Borg dyspnea scale at rest, after three minutes, and after six minutes of starting the test.

Spirometry was performed using a Medikro Pro™ spirometer (Medikro, Finland) under the supervision of a trained study nurse. The study nurse assessed the quality of inhalation each time, and the test was repeated if needed. The spirometry data were analyzed using Medikro Spirometry Software v4.9.0 (Medikro, Finland), and the values were compared to age-, sex-, and height-adjusted reference values[210].

Orthostatic hypotension was evaluated using the orthostatic challenge test, adhering to the European Society of Cardiology (ESC) guidelines[211]In short, patients were asked to lie supine for five minutes before measuring blood pressure with an automatic arm-cuff device. Then, they were asked to change to an upright position, and the blood pressure was measured directly after and two minutes after standing up. An abnormal drop in blood pressure was defined as a decline of more than 20 mmHg in systolic or ten mmHg in diastolic blood pressure or a fall in systolic blood pressure to under 90 mmHg after standing up.

4.3.9 Chest computed tomography (III)

In study III, to assess the lung parenchymal changes caused by the SARS-CoV-2 infection, high-resolution computed tomography (HRCT) of the lungs was taken on a clinical ground from 24/38 (63%) inpatients and 27/48 (56%) outpatients at three months post-infection. In 4/38 (11%) inpatients and 1/48 (2%) outpatients, HRCT was repeated for clinical indication at the six-month follow-up.

Images were taken with a Revolution Apex CT scanner (GE Medical Systems, USA) in a supine position. HCRT protocol with a scan thickness of 0.62 mm and lung windowing was used in expiration and inspiration. Images were reconstructed using the True Fidelity deep learning image reconstruction algorithm (GE Medical Systems, USA), and the images were analyzed by two experienced thoracic radiologists using the Vue PACS image management system (Philips, Netherlands). Evaluation of post-COVID changes was based on previously reported residual lung alterations after SARS-CoV-2 infection[212].

4.3.10 Diagnosis and clustering of PCC

Four study physicians (AH, AV, JO, TF) independently determined the diagnosis of PCC by evaluating the severity of symptoms and possible explanations by other clinical conditions, following the WHO case definition[120]. Patients were categorized into three PCC clusters corresponding to their most dominant self-reported symptoms using the Global Burden of Disease Long COVID Collaborators guidelines[126]. Briefly, persistent fatigue with myalgia or mood swings (cluster 1) included bodily pain (myalgia), weakened muscular strength, numbness and tingling, fatigue, and mood disturbances. Cognitive problems (cluster 2) included attention deficit and mental difficulties, and ongoing respiratory problems (cluster 3) included exertional and resting dyspnea, persistent cough, and thoracic pain. In case of discordance between the experts, the cases were discussed to reach an agreement on the possible existence and categorization of PCC.

4.3.11 Statistical analysis

In studies I and II, data were analyzed with GraphPad Prism (version 8). T cell activity was described using stimulation index (SI), i.e., the ratio of the AIM+ cells after spike peptide pool stimulation to AIM+ cells after DMSO stimulation. If the percentage of AIM+ cells after DMSO stimulation was 0, the SI was marked as the smallest value of the participant. Paired samples with three or more pairs were tested with the Wilcoxon signed-rank test, and unpaired samples were tested with the Mann-Whitney U-test. All tests were two-sided, and p-values <0.05 were considered statistically significant. Correlations were analyzed using Spearman's correlation test.

In study III, data were collected and managed using REDCap electronic data capture tools hosted at Turku University. Continuous variables were summarized with median and interquartile range (lower quartile-upper quartile) due to skewness of data distributions. Categorical variables were reported with counts and percentages. Associations of explanatory variables with PCC were assessed in age- and sex-adjusted logistic regression models and reported in odds ratios (OR) with 95% confidence intervals (CI). The data analysis was generated using SAS software, Version 9.4 of the SAS System for Windows (SAS Institute Inc., Cary, NC, USA). GraphPad Prism version 10.0 (GraphPad Software, USA) was used to analyze EIA data for a four-parameter logistic (4PL) curve model.

5 Results

5.1 Study I

5.1.1 Long-lasting cellular immune responses to COVID vaccination

In study I, we evaluated T cell responses to COVID-19 mRNA vaccination in healthy individuals six months after immunization with two doses of the BNT162b2 mRNA vaccine. We used the AIM flow cytometry assay and measured the levels of cytokines (IL-2, IFN-γ, and TNF-α) and the effector molecule perforin secreted by the activated T cells and other PBMCs and compared the responses to those of COVID-19 convalescent patients and uninfected unvaccinated individuals.

5.1.2 T cell activation after SARS-CoV-2 vaccination among healthy vaccinees and COVID-19 convalescent patients

After activation with the SARS-CoV-2 spike peptide pool, the number of activated CD4+ and CD8+ cells was measured with an 8-color flow cytometry panel. SARS-CoV-2 specific CD4+ cell activation was determined as simultaneous expressions of cellular surface markers CD69 and CD134, and CD8+ cell activation was determined as simultaneous expressions of CD134 and CD137. The highest measured response in the negative control group was defined as a threshold limit.

SARS-CoV-2 specific CD4+ responses (determined as SI > 1.23) to wild-type SARS-CoV-2 peptide pool (Wuhan-Hu-1) were detected in all vaccinees at six weeks (n=20), three months (n=15) and six months (n=17) after the first vaccine dose (Figure 6A). CD8+ responses were detected in 70% of vaccinees at six weeks, 67% at three months, and 53% at six months after the first immunization (Figure 6B). The mean SI values were 8.6–9.5 for CD4+ cells and 1.6–2.6 for CD8+ cells. There was no statistically significant difference in T-cell activation between vaccinated individuals and COVID-19 patients.

To measure the sensitivity of T cells to novel SARS-CoV-2 variants, some samples were also activated with peptide pools derived from the genetic sequence of

Alpha, Beta, Delta, and Gamma variants of SARS-CoV-2. CD4+ responses against all variant peptides were detected in 71% of vaccinees with a mean SI of 7.0–10.0. No significant differences in CD4+ cell activation existed between variant peptide stimulations, except between Alpha and Beta variant peptides ($p<0.01$) six months after vaccination. SARS-CoV-2 specific CD8+ cells were activated in over 50% of vaccinees. Statistically significant differences were measured between the Wuhan-Hu1 and Gamma variant six weeks after vaccination and the Wuhan-Hu-1 and Beta variant six months after vaccination ($p<0.01$). Otherwise, there were no differences in variant peptide stimulations.

To conclude, we showed that BNT162b2 mRNA vaccination generated SARS-CoV-2-specific CD4+ and CD8+ effector cells, which could be activated up to six months after vaccination. The intensity of the response was equal to that generated by SARS-CoV-2 infection.

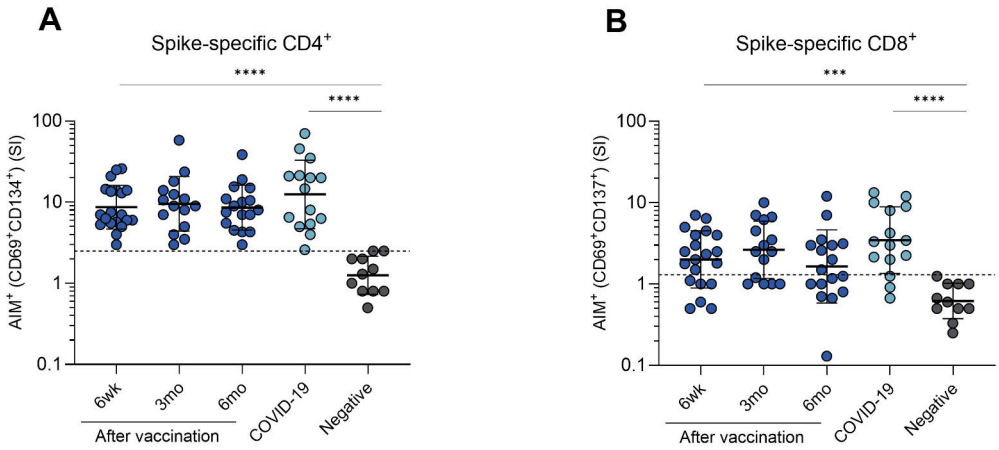

Figure 6. T cell responses against SARS-CoV-2 spike peptides **A.** CD4+ responses defined by the expression of CD69+ and CD134+ and **B.** CD8+ T cell responses defined by the expression of CD69+ and CD137+. The values are represented as stimulation indices, with the highest value of negative samples as the threshold limit. Paired samples were analyzed using the Wilcoxon signed-rank test, and samples with no data on either point were excluded from the analysis. *$p < 0.05$.

5.1.3 Detection of cytokines and effector molecules secreted by stimulated T cells and other PBMCs

Following stimulation with SARS-CoV-2 spike peptide pools, the activated PBMCs produce cytokines and effector molecules to enhance the inflammatory response and proliferation of T cells. The levels of secreted cytokines (IL-2, TNF-α, and IFN-γ) and the effector molecule perforin were measured from the sample supernatants.

The levels of IL-2 and IFN-γ were elevated in vaccinees (329 pg/ml at three weeks, 278 pg/ml at three months, and 378 pg/ml at six months after vaccination) and COVID-19 patients (539 pg/ml) compared to negative controls (29 pg/ml, $p<0.001$). Levels of IFN-γ correlated highly to T cell activation (for CD4+: $r=0.47$, $p=0.0003$ and for CD8+: $r=0.58$, $p<0.0001$), while IL-2 showed less correlation with the activation (for CD4+: $r=0.24$, $p=0.11$ and for CD8+: $r=0.55$, $p<0.0001$) confirming cell-mediated responses to SARS-CoV-2 peptides (Figure 7). An increase in the production of TNF-α and perforin was detected in most COVID-19 patients but only in a few vaccinated individuals. However, the baseline median levels of perforin and TNF-α were high in the samples stimulated with DMSO (perforin 1187 pg/ml and TNF-α 441pg/ml) and negative control samples (perforin 1095 pg/ml and TNF-α 1284) compared to IL-2 and IFN-γ. Furthermore, the measurement of TNF-α was unsuccessful in many samples due to an unexplained aggregation of Luminex beads.

In conclusion, we established that the cellular response generated by BNT162b2 mRNA vaccination was functionally active, producing IFN-γ and IL-2. Furthermore, there were no significant differences in the responses to different variants.

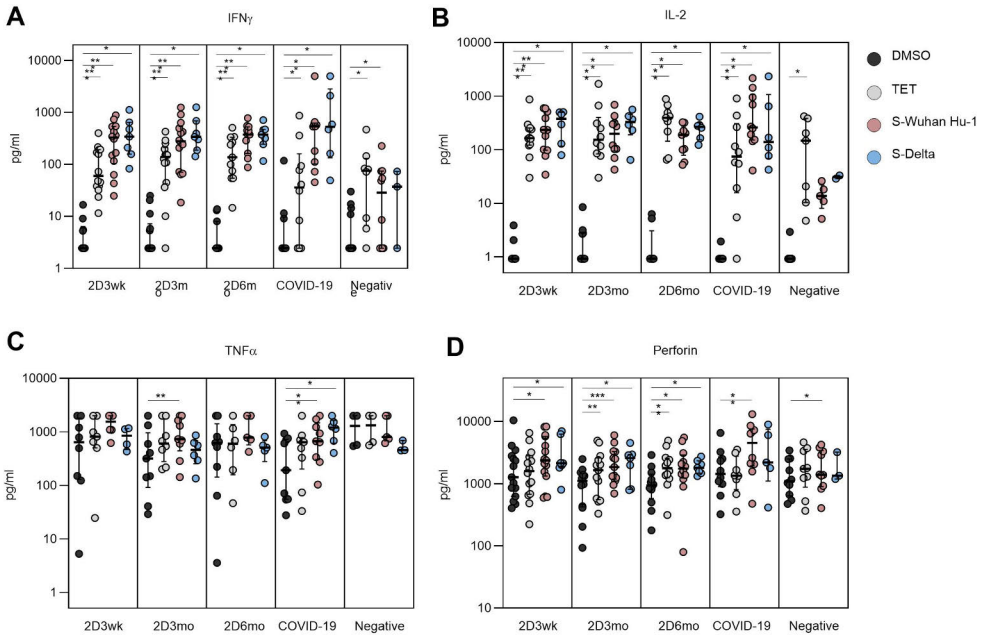

Figure 7. Secretion of IFN-γ, IL-2, TNF-α, and perforin in samples stimulated with tetanus toxoid and peptide pools of S1 subunit of the Wuhan-Hu 1 strain and Delta variant. **A.** Levels of secreted IFN-γ, **B.** IL-2, **C.** TNF-α, and **D.** perforin in supernatants of stimulated PBMCs. Data is represented as median and interquartile range. Wilcoxon signed-rank test was used for statistical analysis. Samples with no data on both data points were excluded from the analysis. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

5.2 Study II

5.2.1 Humoral responses to SARS-CoV-2 vaccination in adult patients with hypogammaglobulinemia

Antibodies against SARS-CoV-2 S1 and N proteins were measured from serum samples from 31 patients with hypogammaglobulinemia and ten immunocompetent HCWs. After the first vaccination, no patient with hypogammaglobulinemia had antibodies against the S1 protein. However, three weeks after the second vaccination, six (24% of samples) patients (three CVID, one SAD, one IgG subclass deficiency [ScD], and one SHG) had measurable levels of anti-S1 antibodies (Figure 8). Furthermore, three months after the second dose, anti-S1 antibodies were still detected in only six (21% of samples) patients (four CVID, one XLA, two SAD, one IgG ScD, and one SHG). Meanwhile, all tested HCWs had high levels (range 65–150 EIA units) of anti-S1 antibodies three weeks and three months after the second vaccination.

After the third and fourth vaccinations, anti-S1 antibodies were detected in all patient groups. Anti-S1 antibodies were detected in 15/21 (71%) (11 CVID, one XLA, one SAD, and one SHG) of the patients three weeks after the third vaccination, and in 14/21 (67%) (10 CVID, one XLA, two SAD, one IgG ScD, and one SHG) of the patients three months after the third vaccination. Three weeks and three months after the fourth vaccination, 14/19 (74%) (11 CVID, one XLA, one IgG ScD, and one SHG) and 11/12 (92%) (nine CVID, one XLA, and one SHG) of the patients had detectable levels of S1 antibodies.

Interestingly, most patients receiving subcutaneous IGRT (81%) had detectable levels of anti-S1 antibodies, while only 43% of patients on intravenous IGRT had detectable anti-S1 antibodies. All in all, we showed that many patients had measurable serum levels of antibodies against SARS-CoV-2. However, we could not differentiate whether the antibodies were generated by B cell response to the vaccination or passively obtained from the IGRT products.

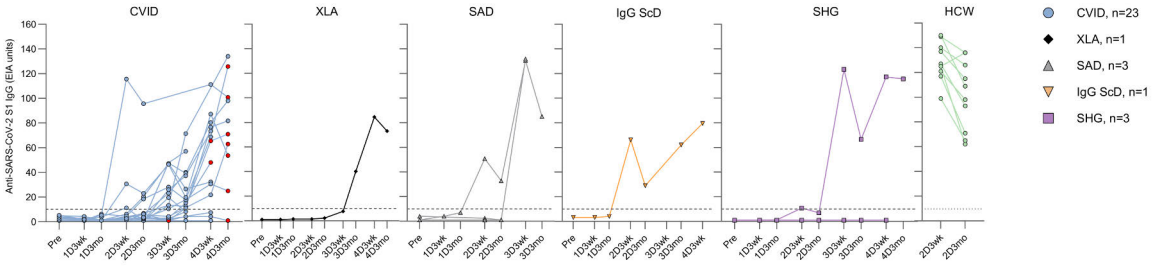

Figure 8. Anti-SARS-CoV-2 S1-specific antibody responses by different types of hypogammaglobulinemia (CVID, XLA, SAD, IgG ScD, and SHG) and HCWs in samples collected before vaccination (Pre) and three weeks and three months after each immunization. Red dots represent samples collected after SARS-CoV-2 infection.

5.2.2 Activation of spike-specific T cells in patients with hypogammaglobulinemia

SARS-CoV-2-specific CD4+ and CD8+ T cell responses were measured using an AIM assay with peptide pools covering the whole S protein of the Wuhan-Hu1 strain, Delta variant, and then circulating Omicron BA.2 variant as stimulants. Similarly, as in study I, CD4+ activation was determined as simultaneous expression of CD69 and CD134, while CD8+ activation was determined as simultaneous expression of CD134 and CD137.

Despite having little to no humoral response after the first and second vaccine doses, CD4+ responses were detected in 12/23 (52%) of patients three weeks and 15/23 (65%) three months after the first vaccine dose (Figure 9A). However, CD8+ responses were detected only in 9/23 (39%) patients three weeks and 11/22 (50%) three months after the first vaccine dose (Figure 9B). Furthermore, CD4+ responses were detected in almost all patients three weeks after the second vaccine dose, and CD8+ response was present in 14/19 (74%) and 16/25 (64%) of the patients three weeks and three months after the second vaccination. The cellular responses in CVID patients were like those of immunocompetent HCWs.

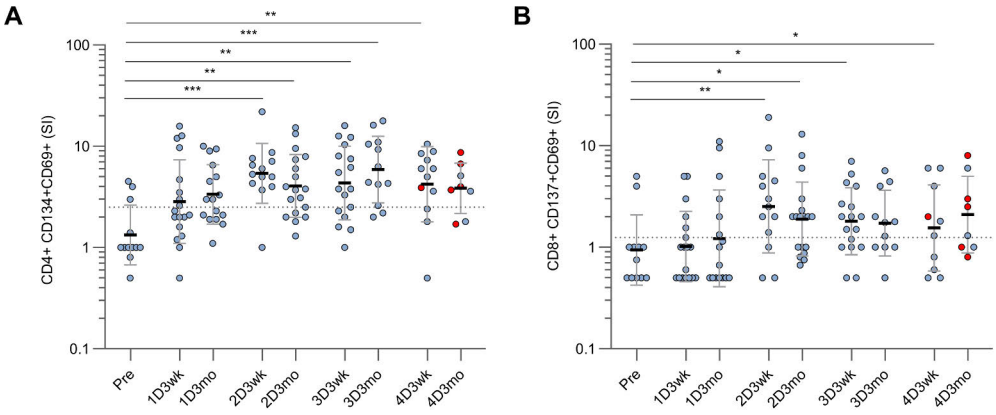

Figure 9. Identification of spike-specific CD4+ and CD8+ T cells in CVID patients. **A.** CD4+ responses, defined by the expression of CD69+ and CD134+ and **B.** CD8+ T cell responses defined by the expression of CD69+ and CD137+. Red dots represent samples collected after SARS-CoV-2 infection. Paired samples were analyzed using the Wilcoxon signed-rank test, and samples with no data on either data point were excluded from the analysis. *p < 0.05.

After the third and fourth vaccine doses, the CD4+ responses were present in 67–78% and CD8+ in 45–69% of CVID patients. However, three months after the second vaccine dose, cellular responses declined more in patients with CVID than in HCWs ($p<0.05$). For four patients (three CVID and one IgG ScD), cellular response declined noticeably (SI > 2.5). However, lasting cellular responses were detected in most patients after the fourth vaccination.

Some samples from CVID patients (n=6) were analyzed with an additional flow cytometry panel covering CD45RA and CCR7 to determine the memory T cell subtypes of the activated T cells. Most memory CD4+ T cells were T_{CM} (52%) and T_{EM} (34%) phenotypes, while most CD8+ cells were T_{EMRA} (83%) and T_{EM} (14%) phenotypes.

In conclusion, our analysis showed that the second vaccination with the BNT162b2 mRNA vaccine generated a lasting memory T cell response in most patients. However, the cellular response declined more in immunocompromised patients compared to healthy vaccinees, providing evidence for the need for subsequent booster immunizations.

5.2.3 Secretion of cytokines and effector molecules by activated PBMCs and T cells

The levels of secreted cytokines (IFN-γ, TNF-α, IL-2, and IL-4) and T cell effector molecules (perforin and granzyme B) were measured from the supernatants of the PBMCs stimulated with Wuhan-Hu-1 spike peptide pools. There was a significant

increase in the levels of IL-2 and IFN-γ after the first and second vaccinations, correlating with the T-cell responses in the AIM assay (Figure 10). The levels of Th2-type cytokine IL-4 increased after the second vaccine dose but remained lower compared to IL-2 and IFN-γ. Patients with CVID had similar cytokine production compared to healthy vaccinees. Three weeks after the third and fourth vaccinations, levels of IL-2 and IL-4 decreased slightly but increased again three months after vaccinations. However, the levels of IFN-γ remained high at all time points and correlated highly with the S-specific CD4+ response ($r=0.606$, $p<0.0001$). In contrast, CD8+ responses correlated most with the production of IL-2 ($r=0.444$, $p<0.0001$). We found a statistically significant difference between pre-and postvaccination samples in the production of granzyme B but not in the production of TNF-alpha and perforin.

Put together, we showed that vaccination with the BNT162b2 mRNA vaccine generated a functionally active T cell response, with the CD4+ response skewed towards a Th1-type antiviral phenotype. The responses to stimulations with different variant peptides did not differ significantly.

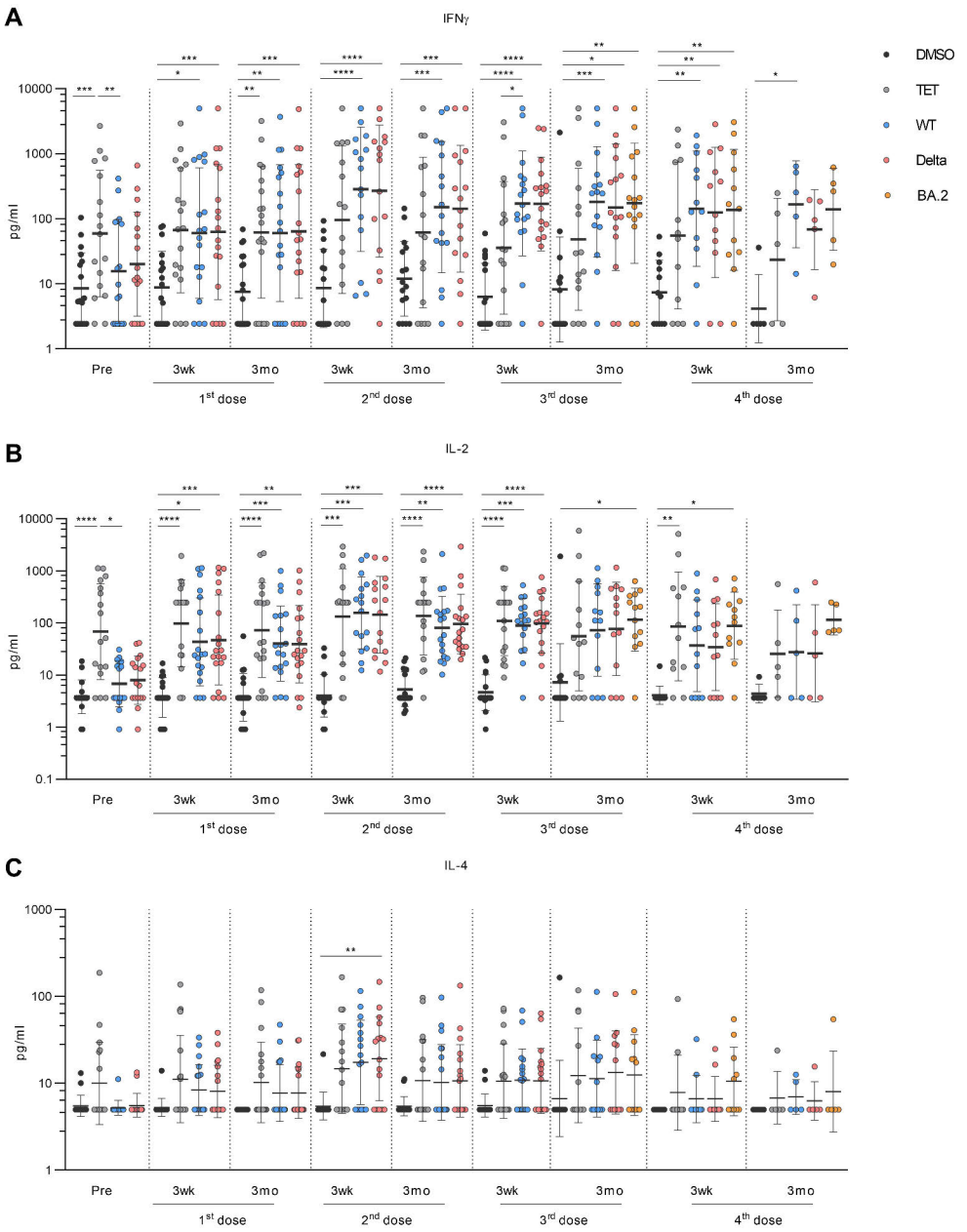

Figure 10. Secretion of cytokines to PBMC supernatants after stimulation with SARS-CoV-2 peptide pools. **A.** Secretion of IFN-γ, **B.** IL-2, and **C.** IL-4 in CVID patients. Tetanus toxoid was used as a positive, and DMSO-treated PBMCs as negative controls. Comparisons between pre-vaccination samples and samples collected after vaccinations were done using the Kruskall-Wallis test, followed by Dunn's multiple comparisons test.

5.3 Study III

5.3.1 Outcomes of patients with prolonged symptoms after COVID-19

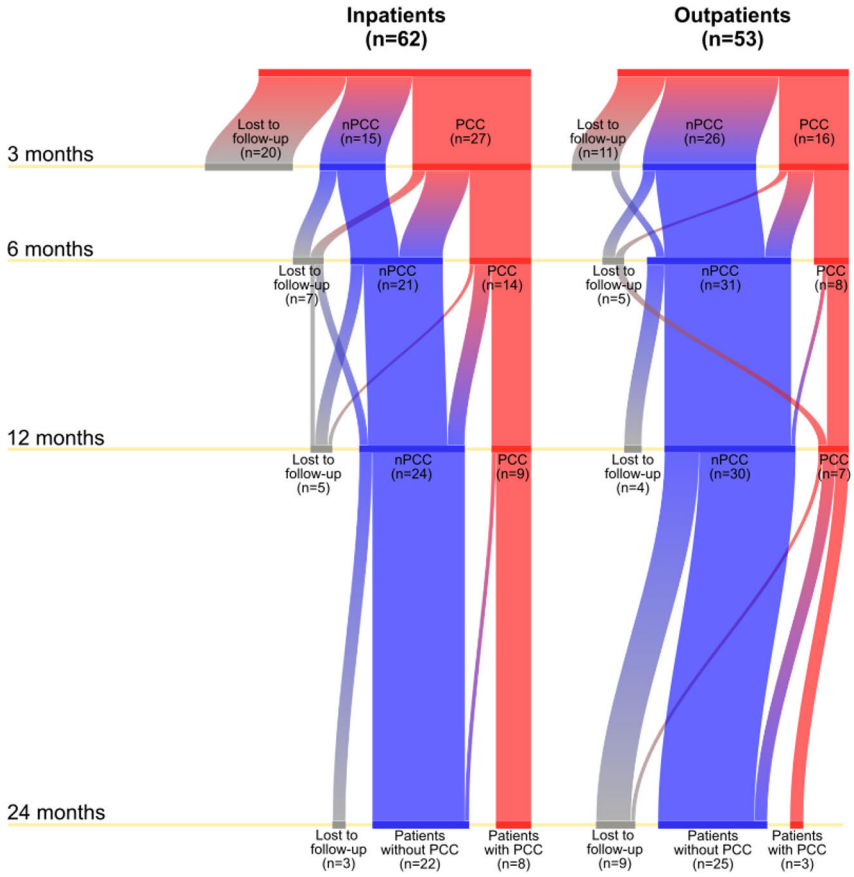

Figure 11. Patient flow throughout the study among the inpatient and outpatient cohorts.

The median age of the inpatients was 52.5 years (95% confidence interval [CI]: 49.9–56.0 years), with 44% (27/62) female, while the median age of the outpatients was 33.3 years (95% CI: 30.7–35.9 years), with 62% (33/53) female. Compared to outpatients, inpatients had higher median BMI and more comorbidities, notably asthma, obstructive sleep apnea, and type 2 diabetes.

Three months post-infection, the prevalence of PCC was 51% (43/84), with 51% (22/43) being female. As risk factors for developing PCC, we identified higher BMI (OR 1.119 [1.013–1.215], p=0.007) and obstructive sleep apnea (OR 5.161 [1.042–

25.56], p=0.04). The severity of acute disease correlated with the prevalence of PCC (Fisher's Exact Test, p=0.04), but the difference was not statistically significant when corrected for sex and BMI (Wald $\chi 2$=3.80, p=0.28). We did not find a correlation between PCC and sex, type 2 diabetes, asthma/COPD, depression, anxiety, or hypertension. The most common self-reported symptom among patients with PCC was fatigue (47%), followed by exertional dyspnea (28%), mood disturbance (26%), muscular weakness and resting dyspnea (23% each), olfactory dysfunction, cognitive decline (21% each), and attention deficit (19%). The specific symptoms are described in Table 5.

Six months post-infection, the prevalence of PCC was 30% (22/74), with fatigue (50%) remaining the most common symptom. There were no differences in orthostatism, 6MWT, or spirometry between patients with PCC and recovered individuals. All patients with PCC had received their first vaccination; of them, 23% (5/22) had been vaccinated within one month before sampling. Among the recovered patients, 88% (45/51) had received their first vaccination, and 33% (17/51) had been vaccinated within a month before sampling.

At one-year follow-up, the prevalence of PCC was 23% (16/70), with fatigue (75%) being the most commonly reported symptom. Patients with PCC had not received recent COVID-19 vaccines, while 5.7% (3/53) of recovered patients were vaccinated within one month. At two-year follow-up, the prevalence of PCC was 18% (11/60), with fatigue in 7/11 patients (64%) as the most common symptom.

At least one persistent symptom (including those not attributed to PCC) was reported by 56% (47/84) of patients at three months, 54% (40/74) at six months, 53% (37/70) at 12 months and 53% (32/60) at 24 months, while at least three persistent symptoms were reported by 25% (21/84) of the patients at three months, 38% (28/74) at six months, 31% (22/70) at 12 months and 25% (15/60) at 24 months.

In conclusion, we found that the prevalence of PCC was relatively high (51%) three months after infection. Fortunately, the prevalence of PCC declined considerably to 18% at 24-month follow-up. The severity of the acute disease increased the risk of developing PCC at three months but did not correlate with the occurrence of PCC after six months.

Table 5. The main symptoms among patients with post-COVID symptoms (PCC) and without post-COVID related symptoms (nPCC). A positive symptom represents an increase of at least four points on the NRS in the reported symptom severity compared to the pre-infection situation.

	3 months		6 months		12 months		24 months	
Symptom	nPCC	PCC	nPCC	PCC	nPCC	PCC	nPCC	PCC
Fatigue	2,4 %	46,5 %	7,7 %	50,0 %	5,6 %	75,0 %	12,2 %	63,6 %
Shortness of breath at rest	0,0 %	23,3 %	5,8 %	4,5 %	0,0 %	25,0 %	0,0 %	0,0 %
Shortness of breath at exertion	4,9 %	27,9 %	1,9 %	36,4 %	5,6 %	50,0 %	6,1 %	36,4 %
Cough	2,4 %	9,3 %	3,8 %	4,5 %	9,3 %	37,5 %	2,0 %	27,3 %
Muscular weakness	2,4 %	23,3 %	5,8 %	27,3 %	7,4 %	62,5 %	6,1 %	36,4 %
Myalgia	0,0 %	7,0 %	3,8 %	18,2 %	1,1 %	50,0 %	10,2 %	27,3 %
Insomnia	2,4 %	7,0 %	9,6 %	18,2 %	7,4 %	31,3 %	12,2 %	18,2 %
Attention deficit	2,4 %	18,6 %	5,8 %	31,8 %	7,4 %	62,5 %	2,0 %	45,5 %
Cognitive decline	0,0 %	20,9 %	5,8 %	27,3 %	1,9 %	43,8 %	2,0 %	27,3 %
Mood disturbance	2,4 %	25,6 %	17,3 %	13,6 %	5,6 %	37,5 %	8,2 %	36,4 %
Anxiety	0,0 %	16,3 %	13,5 %	22,7 %	11,1 %	43,8 %	4,1 %	36,4 %
Olfactory dysfunction	4,9 %	20,9 %	9,6 %	22,7 %	1,9 %	18,8 %	0,0 %	18,2 %
Gustatory dysfunction	4,9 %	9,3 %	1,9 %	22,7 %	5,6 %	25,0 %	0,0 %	18,2 %
Hair loss	4,9 %	14,0 %	1,9 %	13,6 %	3,7 %	12,5 %	0,0 %	27,3 %

5.3.2 Anti-SARS-CoV-2 spike- and nucleoprotein-specific antibodies in patients with PCC and recovered individuals

First, we compared patients experiencing post-COVID symptoms at three months (PCC) with those that were symptomless at three months (nPCC). PCC patients who recovered later during the follow-up were excluded from the first analysis.

At the acute phase, the median serum levels of anti-SARS-CoV-2 IgG antibodies were low in both PCC and nPCC groups. One month after infection, most patients had measurable serum levels of anti-S1 and anti-N antibodies, but the groups showed no significant differences (Figure 12). At three months, the median serum levels of anti-S1 IgG antibodies were higher in the PCC group, but the difference was not statistically significant (33.0 vs. 13.0 EIA units, p=0.09). Six months post-infection, patients from both groups had received their first COVID-19 vaccinations and had significantly increased serum levels of anti-S1 antibodies, but the difference was not significant (100.5 vs. 88.0 EIA units, p=0.15). However, the differences in serum anti-S1 antibody levels were significant at twelve months (94.0 vs. 73.0 EIA units, p=0.045) and 24 months post-infection (90.0 vs. 58.0 EIA units, p=0.025). Meanwhile, while the serum anti-N IgG levels appeared higher in the PCC group, we observed no significant differences during the follow-up (Figure 12B).

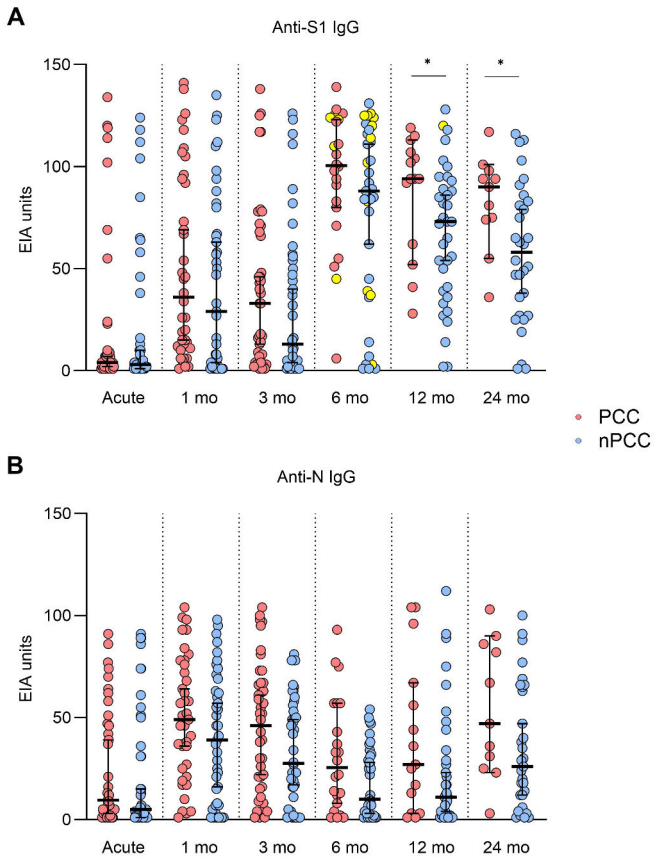

Figure 12. The first analysis of serum anti-SARS-CoV-2 IgG antibody levels in COVID-19 patients with post-COVID symptoms (PCC) and without symptoms (nPCC), where the groups were determined at three months post-infection. Recovering PCC patients were left out of the analysis. **A.** Anti-S1 IgG and **B.** anti-N IgG levels among PCC and nPCC groups. Black lines represent median levels with a 95% CI of the median level. Yellow dots represent samples from patients vaccinated less than a month before sampling. The median antibody levels at each time point were compared with Wilcoxon signed rank test. * $p<0.05$

Then, we did a second analysis with recovering PCC patients as part of the nPCC group. The median serum levels of anti-S1 and anti-N IgG antibodies were higher in the PCC group throughout the follow-up, but the differences were not statistically significant, except for the anti-N IgG at three months post-infection (46.0 vs. 27.0 EIA units, $p=0.048$) (Figure 13). However, although insignificant, the trend was concordant among the inpatients and outpatients (Figure 14). Patients with PCC had more variation in the serum anti-N IgA and IgM antibody levels, but the median levels did not differ significantly.

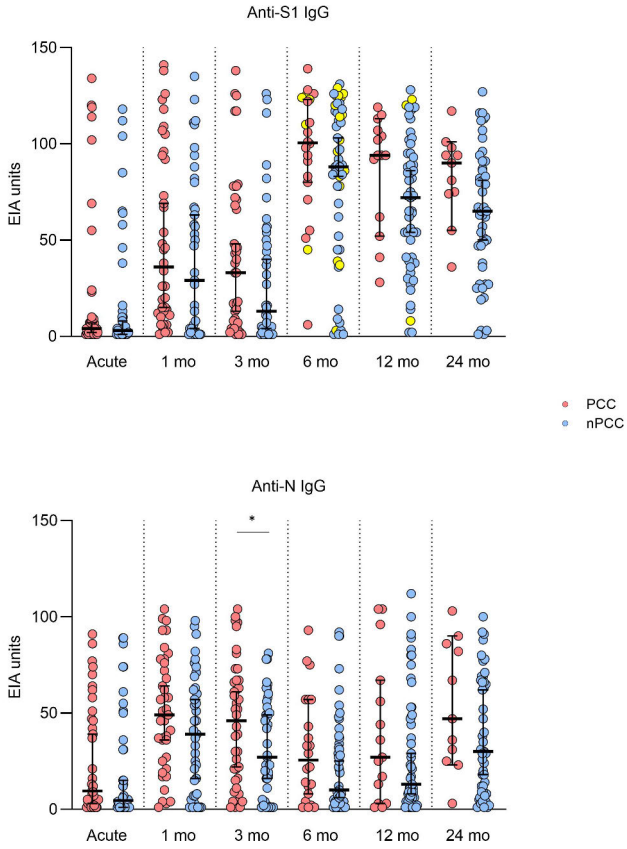

Figure 13. The second analysis of serum anti-SARS-CoV-2 IgG antibody levels in COVID-19 patients with post-COVID symptoms (PCC) and without symptoms (nPCC), where the groups were determined at each time point. **A.** Anti-S1 IgG and **B.** anti-N IgG levels among PCC and nPCC groups. Black lines represent median levels with 95% CI. Yellow dots represent samples from patients vaccinated less than a month before sampling. The antibody levels at each time point were compared with logistic regression adjusted for sex and age. * $p<0.05$

In conclusion, the patients with PCC had significantly higher levels of anti-S1 antibodies twelve and 24 months post-infection compared to patients without post-COVID symptoms (determined at three months). Serum levels of anti-N antibodies were also higher in the PCC group, but the differences were not statistically significant. Moreover, when the recovering PCC patients were considered part of the nPCC group, the differences were not significant, except for anti-N IgG at three months. At six months, most patients had significantly higher serum levels of anti-S1 antibodies, most likely due to vaccinations.

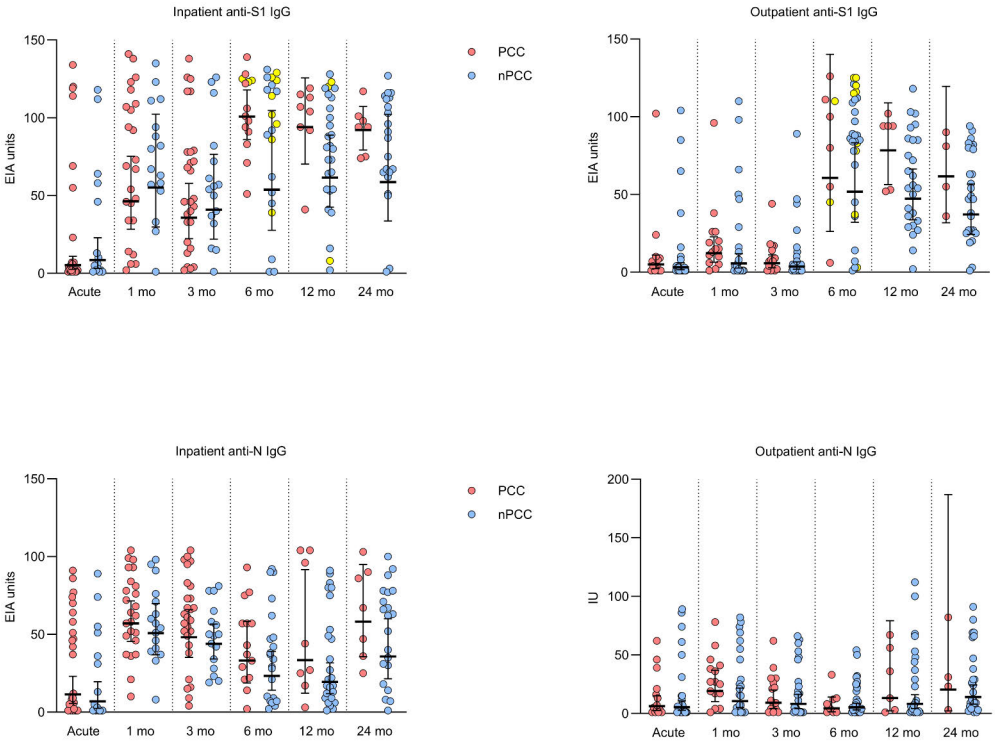

Figure 14. Serum anti-SARS-CoV-2 IgG antibody levels in COVID-19 inpatients (left) and outpatients (right) with post-COVID symptoms (PCC) and without symptoms (nPCC), determined at each time point. Black lines represent median levels with 95% CI. Yellow dots represent samples from patients vaccinated less than a month before sampling. The antibody levels at each time point were compared with logistic regression adjusted for sex and age. * $p<0.05$

5.3.3 Serum levels of sST2, IL-6, hs-CRP, and cortisol in patients with PCC and recovered individuals

For all patients, the median levels of sST2 were 21.3 ng/ml at the acute phase and then declined to a steady level of 12.7–14.0 ng/ml. The median level of IL-6 was also highest at the acute phase at 10.6 pg/ml and then declined to 4.5–5.4 pg/ml, peaking once at 6.1 pg/ml at six months post-infection. The median level of hs-CRP was highest at the acute phase at 20.3 mg/l and then declined to a steady level of 1.6–2.2 mg/l. The median cortisol levels were also highest at the acute phase at 428 nmol/l and steadily declined to 279 nmol/l at 24 months post-infection.

During the acute phase, patients who would later develop post-COVID-related symptoms had significantly higher median levels of pro-inflammatory proteins sST2 (28.2 ng/ml vs. 20.9 ng/ml, p =0.031) and hs-CRP (39.2 mg/l vs. 2.9 ng/l, p=0.002), and significantly lower cortisol levels as compared to recovering patients (307 nmol/l

vs. 354 nmol/l, p=0.005) (Figure 15). Inpatients with PCC had lower cortisol levels in the acute phase, but the difference was insignificant for outpatients. No significant differences in serum levels of IL-6 were measured between the two groups.

Three months after infection, no difference in the serum levels of sST2, cortisol, or hs-CRP was detected between the patients with PCC and recovered patients. Although the levels of IL-6 appeared higher in both inpatient and outpatient cohorts, they did not differ significantly (inpatients: 5.6 pg/ml vs. 5.3 pg/ml, p=0.09; outpatients: 5.4 pg/ml vs. 1.3 pg/ml, p=0.12). Moreover, hs-CRP levels were also marginally higher in the PCC group at 24 months post-infection, but not significantly (2.6 mg/l vs. 1.3 mg/l, p=0.07). Otherwise, the parameters did not differ significantly between the two groups from six months onward.

In conclusion, we noticed that acute phase levels of hs-CRP and sST2 were higher, and cortisol levels were lower in the PCC group compared to the nPCC group, likely due to the more severe acute inflammation and corticosteroid treatment. Although later levels of IL-6 and hs-CRP appeared slightly higher in patients with PCC, we did not observe any significant differences in the measured parameters.

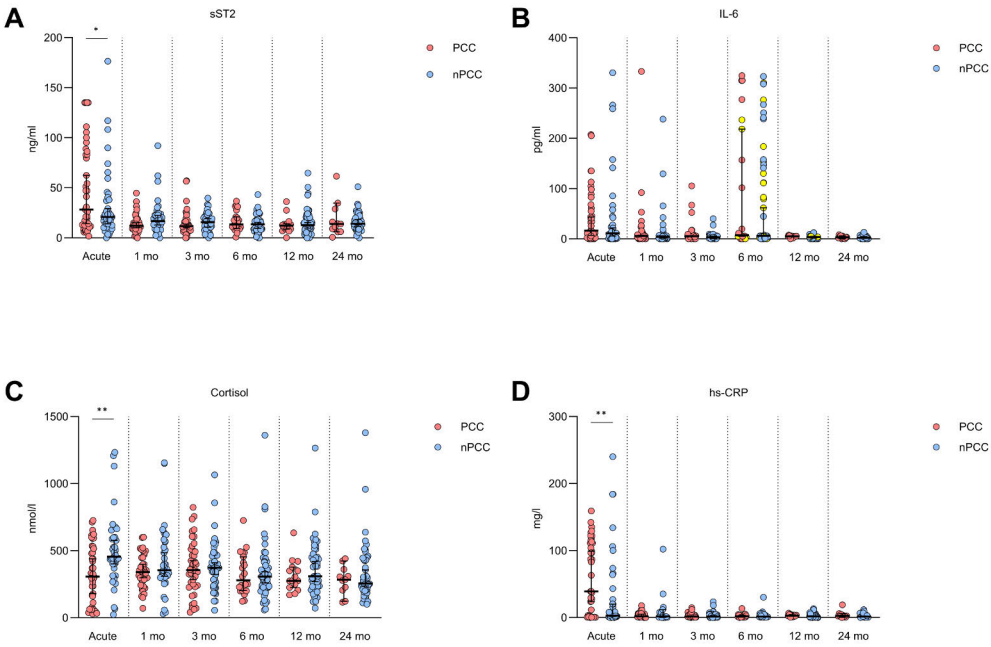

Figure 15. The serum levels of **A.** sST2, **B.** IL-6, **C.** cortisol, and **D.** hs-CRP in patients with PCC and recovered individuals (nPCC). Yellow dots represent samples from patients vaccinated less than one month before sampling. The black lines represent median levels with a 95% CI of the median level. The levels of each parameter were compared with logistic regression adjusted for sex and age. * p<0.05; ** p<0.01

5.3.4 Differences in serum antibody, cortisol, and inflammatory marker levels between PCC clusters

We classified patients into three clusters by their most prevalent symptoms. Cluster 3 patients with persistent respiratory symptoms were often female and had higher BMI than the other two clusters. In contrast, cluster 2 patients with cognitive symptoms were younger than patients in the other two groups. Cluster 3 patients had more restrictive and obstructive spirometry results than those in the other two clusters, but there were no differences in 6MWT or HRCT findings (Table 6).

Table 6. Baseline characteristics and physiological test results among the three PCC clusters.

	3 months			6 months		
	Cluster 1	Cluster 2	Cluster 3	Cluster 1	Cluster 2	Cluster 3
n (%)	13 (30.2 %)	11 (25.6 %)	19 (44.2 %)	8 (36.4 %)	4 (18.2 %)	10 (45.5 %)
Age (years), mean	44.8	38.2	45.8	47.0	38.3	48.0
BMI, mean	29.9	32.0	34.3	31.4	29.4	36.6
Female sex	30.2%	25.6%	44.2%	33.3%	25,0 %	60,0 %
Abnormal HRCT of lungs	33,3% (2/6)	67% (6/9)	41,2% (7/17)			
Physiological tests						
Walking distance (%) in 6MWT, median (IQR)	95.6 (85.5-107.0)	96.9 (90.0-106.0)	97.0 (94.5-106.0)	94.9 (88.3-101.8)	95.5 (91.3-102.5)	96.0 (92.5-100.0)
FVC (Z-value), median (IQR)	0.2 (-0.9 to 0.4)	-0.1 (-0.9 to 0.4)	-2.0 (-2.5 to -0.7)	-0.6 (-1.5 to 0.2)	0.3 (-0.3 to 0.9)	-1.0 (-1.5 to -0.5)
FEV1 (Z-value), median (IQR)	-0.3 (-1.0 to 0.2)	-0.2 (-0.7 to 0.6)	-1.3 (-1.8 to -0.7)	-0.3 (-1.5 to 0.3)	0.2 (-0.1 to 0.7)	-1.1 (-1.7 to -0.4)
PEF (Z-value), median (IQR)	-1.3 (-1.5 to -0.6)	-0.9 (-1.6 to -0.2)	-1.7 (-3.0 to -0.5)	-0.9 (-1.4 to -0.5)	0.0 (-0.6 to 0.7)	-1.0 (-1.9 to 0.2)

At three months, patients with myalgia and fatigue (cluster 1) and patients with ongoing respiratory problems (cluster 3) had higher levels of anti-S1 IgG (Figure 16A). Still, the difference was not significant (33.0 vs. 10.6 EIA units, p=0.14, and 43.3 vs. 10.6 EIA units, p=0.14). At six months, however, the differences in serum anti-S1 IgG levels were significant between clusters 1 and 2 and between clusters 3 and 2 (101.3 vs. 54.5 EIA units, p=0.0122, and 91.3 vs. 54.5 EIA units, p=0.0118, respectively). However, later than six months post-infection, the differences were not significant. The clusters showed no differences in serum anti-S1 IgM and IgA or anti-N IgG antibody levels.

Clusters 1 and 3 had elevated levels of hs-CRP compared to cluster 2 patients. The differences were subtle but consistent and statistically significant at six months (2.0 mg/l vs. 0.7 mg/l, p=0.0168, and 2.8 mg/l vs. 0.7 mg/l, p=0.0152) and 24 months (3.0 mg/l vs. 0.6 mg/l, p=0.0175, and 10.7 mg/l vs. 0.6 mg/l, p=0.0167), represented in the Figure 17D. On the other hand, cluster 2 patients had lower cortisol levels than those in clusters 1 or 3. Twelve months post-infection, the difference was statistically significant between clusters 2 and 1 (179 nmol/l vs. 290 nmol/l, p=0.03) and almost significant between clusters 2 and 3 (179 nmol/l vs. 351 nmol/l, p=0.054). However, for the rest of the follow-up, the differences did not reach statistical significance at any other time point.

Altogether, although the serum levels of hs-CRP, IL-6 and cortisol among samples from PCC clusters 1 and 3 seemed to slightly differ from the samples from cluster 2, the subgroup sizes in our final analysis were too small for any meaningful interpretation.

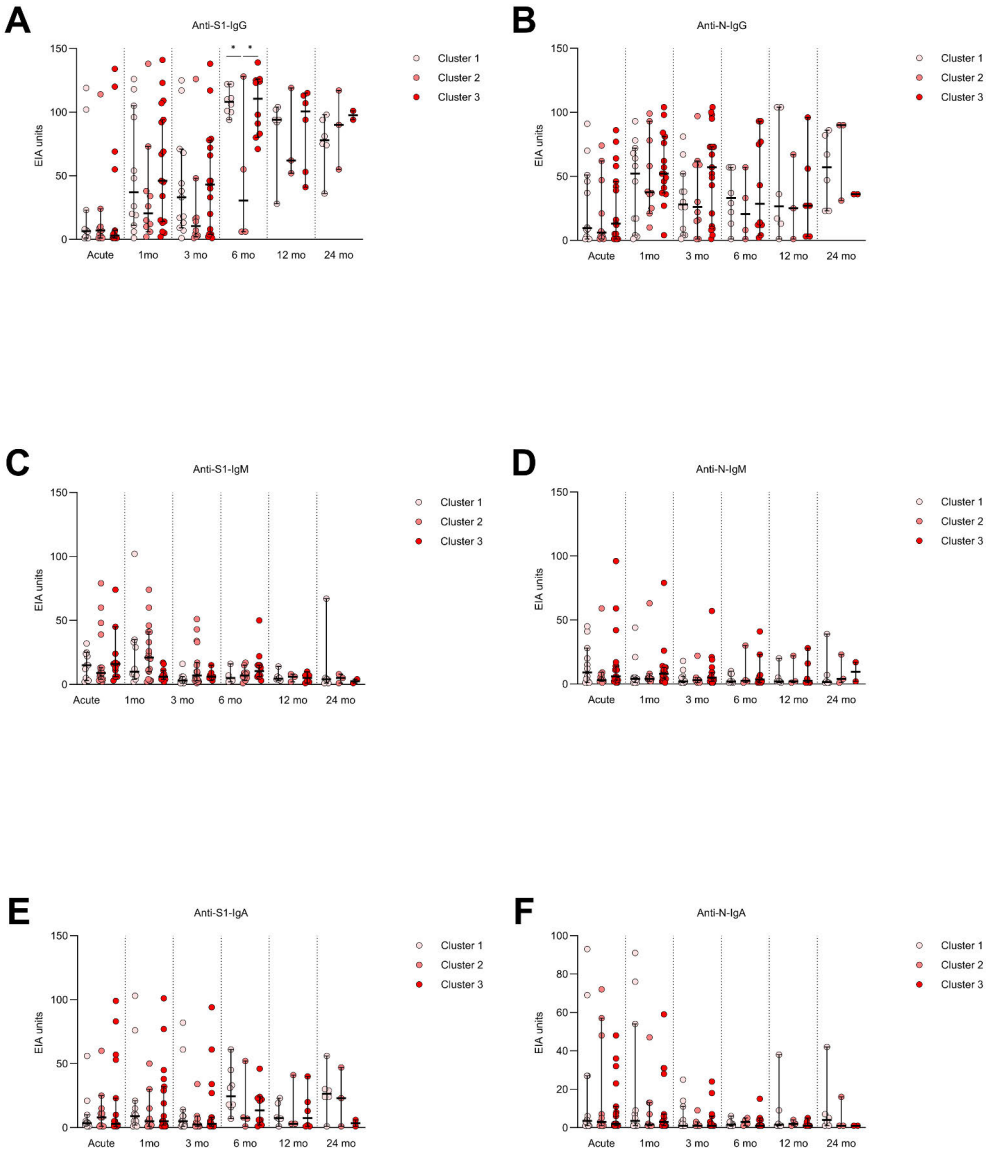

Figure 16. Anti-SARS-CoV-2 S1- and N-specific antibody responses in different clusters of PCC. Levels of **A.** anti-S1 IgG, **B.** anti-N IgG, **C.** anti-S1 IgM, **D.** anti-N IgM, **E.** anti-S1 IgA, and **F.** anti-N-IgA antibody responses. The black lines represent median levels with a 95% CI of the median level. The levels of each parameter were compared with a linear mixed effects model, restricted maximum likelihood (REML) adjusted for sex and age. *p<0.05

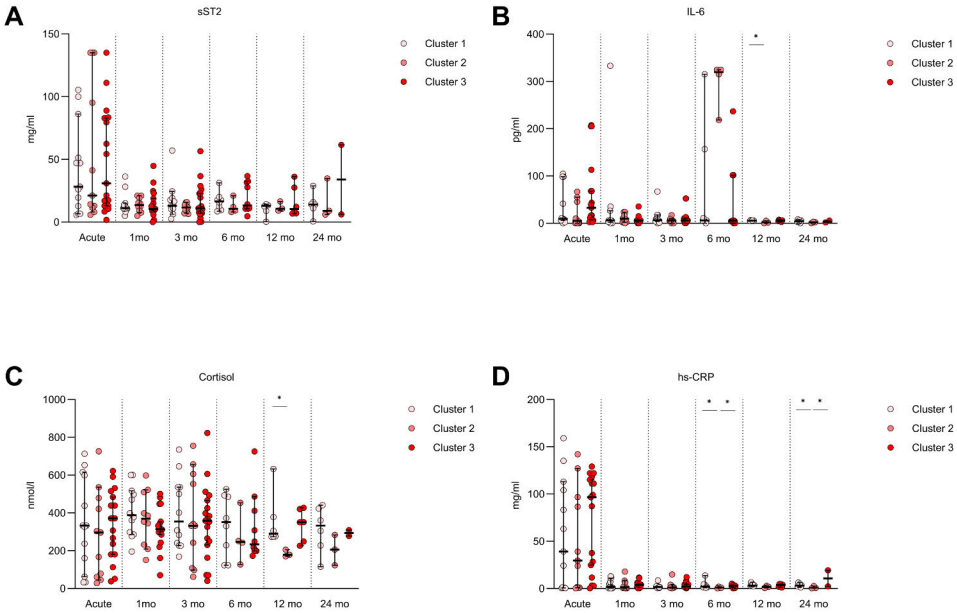

Figure 17. Levels of **A.** sST2, **B.** IL-6, **C.** cortisol, and **D.** hs-CRP in different clusters of PCC. The black lines represent median levels with a 95% CI of the median level. The levels of each parameter were compared with a linear mixed effects model, restricted maximum likelihood (REML) adjusted for sex and age. *p<0.05

6 Discussion

6.1 Cellular immunity following COVID-19 mRNA vaccination

The emergence of novel variants of SARS-CoV-2 with immune-escape mutations meant that the antibodies could no longer as effectively neutralize the new variants[213]. However, the epidemiological data suggested that vaccination protected against mildly symptomatic and severe COVID-19[214]. Previous studies had shown that SARS-CoV-2 infection generates a T cell immune response, likely explaining the immune protection[215]. However, there were limited studies on whether the SARS-CoV-2 vaccination would produce cellular immune responses in healthy individuals or immunocompromised patients. Therefore, it was vital to study cellular immunity generated by SARS-CoV-2 immunization. We studied the responses to the BNT162b2 mRNA vaccine since it was the most frequent COVID-19 vaccine in Finland and Europe at the time[216].

During our study period in the summer and autumn of 2021, the leading regional circulating variant was Delta (B.1.617.2). Therefore, we focused on cellular responses to four locally significant VOCs: Alpha (B.1.17), Beta (B1.351), Gamma (P.1) and Delta[217]. We observed equally strong responses to all circulating variants, proving that the amino acid changes in the VOCs did not alter the T cell activity, consistent with previous studies[218]. Later studies have shown that vaccine-generated T cell responses are not affected by additional viral mutations, such as in Omicron BA.1 and BA.2 subvariants[219].

One of the crucial factors of vaccine efficacy is the ability to generate both humoral and cellular immune responses. We detected CD4+ T cell responses against SARS-CoV-2 spike protein-derived peptides in all healthy vaccinees, while CD8+ T cell responses were detected in 70% of the healthy vaccinees. The responses were similar between vaccinated and convalescent individuals. The data on cytokine production suggested that the CD4+ T cell response was directed towards the Th1 type differentiation with increased mRNA expression and secretion of IFN-γ and IL-2. In contrast to other studies, we did not find increased production of TNF-α or granzyme B, likely due to inadequate stimulation of CD8+ cells. However, our study

showed that vaccinating with the BNT162b2 generates a robust and lasting T cell response that is rapidly activated when encountering the viral epitopes.

For immunocompromised patients, we observed adequate cell-mediated immune responses against the SARS-CoV-2 spike protein, consistent with later studies[220]. Two doses were often required for the response, but the response was maintained with additional booster vaccinations. Other studies have shown that boosters increase the cellular response, suggesting the need for additional immunization[221]. Our data showed that the cellular responses were directed against all tested viral variants. Interestingly, analysis of memory T cell phenotypes in CVID patients revealed that while most CD4+ cells exhibited effector and central memory phenotype, most CD8+ cells were T_{EMRA} phenotype, which is highly cytotoxic but also associated with T cell exhaustion[222].

6.2 Importance of cellular immunity in patients with primary antibody deficiencies

Patients with immunodeficiencies, such as CVID, are at an increased risk of severe COVID-19[223] At the beginning of our study, the commercial IGRT products did not contain protective levels of anti-SARS-CoV-2 antibodies, or the antibodies did not protect against the circulating Omicron variant[224]. Therefore, patients with hypogammaglobulinemia relied primarily on cellular immune responses for protection against severe COVID-19. We found detectable but inconsistent levels of anti-S IgG antibodies after the third and fourth boosters in the sera of the CVID patients. However, our patient with XLA, who cannot produce antibodies, also had detectable serum levels of anti-S1 antibodies. Therefore, the measured serum antibodies are most likely derived exogenously from the IGRT products.

Patients with PAD were recommended to be vaccinated against SARS-CoV-2, but there was limited information on the cellular immune responses to the vaccination. We showed that immunodeficient patients develop lasting T cell responses and benefit from immunization with the COVID-19 mRNA vaccine. However, as most of the CD8+ T cells generated by vaccination were of the exhausted T_{EMRA} phenotype, the number of cytotoxic T cells might be compromised if the patients get vaccinated and infected multiple times with SARS-CoV-2. Consequently, it is vital to study memory T cell responses generated by hybrid immunity among immunocompromised patients in the future[225].

6.3 Post-COVID-19 condition

Consistent with previous meta-analyses[181,226], we found that severe COVID-19 predisposes patients to persistent post-COVID symptoms. However, milder cases

also exhibited similar symptoms, albeit at a lower frequency, consistent with prior Finnish research[128]. For 2–5% of patients, these symptoms significantly impacted the quality of life., However, most patients experienced symptom resolution within 1-2 years of follow-up. Our study cohort consisted of unvaccinated individuals infected with Alpha and Delta variants, making these findings particularly relevant to the context of the early pandemic. As vaccination and infection with more recent variants like Omicron are associated with a lower risk of PCC, these results may not directly apply to later stages of the pandemic[227,228].

We observed that patients with PCC had significantly elevated serum levels of anti-S1 antibodies at 12 and 24 months post-infection compared to those without COVID-related symptoms at three months. Although the differences in serum anti-N antibody levels were not statistically significant, the PCC group had consistently higher median levels throughout the study period. This trend may reflect a more robust humoral response due to the increased severity of acute disease[229]. Interestingly, elevated antibody levels were also noted in our outpatient cohort, aligning with prior research showing higher serological responses in milder cases[181]. These findings suggest ongoing immune activation, potentially linked to subclinical viral persistence, as supported by emerging evidence of prolonged SARS-CoV-2 RNA presence in multiple tissues[190,191]. Persistent viral reservoirs may account for sustained higher serum antibody levels, indicating unresolved viral activity that could contribute to PCC pathophysiology. However, further research with tissue biopsies is necessary to confirm this hypothesis.

The clinical overlap between PCC and myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS) suggests that insights from ME/CFS research could be valuable. However, the post-infectious hypothesis of ME/CFS is questioned at best due to inconsistent data quality[230], and direct extrapolation to PCC should be approached with caution.

Some studies have shown that patients with ME/CFS have defects in NK cells and abnormalities in T cell function with elevated levels of cytokines and altered inflammatory proteins[231–233]. Improvement in cell-mediated immunity is associated with alleviating post-infection fatigue symptoms in patients with PCC and ME/CFS.[198,234]. In our cohort, patients who developed PCC exhibited higher levels of inflammatory markers (sST2, IL-6, and hs-CRP) during the acute phase, correlating with disease severity. These markers declined within a month, likely reflecting effective treatment during hospitalization, and normalized within three to six months. Fatigue and myalgia, prominent symptoms in Cluster 1, may be linked to ongoing low-grade inflammation, though our small sample size limits definitive conclusions[235] . Respiratory symptoms were associated with reduced pulmonary function but showed no correlation with HRCT findings, suggesting that

inflammation rather than structural lung damage may be the primary driver. Notably, respiratory symptoms improved in most patients within a year.

Cognitive impairments in PCC could result from various mechanisms, including hypocortisolism, impaired blood-brain barrier function[236] or functional neurological disorders[237]. Activation of oxidative and nitrosative stress and neuroinflammatory pathways in patients with ME/CFS have been shown to disrupt the hypothalamic-pituitary-adrenal (HPA) axis, resulting in lower baseline glucocorticoid levels[238]. However, the quality of the studies has been challenged, and the replicability of these findings has been limited[239]. In our study, we did not observe any differences in the serum cortisol levels between the two groups. The overall cortisol levels of patients with PCC were significantly lower in the acute phase – especially among inpatients with a more severe disease form. These patients usually received corticosteroids during hospitalization, suppressing endogenous cortisol production. Later in the follow-up, these differences in serum cortisol levels diminished. Therefore, it is unlikely that hypocortisolism would explain these post-infectious symptoms. However, a subgroup of PCC patients with cognitive problems seemed to have lower cortisol levels than recovered individuals or patients with myalgic or respiratory symptoms. Still, the subgroups were far too small to make any firm conclusion.

In summary, our findings provide valuable insights into the immunological aspects of PCC, highlighting potential mechanisms such as persistent viral replication and immune dysregulation. Future research should focus on evaluating these hypotheses in vaccinated cohorts and with newer variants to better understand the evolving risk profile of PCC. Understanding these mechanisms could aid in developing targeted interventions to improve long-term outcomes.

6.3.1 Treatment and management of PCC

Effective treatment of PCC remains challenging due to its uncertain, multifactorial pathogenesis. Standardized diagnostic criteria are crucial given the overlap of PCC symptoms with other conditions. Moreover, there may be PCC subtypes with different underlying mechanisms suggesting the need for tailored therapeutic strategies rather than a single, uniform approach[240].

Fatigue is a central symptom of PCC, and rehabilitation often focuses on increasing physical activity. However, strict exercise without pacing may exacerbate symptoms, particularly in patients with severe fatigue[158]. Cognitive behavioral therapy (CBT) and graded exercise therapy (GET), widely used for chronic fatigue syndrome (ME/CFS), have shown mixed results. While early studies suggested some benefits, recent studies have questioned the efficacy of these approaches in ME/CFS and PCC, highlighting the need for alternative strategies[241].

Vaccination has been associated with a reduced risk of developing PCC, though its protective effect after infection remains uncertain. Importantly, vaccination does not increase the risk of developing PCC[242].

Pharmacological options trialed in ME/CFS may also be relevant for PCC. Beta-blockers are used for postural tachycardia, while intravenous immunoglobulin (IVIG) targets immune dysregulation. Newer therapies, like BC 007, aim to neutralize anti-GPCR antibodies, but these require further validation[243]. Low-dose naltrexone has shown promise in off-label use but lacks high-quality RCT evidence[244].

Addressing chronic inflammation from viral persistence is an emerging area of research. Epipharyngeal zinc chloride irrigation has shown preliminary success in clearing residual viral RNA[245], while intravenous RNase therapy did not yield benefits[246]. Nirmatrelvir/ritonavir reduced the risk of PCC in the acute phase[247], but remdesivir did not alter long-term outcomes, and nirmatrelvir/ritonavir showed no benefit in the chronic phase[248].

In conclusion, diverse therapeutic approaches are being explored with limited results. The heterogeneity of PCC suggests a need for personalized treatments. Rigorous RCTs and further research into specific subtypes and mechanisms will be critical for developing effective, evidence-based guidelines.

6.4 Research of post-viral conditions in the future

Patients with post-acute infectious disease symptoms are a heterogeneous group with multiple possible etiologies. Some may experience symptoms for psychosocial reasons (functional disorders) or metabolic reasons. In contrast, others may have accumulated damage caused by the acute infection or unresolved long-term inflammation from a dysregulated immune system. Therefore, it is challenging to study the pathophysiology of these disorders simultaneously.

Many post-acute infection syndromes have been described, but little is known about their pathophysiology. However, research into PCC has reignited the research on post-viral syndromes in general. Differentiating between subtypes of post-viral syndrome patients is essential to determine the biomedical background and, hopefully, develop effective diagnostics and treatments for these debilitating disorders.

Future research should focus on categorizing patients based on their most severe and limiting symptoms, which will require extensive, well-characterized cohort studies. Multidisciplinary approaches integrating proteomics, metabolomics, genomics, and advanced RNA analysis from tissue samples, combined with comprehensive clinical evaluation, are essential for uncovering distinct

pathophysiological mechanisms. Such efforts will be crucial in advancing our understanding of post-viral conditions.

6.5 Limitations of the study

We used cryopreserved PBMCs to measure the cellular responses, which could lead to decreased cell viability and loss of infrequent memory cell populations. On the other hand, cryopreserved cells enabled us to simultaneously analyze all longitudinal samples, minimizing batch effects and increasing the reliability of our results. In the cell stimulations, we optimized for CD4+ activation, thus limiting the detection of CD8+ responses. We used relatively short incubation times of 48h and 15-mer peptide pools for cellular stimulations, while longer stimulations with 9-10-mer peptides are more suitable for CD8+ activation through MHC I[249].

The study populations across all three studies were relatively small, particularly in the subgroup analyses of study III. This limited our statistical power and restricted the range of advanced statistical methods we could employ, potentially affecting the robustness of some findings. Additionally, in study I, we only assessed immune responses to the BNT162b2 mRNA vaccine, limiting the generalizability of our results to other vaccine types. In studies II and III, we could not control the timing, administration, or types of vaccines received by participants, leading to variability in vaccination schedules, which may have influenced the observed immune responses.

In study III, the retrospective data collection for acute phase symptoms and pre-existing conditions may have introduced recall bias or inaccuracies in symptom reporting. Furthermore, the absence of internationally standardized diagnostic criteria for PCC likely contributed to variability in diagnosis and symptom characterization. This limitation may have affected the consistency of the PCC definition across participants. Lastly, we did not include an age- and gender-matched negative control group, which limits our ability to assess the potential psychosocial impact of the pandemic on reported symptoms[250].

7 Summary and conclusions

This dissertation aimed to investigate the immunological responses following SARS-CoV-2 mRNA vaccination and infection, as well as the pathophysiology of post-COVID-19 condition (PCC). Our research provides valuable insights into immunological memory formation and protection against SARS-CoV-2 infection, and the possible complex mechanisms underlying PCC.

Study I demonstrated that the BNT162b2 mRNA vaccine generated robust CD4+ and CD8+ T cell responses, even in the presence of immune-escape mutations found in circulating variants like Delta. The antiviral Th1 phenotype, characterized by increased IFN-γ and IL-2 production, indicates a strong cellular immune response. This robust T cell activity likely contributes to protection against severe disease, even as the efficacy of neutralizing antibodies declines.

Study II showed that patients with primary antibody deficiencies, including those with common variable immunodeficiency, developed detectable T cell responses following vaccination, likely protecting against severe COVID-19, mainly when boosted with additional vaccine doses. However, the prevalence of possibly exhausted T_{EMRA} memory CD8+ T cells highlights the need for ongoing research into memory T cell function and hybrid immunity in these vulnerable populations.

Study III found that severe COVID-19 significantly predisposes patients to develop PCC, with symptoms persisting for up to 24 months post-infection. Fortunately, most patients experienced gradual symptom alleviation over time. Elevated serum levels of anti-S1 antibodies and inflammatory markers in PCC patients suggest ongoing immune activation, potentially linked to persistent viral reservoirs. These findings suggest further research into the mechanisms driving persistent symptoms and immune dysregulation in PCC.

The findings presented in this dissertation have significant implications for clinical practice and public health. The demonstrated efficacy of SARS-CoV-2 mRNA vaccines in eliciting robust cellular responses supports their continued use, particularly in immunocompromised individuals who may depend more on cellular immunity than humoral responses for protection. Understanding the heterogeneity of PCC and the potential role of persistent viral replication and immune dysregulation can inform more targeted diagnostic and therapeutic strategies.

In conclusion, this dissertation advances our understanding of immune responses following COVID-19 vaccination and provides insights into the complex pathophysiology of post-COVID condition (PCC). By demonstrating the robustness of vaccine-induced cellular immunity and identifying potential mechanisms driving persistent symptoms, these findings lay the groundwork for optimizing vaccination strategies across diverse patient groups. Future research on PCC should prioritize the role of cellular immunity, immunological dysfunction, and persistent viral activation, as well as explore personalized approaches to improve patient outcomes and mitigate the long-term impact of COVID-19 on public health.

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Antti Hurme

Antti Hurme

Growing up as the third child in a family of five brothers, I developed a competitive yet compassionate and cooperative spirit. From a young age, I was driven by curiosity to uncover the root causes of the issues around me, never satisfied with the explanation, "It's just the way it is." I believe the scientific process is the best path through the unknown. It teaches humility by recognizing mistakes and offers the beauty of discovering pieces of the truth.

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