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Analyse der Entwicklung der SARS-CoV-2-Pandemie in München, von ihrem Beginn bis zur Entstehung der Omikron Variante im Hinblick auf Veränderungen der Prävalenz und Unterschiede zwischen Durchbruchinfektionen und nicht-Durchbruchinfektionen

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Erstes Gutachten:	PD Dr. Noemi Castelletti
Zweites Gutachten:	Prof. Dr. Katja Radon
Drittes Gutachten:	PD Dr. Albrecht von Brunn

Dekan:	Prof. Dr. med. Thomas Gudermann

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List of Abbreviations

• COVID-19	Coronavirus Disease
• SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
• KoCo19	Prospektive Kohorte COVID-19 München
• CoVaKo	COVID Vakzin Konsortium
• KoCoImmu	Prospektive COVID-19 Kohorte München - Immunologie
• BTI	Breakthrough infection
• Non-BTI	Non breakthrough infection
• PCR Test	Polymerase chain reaction Test
• Anti-N-antibody/ Anti-N	Anti-Nucleocapsid-antibody
• Anti-S-antibody/ Anti-S	Anti-Spike-antibody
• VOI	Variant of interest
• VOC	Variant of concern
• DBS	Dried blood sample

List of Publications

2024

A Dried Blood Spot protocol for high-throughput quantitative analysis of SARS-CoV-2 RBD serology based on the Roche Elecsys system. Castelletti N*, Paunovic I*, Rubio-Acero R*, Beyerl J, **Plank M**, Reinkemeyer C, Kroidl I, Noreña I, Winter S, Olbrich L, Janke C, Hoelscher M, Wieser A; KoCo19/ORCHESTRA Working group. Microbiol Spectr. 2024 Mar 1:e0288523. doi: 10.1128/spectrum.02885-23. Epub ahead of print. PMID: 38426747.

2023

Clinical and immunological benefits of full primary COVID-19 vaccination in individuals with SARS-CoV-2 breakthrough infections: A prospective cohort study in non-hospitalized adults. Prelog M*, Jeske SD*, Asam C*, Fuchs A*, Wieser A, Gall C, Wytopil M, Mueller-Schmucker SM, Beileke S, Goekkaya M, Kling E, Geldmacher C, Rubio-Acero R, **Plank M,** Christa C, Willmann A, Vu M, Einhauser S, Weps M, Lampl BMJ, Almanzar G, Kousha K, Schwägerl V, Liebl B, Weber B, Drescher J, Scheidt J, Gefeller O, Messmann H, Protzer U, Liese J, Hoelscher M, Wagner R, Überla K, Steininger P; CoVaKo Study Group. J Clin Virol. 2023 Nov 28;170:105622. doi: 10.1016/j.jcv.2023.105622. Online ahead of print.

The Prospective COVID-19 Post-Immunization Serological Cohort in Munich (KoCo-Impf): Risk Factors and Determinants of Immune Response in Healthcare Workers. Reinkemeyer C*, Khazaei Y*, Weigert M, Hannes M, Le Gleut R, **Plank M,** Winter S, Noreña I, Meier T, Xu L, Rubio-Acero R, Wiegrebe S, Le Thi TG, Fuchs C, Radon K, Paunovic I, Janke C, Wieser A, Küchenhoff H, Hoelscher M, Castelletti N; KoCo-Impf/ORCHESTRA working group. Viruses. 2023 Jul 18;15(7):1574. doi: 10.3390/v15071574.

The representative COVID-19 cohort Munich (KoCo19): from the beginning of the pandemic to the Delta virus variant. Le Gleut R*, **Plank M*,** Pütz P, Radon K, Bakuli A, Rubio-Acero R, Paunovic I, Rieß F, Winter S, Reinkemeyer C, Schälte Y, Olbrich L, Hannes M, Kroidl I, Noreña I, Janke C, Wieser A^{+}, Hoelscher M^{+}, Fuchs C^{+}, Castelletti N^{+}; KoCo19/ORCHESTRA-study group. BMC Infect Dis. 2023 Jul 13;23(1):466. doi: 10.1186/s12879-023-08435-1.

Impact of Omicron Variant Infection on Assessment of Spike-Specific Immune Responses Using the EUROIMMUN Quan-T-Cell SARS-CoV-2 Assay and Roche Elecsys Anti-SARS-CoV-2-S. Ahmed MIM*, **Plank M***, Castelletti N, Diepers P, Eser TM, Rubio-Acero R, Noreña I, Reinkemeyer C, Zapf D, Hoelscher M, Janke C, Wieser A^{+}, Geldmacher C^{+}, On Behalf Of The KoCo/Orchestra Study Group. Diagnostics (Basel). 2023 Mar 8;13(6):1024. doi: 10.3390/diagnostics13061024.

2022

Enhanced Spike-specific, but attenuated Nucleocapsid-specific T cell responses upon SARS-CoV-2 breakthrough versus non-breakthrough infections. Ahmed MIM*, Diepers P*, Janke C, **Plank M,** Eser TM, Rubio-Acero R, Fuchs A, Baranov O, Castelletti N, Kroidl I, Olbrich L, Bauer B, Wang D, Prelog M, Liese JG, Reinkemeyer C, Hoelscher M, Steininger P, Überla K, Wieser A, Geldmacher C. Front Immunol. 2022 Dec 13;13:1026473. doi: 10.3389/fimmu.2022.1026473. eCollection 2022.

1. Contribution to the Publications

1.1 Contribution to Paper I

The manuscript titled “The representative COVID-19 cohort Munich (KoCo19): From the Beginning of the Pandemic to the Delta Variant” aims to estimate the cumulative seroprevalence of SARS-CoV-2 infections in the general population of Munich from April 2020 to November 2021. It tracks the progression of the pandemic, assesses the impact of vaccination on antibody development, and investigates the factors influencing infection risk. I led in managing the KoCo19 cohort directly at the study site. My responsibilities included organising the KoCo19 study during and between sampling rounds; actively participating in and leading the coordination of study rounds and cohort maintenance; adjusting the study questionnaires; organising and coordinating the DBS kit shipments to participants; and communicating with participants in various forms. For example, I managed the study hotline and the process of creating and sending result letters to inform participants about their blood sample results. Direct engagement with participants and collaboration with both our institute and external partners was crucial for ensuring successful and well-executed study rounds.

In terms of manuscript preparation, I made significant contributions to writing the manuscript, offering insights and perspectives based on the data collected. Additionally, I conducted literature research, coordinated with co-authors, and played a pivotal role in the submission, revision, and resubmission of the manuscript. I was also actively involved in interpreting the results and conducting preliminary research, ensuring a comprehensive and accurate representation of our findings. The shared first authorship results from the division of work for the project between Ronan Le Gleut and myself. As outlined, the division of the first authorship reflects the division of work throughout the study and the manuscript preparation. While I took on the bulk of the practical work including planning and organisation, Ronan Le Gleut primarily focused on statistical analyses and related tasks, without direct involvement in the study implementation. The collaboration led to the successful publication of the study findings.

1.2 Contribution to Paper II

The Corona-Vakzin-Konsortium (CoVaKo) project is supported by the Bavarian Ministry for Science and Art. In a collaborative effort, all Bavarian University Hospitals aimed to compare the clinical and serological characteristics between unvaccinated and fully vaccinated individuals. For this study collected data during the Alpha and Delta waves of breakthrough (BTI) and non-breakthrough infections (non-BTI).

As part of the LMU study site, my primary responsibilities included participant recruitment in cooperation with the local health authorities and our partner study sites, organisation, and communication. I served as the primary point of contact for participants, guiding them through the recruitment and study process. I also conducted household visits to collect questionnaire data. Furthermore, it included result communication from our site to the participants, via personalised result letters. I also ensured seamless collaboration with our partner universities across Bavaria. For our site, I coordinated efforts to synchronize procedures and data collection methods across the sites, as well as sample sharing, contributing to the study's cohesive and standardised approach. I collaborated with the design of a streamlined data flow system for collaboration with

other study sites, ensuring efficient data collection and analysis. I contributed to the design planning of the analysis. Nevertheless, a study of this magnitude cannot be conducted alone and requires collaboration from a vast number of contributors. Hence, the large number of authors.

1.3 Contribution to Paper III

The manuscript titled “Impact of Omicron Variant Infection on Assessment of Spike-Specific Immune Responses Using the EUROIMMUN Quan-T-Cell SARS-CoV-2 Assay and Roche Elecsys Anti-SARS-CoV-2-S” sought to assess the Quan-T-Cell SARS-CoV-2 assay's performance by comparing IFNy concentrations between the original "Wuhan" and Omicron-based stimulator tubes. Additionally, it examined the influence of Omicron infections on the Roche Elecsys anti-SARS-CoV-2 anti-S assay in comparison to earlier pandemic phases.

Prior to the start of the data collection, I led the adjustment of the study protocol and updated, and adjusted the questionnaire that was used during the visits. Additionally, I led, and carried out participant recruitment in cooperation with health authorities, organised visits including the actual sample collection and ensured smooth and complete data collection, right with the start of the Omicron wave in Germany. We were among the first to collect samples from Omicron-infected individuals. In the context of manuscript preparation, I conducted the literature research, significantly contributed to manuscript preparation, took part in the analysis, and actively coordinated the work with co-authors throughout the submission, revision, and resubmission phases of the manuscript to the journal. These aspects underline my role in the active sample collection, writing of the manuscript, and overall execution of the study. However, Mohamed I. M. Ahmed contributed significantly to the study through his work in the lab and involvement in manuscript and graphics creation. For this reason, we share the first authorship.

2. Introduction

2.1 Background

2.1.1 Chronology of the COVID-19 Pandemic

In January 2020, initial reports emerged of a novel respiratory disease suggesting confirmed cases in Wuhan, China (1–3). Shortly after, the first cases outside China were confirmed in Thailand, followed by Japan and South Korea (1,4–6). The first COVID-19 death was reported on January 11, 2020 (7). By late January 2020, confirmed cases emerged in the US and France, marking the virus's entry into Europe (6,8). Our institute reported the first case in Munich, Germany shortly after, leading to the identification of a cluster of over 10 infected individuals (9). Virus transmission increased rapidly, driven by spreading events at private or public gatherings (10). In Germany, the first such event was a carnival festivity (11,12), followed by skiing tourists in Ischgl and football matches in Italy (13,14). Subsequently, the WHO declared COVID-19 a pandemic in March 2020 (15).

Early in the pandemic, uncertainty prevailed, particularly regarding the potential for asymptomatic transmission of COVID-19 (16). In the absence of vaccines and specific medications, non-pharmaceutical interventions like facemasks, social distancing, and lockdowns were the primary measures implemented (17–20). In Germany, the first lockdown spanned from March until May 2020, followed by subsequent partial and complete lockdowns (17,19,21,22). Facemasks became mandatory in April 2020 for certain situations (18). The second lockdown extended from December 2020 until May 2021 (19,23). Later strategies, such as the “Bundesnotbremse” and “Infektionsschutzmaßnahmen” were introduced (24–26). These regulations were largely guided by incidence rates, which were also pivotal in the public discourse (27–29). This rate, used to gauge healthcare system burden and trigger interventions, was derived from PCR and antigen testing (30–33). These tests were not always universally available, and factors such as timing and viral load affected their accuracy (34). Consequently, asymptomatic cases and mild infections were often missed, leading to underreporting in official statistics. Nevertheless, expanding COVID-19 testing services was vital for controlling incidence rates. Initially, testing was limited to certain high-risk groups (e.g. symptomatic individuals and high-risk groups) (35–38). This changed with increasing capacities, leading to the development of an official testing strategy (39–41). By July 2020, capacities had expanded to the extent that all citizens could be tested free of charge (42). Concurrently, vaccine development progressed rapidly. By December 2020, the first vaccine was administered in Germany (6,43). Initially, vaccine shortages necessitated strict prioritization (44–46). With increased availability and accumulating evidence regarding safety and effectiveness, recommendations and approvals were adapted accordingly (47,48).

The emergence of new virus variants altered the pandemic’s trajectory. They contributed to multiple waves of infections in Germany. In Munich, 6 big waves of infections could be identified, from January 2020 to April 2022 (as displayed in **Figure 1**) (49,50).

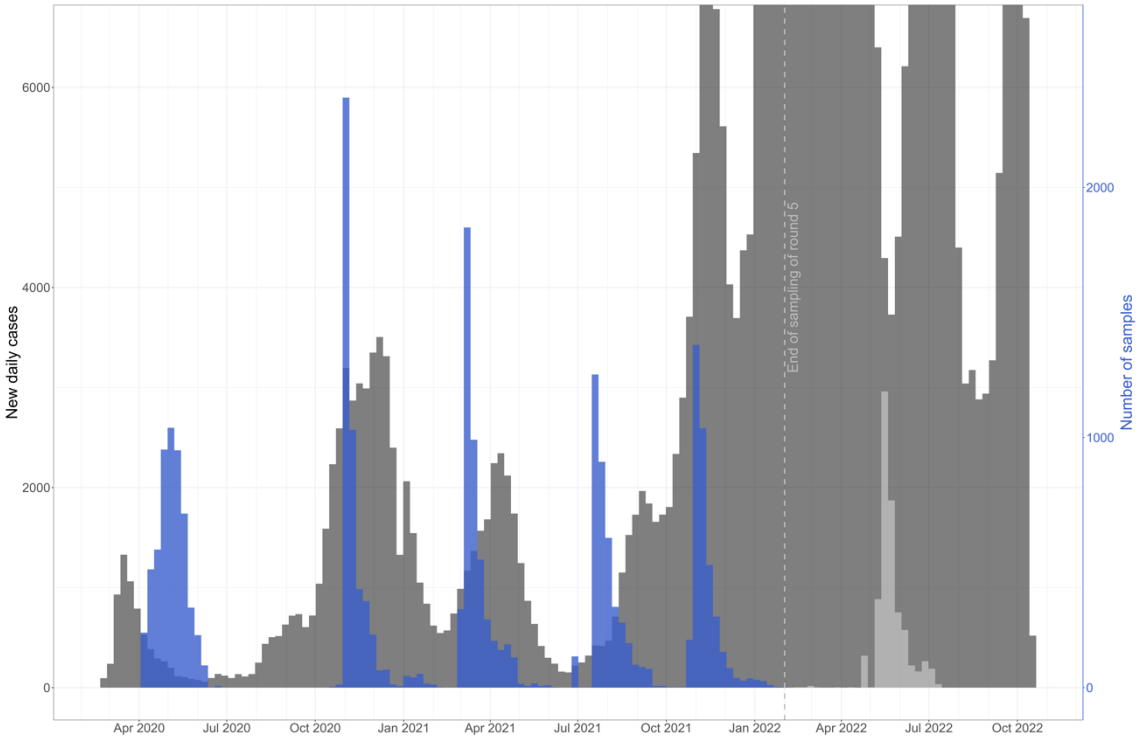

Figure 1: Development of COVID-19 infection numbers in Munich with regard to KoCo19 sample collection. In black, daily infections as reported by the Robert-Koch Institute (RKI). In blue, daily collected samples from baseline and to the fifth round of KoCo19 after or during infection peaks. In light grey, the sixth round in mid-2022, which was not included in the publication and doctoral project. Adapted and reprinted from (51).

While various variants of interest and concern (VOI and VOC) emerged, only a few became predominant globally. The initial virus strain – the wild type – was superseded by the Alpha variant in early 2021 (52). During this period, vaccination efforts were already well underway, with over 15 million doses administered to the German population by mid-April 2021 (53). The Delta variant subsequently emerged, overtaking Alpha in mid-2021 (54). By the end of 2021 and the beginning of 2022, Omicron had become the dominant variant, raising significant concern due to its increased transmissibility (47,55–61). At that time, several effective vaccines were widely available in Germany. More than half of the population had received at least one dose (53). These factors contributed to a shift in infection patterns. The study found a rise in breakthrough infections (BTIs), likely due to the Omicron variant's ability to evade vaccine induced immunity (62–64). Although these cases were generally less severe, their sheer number was causing concern (65,66). In response, a third booster shot was promoted, and the PCR testing strategy was adjusted to fit the increased demand (67,68).

In 2022, after the peak of the Omicron wave, several restrictions were relaxed, including the shortening of the isolation period in case of an infection. Additionally, hotspot rules were implemented to focus measures on regions with high incidence rates (68). By April 2023, all regulations in Germany had been lifted (69). In May 2023, the WHO declared COVID-19 no longer a global public health emergency (70).

Given these dynamics, the need for comprehensive research to understand the effect of public health measures, the prevalence of antibodies in the population, vaccine effectiveness, and the impact of emerging variants on laboratory methods became evident. At our institute, several pivotal studies were conducted to address these gaps, among those were:

- **"Prospektive Kohorte COVID-19 München (KoCo19)"**: Aimed at providing a comprehensive overview of the pandemic's progression in Munich
- **"COVID Vakzin Konsortium (CoVaKo)"**: Focused on analysing the effectiveness of COVID-19 vaccines

- **“Prospektive Kohorte COVID-19 München – Immunologie (KoCoImmu)”**: Investigated the factors underlying the clinical progression and subsequent the transmission of COVID-19; the effect of the Omicron variant on laboratory methods and immune responses

2.1.2 Immunological Insights into SARS-CoV-2

SARS-CoV-2 - the causative agent of COVID-19 – belongs to the family of Coronaviridae (71). The virus size measures from 80-140 nm, with single-stranded RNA enclosed within a membrane with spike proteins (71–75). The spike protein, approximately 20-25 nm in size (see **Figure 2**), is crucial in the virus's interaction with host cells (72).

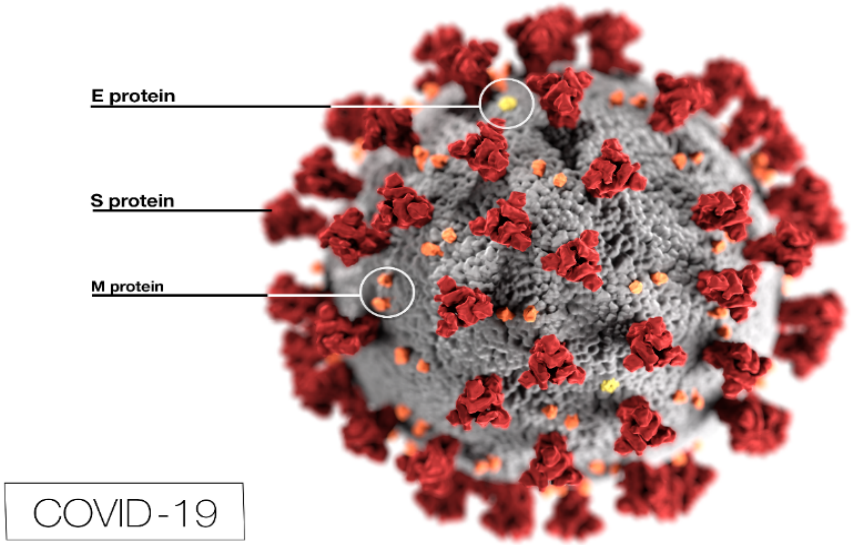

Figure 2: The first public domain picture of SARS-CoV-2 created by the Centers for Disease Control and Prevention. The proteins on the surface are displayed, the biggest one – the spike protein – in red is crucial for the virus ability to infect host cells. On the inside, there is a single-stranded RNA. Reprinted from (76).

SARS-CoV-2 primarily infects respiratory epithelial cells. Upon encountering them, SARS-CoV-2 uses its spike proteins to bind to angiotensin-converting enzyme 2 receptors on the cell surface, facilitating viral entry (2,71,75,77,78). The spike proteins consist of two parts: S1, which contains the receptor binding domain, responsible for binding to the host cell, and S2, which facilitates the fusion with the host cell membrane (79–84). Once bound to the cell, the virus releases its RNA genome (75,85).

The exact origin of SARS-CoV-2 remains uncertain. The most likely case is zoonotic transmission, from an animal, possibly involving an intermediate host, to the first human case (86,87). SARS-CoV-2 shares similarities with SARS-CoV (88–90). Although, it exhibits higher transmissibility compared to SARS-CoV (91). Like many RNA viruses, SARS-CoV-2 undergoes genetic changes, leading to the development of new variants (92–94). These variants arise through mutations in the viral genome, particularly in regions encoding key proteins such as the spike protein (95–99). Variants of concern (VOCs) exhibit significant changes in transmissibility, virulence, or the effectiveness of public health measures and vaccines compared to others (94). Understanding the genetic diversity of the virus is crucial for effective surveillance, prevention, and control of COVID-19.

The WHO developed a scale to classify SARS-CoV-2 infection severity based on minimal common outcome methods. It categorizes cases as uninfected, ambulatory mild disease, hospitalized with moderate disease, hospitalized with severe disease, or dead (100). Common

symptoms including cold-like symptoms, fatigue, and muscle or headaches, typically manifesting 5-6 days post-exposure (5,101). Additionally, loss of smell and taste has been reported, occasionally persisting beyond the acute infection (102). Mutations can influence symptom severity and presentation, highlighting the virus's dynamic nature (102,103). Vaccination against COVID-19 has proven effective in reducing disease severity, mortality, and the risk of long COVID, even newer mutations across diverse populations (104–110).

Upon infection, the immune system is activated. Initially, the innate immune response recognises viral components, leading to the production of interferons and pro-inflammatory cytokines (111–116). The main goal of this response is to minimize viral replication, eliminate infected cells, and activate the adaptive immune system (111,115–117). The adaptive immune system can be divided into three main components: B cells, which produce antibodies, CD4+T cells, which can act as effectors and CD8+T cells, which terminate infected cells (118). Once activated, SARS-CoV-2 specific antibodies, as well as CD4+T and CD8+T cells, are produced (115,118,119). Beyond the acute reaction, long-term immunity is enabled by memory T cells (120). **Figure 3** illustrates the infection and immune response process.

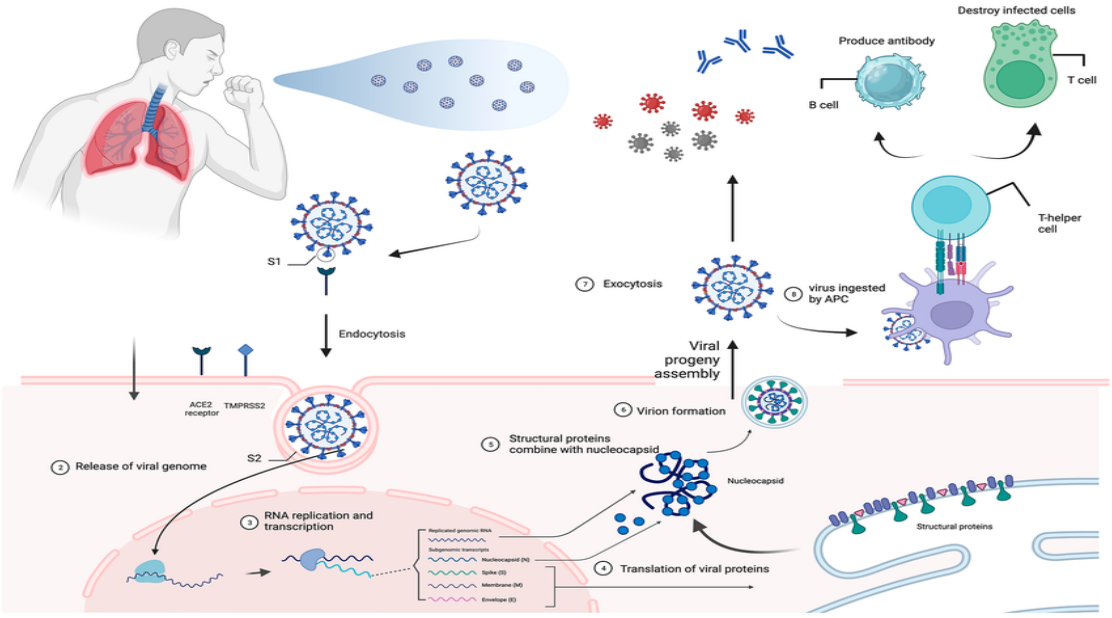

Figure 3: Progression of infection, from the fusion of SARS-CoV-2 with the host cell to the subsequent immune response of the host. After the infection, the immune response is triggered and antibodies develop. Reprinted from (121).

There are different SARS-CoV-2 antibodies produced by the immune system. For this thesis, the most relevant antibodies are those targeting the Spike (S) and Nucleocapsid (N) proteins. Anti-Spike antibodies (anti-S) target the virus's spike proteins (122). In contrast, anti-Nucleocapsid-antibodies (anti-N) target the nucleocapsid protein inside of the virus membrane (123). The presence and concentration of these antibodies can be measured in blood samples, after an infection or vaccination to assess the effect (124–126).

Vaccines have played a pivotal role in combating the virus and pandemic. Novel mRNA vaccines elicit an immune response against the virus's spike proteins (127–135). By enabling the immune system to produce antibodies against the spike protein prior to infection, its ability to prevent infection or severe disease is increased (136–139). Numerous studies have demonstrated their safety and efficacy in providing robust protection against severe manifestations of the disease (136,140–142). However, vaccines do not offer complete protection from infection, resulting in BTIs (143–146).

2.2 Rationale and Study Objectives

2.2.1 Rationale

Facing the challenges described in the previous chapters, numerous studies aimed at understanding the virus were carried out. This thesis focuses on three studies undertaken at our institute.

“The representative COVID-19 cohort Munich (KoCo19)”, commenced in April 2020 (147). To our knowledge, it is the last ongoing representative cohort study focused on COVID-19 in Germany. Baseline data, including blood samples and questionnaires, were collected from April to June 2020 (147). Using a random walk approach to select participants and implementing statistical methods, the cohort of 5313 participants is representative of Munich (51,147). Participants older than 13 years were included into sample collection (147). The study aimed to better understand the true number of infections within the cohort based on the seroprevalence of antibodies and identify risk factors for infection (147). Our strategy relied on detecting antibodies induced by SARS-CoV-2 infection or vaccination. This approach is advantageous, especially compared to PCR-based studies, as it can identify past infections and asymptomatic cases can be found.

In this context, the presence of anti-N-antibodies points towards a past COVID-19 infection. The detection of anti-S-antibodies, allows us to conclude an infection and vaccination (148,149). Questionnaires complemented antibody testing by providing dates of vaccinations and infections. Initially, whole blood samples were collected to analyse the seroprevalence of SARS-CoV-2 specific antibodies. Through extensive validation, it was possible to transfer the measurements to dried blood samples (DBS) consisting of capillary blood from the finger, captured on filter papers (Anti S/N Paper). This approach has the great advantage that the blood can be self-sampled by the participants, significantly reducing the required resources from the study site. At baseline, around 50 fieldworkers worked several weeks to include participants and take samples (147). This effort was significantly reduced in the follow-ups by switching to the DBS method. Follow-ups were conducted after major infection waves (see **Figure 1**) (51). The periods and context of the follow-ups are described in **Figure 4**.

Besides this, the institute was involved in COVID-19 research via the Corona-Vakzin-Konsortium (CoVaKo) to study the effectiveness and safety of COVID-19 vaccinations. This collaboration among all Bavarian university hospitals aimed to understand the disease's clinical course and the immune response (150). Factors such as disease severity, symptomatology, and immune response were compared between BTIs and non-BTIs. Recruitment commenced in April 2021 and lasted until November (**Figure 5** shows the context of the time period). A total of 300 individuals above 18 years, who tested positive for COVID-19, were enrolled shortly after their initial positive PCR test. Subsequently, the initial visit was scheduled as soon as possible. Following the initial visit, there were three more visits with approximately seven-day intervals between them. Visits five and six could occur after six and nine months, respectively (150).

Recruitment was particularly challenging for non-BTI cases, and each visit required significant resources in outreach and laboratory work. Consequently, the participant number was smaller (300 participants in total with 50 at our study site) compared to KoCo19. In total, up to six visits were possible per participant. Participants were divided into BTI and non-BTI cases. During visits, medical data, vital parameters, swabs, and blood samples were collected, and the severity of symptoms was categorized (150). Based on the collected blood samples, viral load, and antibody

levels were measured for each visit. The analysis of the SARS-CoV-2-specific antibody response involved determining the concentrations of antibodies directed against the spike-antigen and nucleocapsid-antigen (150).

Due to the frequent mutations of SARS-CoV-2, continual adaptation and validation of diagnostic tools were necessary. The KoCoImmu study, a sub-study of KoCo19, addressed this need by targeting the recruitment of individuals infected with the Omicron variant. The study aimed to identify factors underlying the clinical progression and subsequent transmission of COVID-19 (151). Participants were recruited from December 2021 to March 2022, as illustrated in **Figure 6** depicting the pandemic situation during this period. A total of 37 participants, all aged 18 or older, were recruited and categorized into BTI and non-BTI. Potential participants either contacted the study team directly or were referred by health authorities to it. Following a PCR-confirmed Omicron infection, a visit was scheduled for approximately 21 days later. During these visits, whole blood and DBS were collected (151).

The Quan-T-Cell SARS-CoV-2 Interferon gamma release assay (IGRA) was employed using both an original Wild type and an adapted Omicron-based IGRA tube to assess differences. Additionally, anti-S-antibodies were measured to evaluate the immune response (151). This approach allowed us to analyse the impact of Omicron's numerous mutations on the results obtained from standard procedures used in our COVID-19 labs. However, recruiting non-BTI and never before infected cases proved challenging. Combined with the substantial amount of work required for each participant encompassing both field and laboratory efforts, the study ultimately had a relatively small sample size. This approach was also adopted to ensure the timely completion of the study.

These studies just described underscore the paramount importance of research aimed at unravelling the complexities of the pandemic and advancing knowledge. Such research plays a pivotal role in shaping decisions made by policymakers, researchers, and individuals.

2.2.2 Objectives

The overarching objective of this doctoral project is to integrate findings from the three key studies described in the previous chapter, conducted at our institute, to construct a cohesive narrative about the progression of the COVID-19 pandemic and its impact in Munich.

Given the rapidly expanding field of COVID-19 research, it is imperative to approach the topic from various perspectives to understand the virus's spread, the impact of emerging variants, and the differences between BTI and non-BTI cases.

The primary aim of this thesis is to synthesize various strands of COVID-19-related research conducted at the institute to provide comprehensive insights. The objectives are structured around the following key questions:

1. **How has the number of infections, as indicated by seroprevalence among KoCo19 study participants, evolved throughout the COVID-19 pandemic in Munich?**
    - What were the temporal changes in seroprevalence observed in the study population?
    - How did different risk factors influence seroprevalence across the study period?
    - What impact did vaccination status have on seroprevalence rates?

2. **How do discrepancies manifest between BTIs and non-BTIs, and how can these distinctions be characterized?**
    - What clinical symptom differences were observed between BTIs and non-BTIs?
    - How did the immunological responses, including antibody levels and neutralization capacity, differ between BTIs and non-BTIs?
3. **What impact have emerging SARS-CoV-2 variants had on the study population and research methodologies?**
    - How did new variants affect infection rates and immune responses?
    - How did the performance of various laboratory methods compare when assessing immune responses to different variants?

This approach ensures that the thesis reflects a comprehensive and multi-faceted understanding of the COVID-19 pandemic in Munich, drawing from the collaborative efforts of multiple studies.

2.3 Publications and their Contribution to the Research Questions

The following section will provide contextualisation for the individual parts of the doctoral project.

2.3.1 Paper I

The representative COVID-19 cohort Munich (KoCo19): from the beginning of the pandemic to the Delta virus variant.

The KoCo19 study commenced in April 2020 with the primary objective of developing a robust tool capable of accurately assessing SARS-CoV-2 prevalence in the population. This approach was crucial for understanding the true number of infections, including asymptomatic cases, within the cohort and identifying risk factors for infection.

The pandemic's trajectory was highly uncertain and challenging to predict. In retrospect, we now understand that the period until the 4th follow-up of the study witnessed the emergence of multiple predominant VOCs and the commencement of the vaccine campaign (for further information see **Figure 4)** (43,152). During this time, we developed a reliable tool capable of accurately assessing SARS-CoV-2 antibody prevalence in the population via blood tests (147). As described, the method relied on the measurement of SARS-CoV-2 specific antibodies. By detecting these antibodies, it was possible to detect past infections and gather information on the effect of vaccinations. Our sampling strategy ensured data collection at critical time points. This way we contributed valuable information on true infection numbers and could compare official PCR-based numbers to our values. This is the biggest advantage of this approach.

The KoCo19 study results directly contribute to the project's objectives by:

- **Accurate estimation of infection numbers**: Providing a more accurate estimation of infection numbers in Munich and highlighting underreporting. Furthermore, our data suggested lower infection prevalence in the vaccinated population compared to the non-vaccinated
- **Understanding seroprevalence factors**: Classifying participants into various groups, enhancing our understanding of seroprevalence factors

- **Identifying disparities**: Collecting data on vaccination rate disparities between our cohort and the general population, revealing noteworthy differences
- **Impact of infection waves**: Depicting the impact of infection waves on antibody levels in the population

These findings addressed key research questions related to the evolution of seroprevalence, underreporting, and the effects of different virus variants on the population and research methodologies.

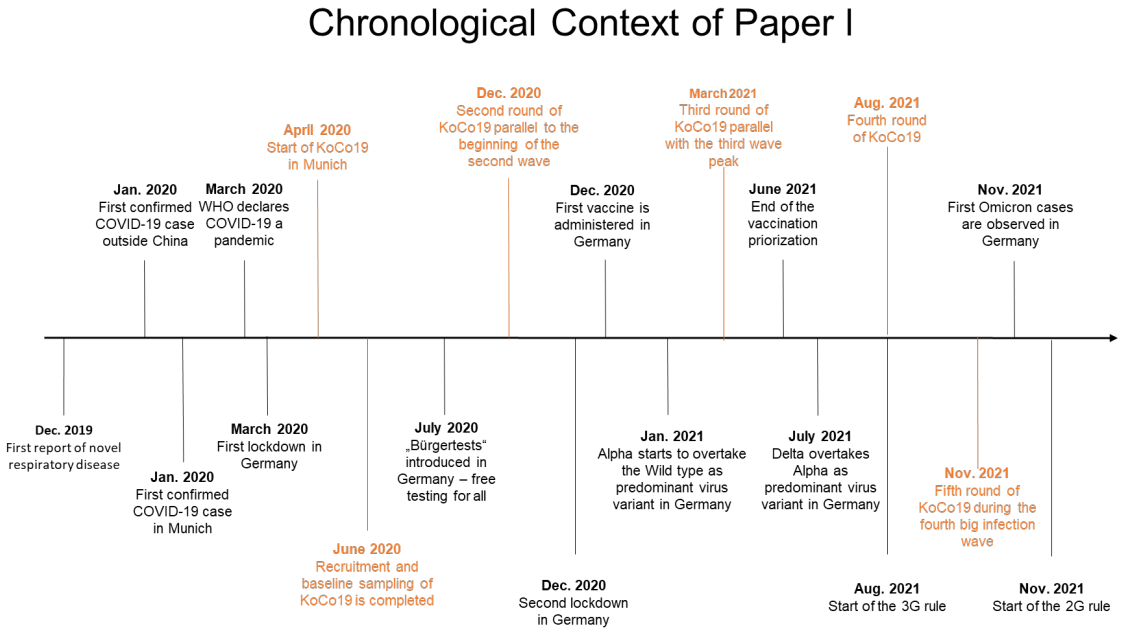

Figure 4: The timely context of KoCo19. Important events that had an influence on the progression of the pandemic, such as lockdowns, the emergence of new VOCs and the first vaccination are shown in black. The sampling time periods are displayed in orange. Further, the individual rounds of the study are displayed. Self-created graphic.

2.3.2 Paper II

Clinical and immunological benefits of full primary COVID-19 vaccination in individuals with SARS-CoV-2 breakthrough infections: A prospective cohort study in non-hospitalized adults.

Given the rising number of BTIs, it became increasingly relevant to examine the clinical course and immune response in more detail. To address this need, the CoVaKo study was conducted. Comparing immune responses between non-BTIs and BTIs was essential for identifying potential disparities, thereby enhancing our understanding of the virus. This project commenced in April 2021, with the goal of enrolling individuals who had tested positive for COVID-19 via PCR test. Each participant could undergo up to six visits over a period of six to nine months (150). During the study period, the Alpha and Delta VOC were predominant, and the vaccination rate was steadily increasing (more information on the context can be found in **Figure 5**).

Recruitment efforts targeted both BTIs and non-BTIs, allowing for comprehensive comparisons across various parameters. This approach aimed to represent the clinical course of an infection and illustrate immunological aspects. The objective was to construct a detailed comparison of symptoms and their severity across the two groups, alongside an evaluation of their immunological responses. This comprehensive approach enabled a more nuanced understanding.

The study results directly contribute to the project's objectives by:

- **Clinical and immunological understanding of BTIs**: Providing a detailed understanding of BTIs from both clinical and immunological perspectives
- **Disparities between BTIs and non-BTIs**: Assessing disparities in the clinical and immunological course of infection between BTIs and non-BTIs
- **Impact of VOCs on infections**: Elucidating the impact of VOCs on infections, their contribution to BTIs, and their role in altering infection dynamics

These findings addressed key research questions related to the differences in clinical symptoms, immune responses, and the effects of different virus variants on BTIs and non-BTIs

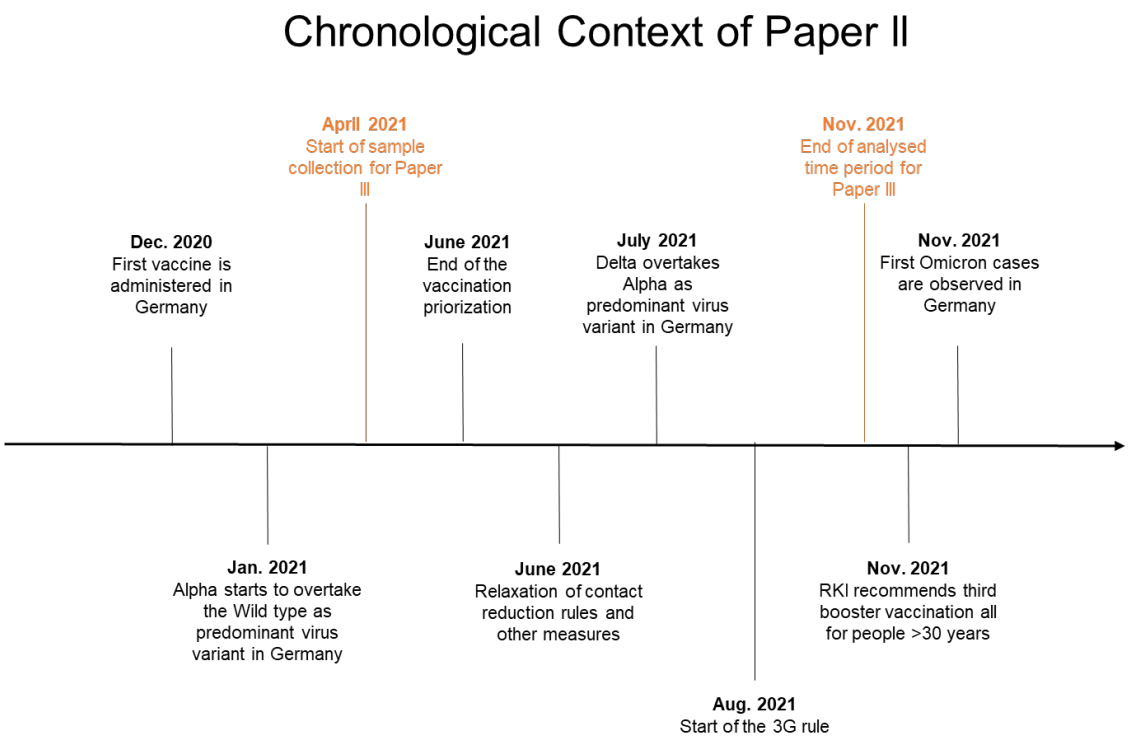

Figure 5: The timely context of the CoVaKo study. In orange the start and end of the sampling period is marked, in black important developments influencing the study are mentioned. Self-created graphic.

2.3.3 Paper III

Impact of Omicron Variant Infection on Assessment of Spike-Specific Immune Responses Using the EUROIMMUN Quan-T-Cell SARS-CoV-2 Assay and Roche Elecsys Anti-SARS-CoV-2-S.

In December 2021, incidence rates were slightly declining from the Delta wave but reached new heights in January 2022 due to the Omicron variant (compare **Figure 1**) (153–156). During this period, vaccines and extensive testing capacities were widely available, and Omicron VOC became predominant in late 2021 (see **Figure 6** for context). A rapid response to this new development was essential. Consequently, we began recruiting Omicron infected participants for the KoCoImmu study in early December 2021 on. The goal was to examine the impact of the numerous mutations in the spike protein on our methods and to determine whether there were differences between the immune responses of BTIs and non-BTIs. This approach allowed us to analyse the performance of our methods and compare results between different groups.

Specifically, antibody and T cell response measurements were utilised throughout the KoCo19 and its sub-studies as well as the CoVaKo study, to analyse the immune response (51,157). These established methods enabled comprehensive and consistent analysis of the immune

response across different phases of the pandemic, providing valuable insights into the effectiveness of vaccines and the impact of new variants. Assessing the effect of Omicron on these methods was especially crucial to ensure their continued reliability and accuracy. The KoCoImmu study results directly contribute to the project's objectives by:

- **Robustness of laboratory methods**: Demonstrating that our laboratory methods remain effective in yielding reliable results despite the mutations in the Omicron variant
- **Understanding immune response**: Providing a detailed comparison of antibody levels between BTIs and non-BTIs after Omicron infection, enhancing our understanding of the immune response to different variants
- **Impact of variants on research**: Highlighting the continued relevance and adaptability of our research methods in the face of evolving viral variants

These findings addressed key research questions related to the robustness of our methodologies and the differential immune responses elicited by BTIs and non-BTIs with regard to the newest VOC, thereby contributing to a comprehensive understanding of the pandemic's progression and the ongoing challenges posed by emerging variants.

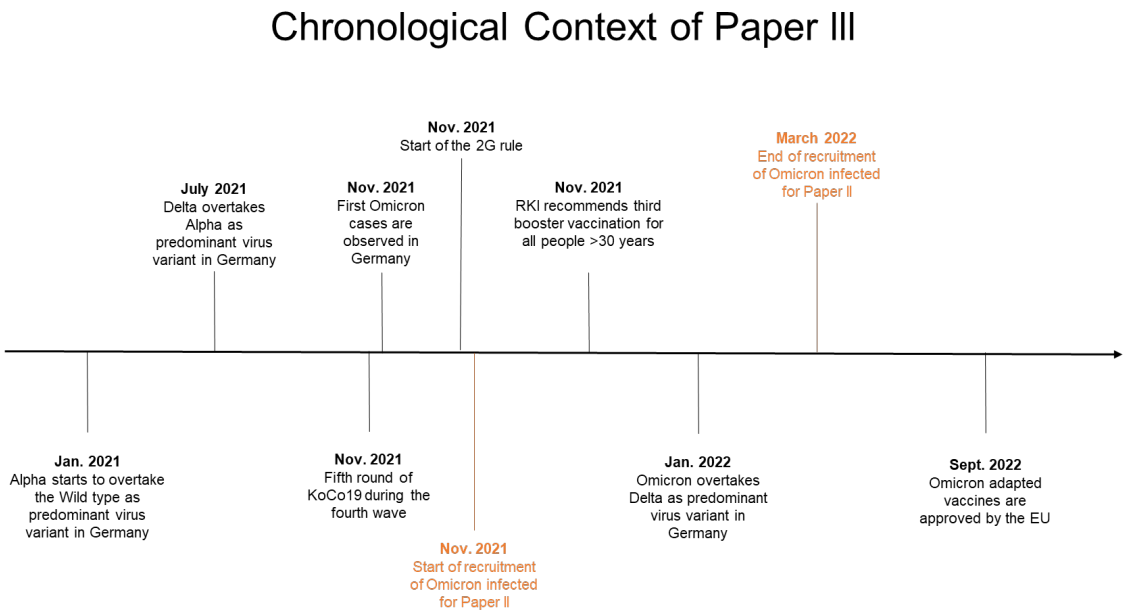

Figure 6: The timely context of the KoCoImmu Omicron focused study. In orange the start and endpoint of the sampling period is displayed. In black development of the pandemic, influencing the study is described. Self-created graphic.

3. Summary

The emergence of COVID-19 as a pandemic in 2020 presented unprecedented challenges to the global community. Rapid and extensive research was crucial to navigate this crisis. This doctoral project was embedded in three of the COVID-19 related studies conducted at our institute. The overarching objective it was to combine their results into a comprehensive overview of the progression of the COVID-19 pandemic in Munich, from its inception to the emergence of the Omicron variant. To achieve this, the KoCo19 study explored the evolution of infection numbers, throughout the pandemic in Munich via blood samples. It also examined the impact of emerging SARS-CoV-2 variants on both the population and the methodological laboratory frameworks employed. Furthermore, the study investigated how discrepancies manifested between BTIs and non-BTIs, and through what avenues these distinctions could be described. The resulting publication "*The representative COVID-19 cohort Munich (KoCo19): from the beginning of the pandemic to the Delta virus variant" is a part of this dissertation.*

The KoCo19 study monitored the seroprevalence of COVID-19 antibodies among over 5,000 participants from the onset of the pandemic until after the Delta wave. Using the Roche Elecsys® Anti SARS CoV 2 anti-N assay to detect past infections and the anti-S assay to identify preceding vaccinations or infections, we were able to differentiate the sources of antibodies in the population. This study comprised five rounds (baseline and four follow-ups) at critical points of the pandemic, producing representative results for Munich's population. The cumulative incidence rose significantly from 1.6% in May 2020 to 14.5% by November 2021. Risk factors identified included being born outside Germany, working in high-risk jobs, and lower living area per inhabitant. The study also revealed significant underreporting of infections in official numbers, with higher infection prevalence in the unvaccinated population. By November 2021, 86.8% of the Munich population had developed anti-S and/or anti-N-antibodies.

Conducted from April to November 2021 across Munich and five other Bavarian centers, the CoVaKo study assessed the impact of COVID-19 vaccination on symptoms and immunogenicity of SARS-CoV-2 VOC infections. The resulting publication, "*Clinical and immunological benefits of full primary COVID-19 vaccination in individuals with SARS-CoV-2 breakthrough infections: A prospective cohort study in non-hospitalized adults"* constitutes the second *part* of this project. During this period, most adults had received at least one vaccine dose, and the predominant VOCs were Alpha and Delta. The study included 212 BTIs and 88 non-BTIs, recruited within 14 days of PCR-confirmed infection, followed by weekly visits for sample and questionnaire data collection. Results indicated that full primary vaccination significantly reduced symptom severity and duration in BTI cases for five symptoms. Fully vaccinated BTIs exhibited higher relative avidity index and anti-S-IgG avidity at all time points, with higher anti-S-antibody levels across all visits compared to non-BTIs. These findings suggest that two-dose vaccination reduces symptom frequency in BTI cases and elicits a more rapid and sustained neutralization capacity against the infecting VOC compared to unvaccinated individuals.

The third project is based on the KoCoImmu study. It led to the publication "*Impact of Omicron Variant Infection on Assessment of Spike-Specific Immune Responses Using the EUROIMMUN Quan-T-Cell SARS-CoV-2 Assay and Roche Elecsys Anti-SARS-CoV-2-S"* which is included in this thesis. Data collection coincided with the spread of Omicron in Munich from December 2021 to March 2022. Previously uninfected participants were recruited (BTI n = 20, non-BTI n = 17), and samples were taken approximately 21 days post-positive test. Potential participants either

were reported to the study management via local health authorities or contacted us directly. Blood samples were analysed using the Quan-T-Cell SARS-CoV-2 assays (EUROIMMUN), comparing the Wild type SARS-CoV-2 IGRA tube with a new Omicron-adapted version. Both tests yielded comparable results, with 19 out of 21 samples showing a positive IFNγ response to the original antigen. The Roche Elecsys anti-SARS-CoV-2 anti-S1 test (also used in KoCo19) revealed that Omicron non-BTIs had significantly lower median Spike-specific antibody concentrations, with eight individuals not meeting the positivity cut-off. The assay measures antibodies Spike-specific antibodies based on the original wild type antigen. In contrast, BTIs had detectable antibodies in all measurements, with a 400-fold higher median specific antibody concentration compared to controls. These results indicate that the Omicron-adapted IGRA tubes did not enhance the Quant-T Cell-SARS-CoV-2 assay's performance, and the serological test displayed significant differences in antibody response between BTIs and non-BTIs. Both CoVaKo and the KoCoImmu Omicron approach had much smaller sample sizes than the KoCo19 study. This has several reasons. First, the substantial amount of work required for each participant, encompassing both field and laboratory efforts, made these studies much more labour intensive than the DBS based approach. Furthermore, both studies focused on differences between BTI and non-BTIs. Especially during the later stages of the pandemic, unvaccinated individuals were often unwilling to participate in research for various reasons.

To summarize, we estimated the number of infections within the Munich population using a serological approach and revealed significant discrepancies with the officially reported numbers. Our research highlighted the importance of reassessing measurement tools in the context of VOCs, such as Omicron, which significantly influenced infection rates, immune responses, and vaccine efficacy. Additionally, we observed notable differences between BTIs and non-BTIs in terms of clinical and immunological features. Fully vaccinated individuals experienced milder symptoms and exhibited stronger immune responses compared to unvaccinated individuals, underscoring the benefits of vaccination and the need for tailored public health strategies.

In conclusion, the findings presented in this thesis underscore the critical importance of robust research endeavours in understanding the COVID-19 pandemic. Although the pandemic has ended, COVID-19 remains a global concern, necessitating continued research and vigilance.

4. Zusammenfassung

Das Auftreten der COVID-19 Pandemie im Jahr 2020 stellte die globale Gemeinschaft vor beispiellose Herausforderungen. Rasche und umfassende Forschung war entscheidend, um diese Krise zu bewältigen. Das vorliegende Promotionsprojekt war in drei der am Institut durchgeführten COVID-19-bezogenen Studien eingebettet. Das übergeordnete Ziel dieser Dissertation war es, deren Ergebnisse zu einer umfassenden Übersicht über den Verlauf der COVID-19-Pandemie in München, von ihrem Beginn bis zum Auftreten der Omikron-Variante, zu kombinieren.

Um dies zu erreichen, wurde die Entwicklung der Infektionszahlen während der Pandemie in München anhand von Blutproben beobachtet. Des Weiteren, wurde die Auswirkungen aufkommender SARS-CoV-2-Varianten sowohl auf die Bevölkerung als auch auf die verwendeten methodischen Laborrahmen untersucht. Darüber hinaus wurde analysiert, wie sich Unterschiede zwischen Durchbruchsinfektionen (BTIs) und Nicht-BTIs manifestierten und auf welche Weise diese Unterschiede beschrieben werden konnten.

Die KoCo19-Studie überwachte die Seroprävalenz von COVID-19-Antikörpern bei über 5.000 Teilnehmern vom Beginn der Pandemie bis nach der Delta-Welle. Mit dem Roche Elecsys® Anti-SARS-CoV-2 anti-N Assay zur Erkennung vergangener Infektionen und dem Anti-S Assay zur Identifizierung früherer Impfungen oder Infektionen konnten wir die Quellen der Antikörper in der Bevölkerung differenzieren. Die daraus resultierende Publikation "The representative COVID-19 cohort Munich (KoCo19): from the beginning of the pandemic to the Delta virus variant" ist Teil dieser Dissertation. Diese Studie umfasste fünf Runden (Baseline und vier Follow-ups) zu kritischen Zeitpunkten der Pandemie und lieferte repräsentative Ergebnisse für die Münchner Bevölkerung. Die kumulative Inzidenz stieg signifikant von 1,6 % im May 2020 auf 14,5 % im November 2021. Identifizierte Risikofaktoren umfassten die Geburt außerhalb Deutschlands, Arbeiten in Hochrisikoberufen und eine geringere Wohnfläche pro Einwohner. Die Studie zeigte auch eine erhebliche Unterberichterstattung der Infektionen in den offiziellen Zahlen, mit einer höheren Infektionsprävalenz in der ungeimpften Bevölkerung. Bis November 2021 hatten 86,8 % der Münchner Bevölkerung Anti-S- und/oder Anti-N-Antikörper entwickelt.

Die von April bis November 2021 in München und fünf weiteren bayerischen Zentren durchgeführte CoVaKo-Studie bewertete die Auswirkungen der COVID-19-Impfung auf Symptome und Immunogenität von SARS-CoV-2 VOC Durchbruchsinfektionen. Die daraus resultierende Publikation "Clinical and immunological benefits of full primary COVID-19 vaccination in individuals with SARS-CoV-2 breakthrough infections: A prospective cohort study in non-hospitalized adults" stellt den zweiten Teil dieses Projekts dar. In diesem Zeitraum hatten die meisten Erwachsenen mindestens eine Impfdosis erhalten, und die vorherrschenden VOCs waren Alpha und Delta. Die Studie umfasste 212 BTIs und 88 Nicht-BTIs, die innerhalb von 14 Tagen nach PCR-bestätigter Infektion rekrutiert und wöchentliche Besuche zur Proben- und Fragebogendatenerhebung unterzogen wurden. Die Ergebnisse zeigten, dass die vollständige Grundimmunisierung die Schwere und Dauer der Symptome bei BTI-Fällen für fünf Symptome signifikant reduzierte. Vollständig geimpfte BTIs wiesen zu allen Zeitpunkten einen höheren relativen Aviditätsindex und eine höhere Anti-S-IgG-Avidität auf, mit höheren Anti-S-Antikörperspiegeln über alle Besuche hinweg im Vergleich zu Nicht-BTIs. Diese Ergebnisse deuten darauf hin, dass eine Zweifachimpfung die Symptomhäufigkeit bei BTI-Fällen reduziert und eine schnellere und anhaltendere Neutralisierungskapazität gegen das infizierende VOC im Vergleich zu ungeimpften Personen hervorruft.

Das dritte Projekt basiert auf der KoCoImmu-Studie. Es führte zur Veröffentlichung "Impact of Omicron Variant Infection on Assessment of Spike-Specific Immune Responses Using the EUROIMMUN Quan-T-Cell SARS-CoV-2 Assay and Roche Elecsys Anti-SARS-CoV-2-S", die in diese Dissertation aufgenommen wurde. Die Datenerhebung fiel mit der Ausbreitung von Omikron in München von Dezember 2021 bis März 2022 zusammen. Zuvor nicht infizierte Teilnehmer wurden rekrutiert (BTI n = 20, Nicht-BTI n = 17), und Proben wurden etwa 21 Tage nach dem positiven Test entnommen. Potenzielle Teilnehmer wurden entweder von den örtlichen Gesundheitsbehörden an die Studienleitung gemeldet oder kontaktierten uns direkt. Blutproben wurden mit den Quan-T-Cell SARS-CoV-2-Assays (EUROIMMUN) analysiert, wobei die Wildtyp-SARS-CoV-2 IGRA-Röhre mit einer neuen Omikron-adaptierten Version verglichen wurde. Beide Tests ergaben vergleichbare Ergebnisse, wobei 19 von 21 Proben eine positive IFNγ-Antwort auf das ursprüngliche Antigen zeigten. Der Roche Elecsys anti-SARS-CoV-2 anti-S1 Test (ebenfalls in KoCo19 verwendet) zeigte, dass Omikron Nicht-BTIs signifikant niedrigere mediane Spike-spezifische RBD-Antikörperkonzentrationen aufwiesen, wobei acht Personen den Positivitäts-Grenzwert nicht erreichten. Der Assay misst Spike-spezifische Antikörper basierend auf dem ursprünglichen Wildtyp-Antigen. Im Gegensatz dazu hatten BTIs in allen Messungen nachweisbare Antikörper, mit einer 400-fach höheren medianen spezifischen Antikörperkonzentration im Vergleich zu den Kontrollen. Diese Ergebnisse zeigen, dass die Omikron-adaptierten IGRA-Röhren die Leistung des Quan-T-Cell SARS-CoV-2 Assays nicht verbesserten und der serologische Test signifikante Unterschiede in der Antikörperantwort zwischen BTIs und Nicht-BTIs aufwies. Sowohl CoVaKo als auch der KoCoImmu Omikron-Ansatz hatten deutlich kleinere Stichprobengrößen als die KoCo19-Studie. Dies hat mehrere Gründe. Erstens erforderte jeder Teilnehmer einen erheblichen Arbeitsaufwand, der sowohl Feld- als auch Laboraufgaben umfasste, was diese Studien viel arbeitsintensiver machte als den auf DBS basierenden Ansatz. Darüber hinaus konzentrierten sich beide Studien auf Unterschiede zwischen BTI und Nicht-BTI. Besonders in den späteren Phasen der Pandemie waren ungeimpfte Personen aus verschiedenen Gründen oft nicht bereit, an Forschungsprojekten teilzunehmen.

Zusammenfassend schätzten wir die Anzahl der Infektionen innerhalb der Münchner Bevölkerung mithilfe eines serologischen Ansatzes und enthüllten erhebliche Diskrepanzen mit den offiziell gemeldeten Zahlen. Unsere Forschung hob die Bedeutung der Neubewertung von Messinstrumenten im Kontext von besorgniserregenden Varianten (VOCs) wie Omikron hervor, die die Infektionsraten, Immunantworten und Impfeffizienz erheblich beeinflussten. Darüber hinaus beobachteten wir bemerkenswerte Unterschiede zwischen Durchbruchsinfektionen (BTIs) und Nicht-BTIs in Bezug auf klinische und immunologische Merkmale. Vollständig geimpfte Personen erlebten mildere Symptome und zeigten stärkere Immunantworten im Vergleich zu ungeimpften Personen, was die Vorteile der Impfung und die Notwendigkeit maßgeschneiderter Strategien im Bereich der öffentlichen Gesundheit unterstreicht.

Abschließend unterstreichen die in dieser Dissertation präsentierten Ergebnisse die kritische Bedeutung robuster Forschungsbemühungen zum Verständnis der COVID-19-Pandemie. Obwohl die Pandemie beendet ist, bleibt COVID-19 ein globales Anliegen, das fortgesetzte Forschung und Wachsamkeit erfordert.

5. Paper I

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RESEARCH Open Access

The representative COVID-19 cohort Munich (KoCo19): from the beginning of the pandemic to the Delta virus variant

Ronan Le Gleut[1,2†], Michael Plank[3†], Peter Pütz[4], Katja Radon[5,6,7], Abhishek Bakuli[3], Raquel Rubio-Acero[3], Ivana Paunovic[3], Friedrich Rieß[3], Simon Winter[3], Christina Reinkemeyer[3], Yannik Schälte[1,8,9], Laura Olbrich[3], Marlene Hannes[3], Inge Kroidl[3], Ivan Noreña[3], Christian Janke[3], Andreas Wieser[3,10,11,12†], Michael Hoelscher[3,6,10,11†], Christiane Fuchs[1,2,8,13†], Noemi Castelletti[3,11,14*†] and the KoCo19/ORCHESTRA-study group

Abstract

Background Population-based serological studies allow to estimate prevalence of SARS-CoV-2 infections despite a substantial number of mild or asymptomatic disease courses. This became even more relevant for decision making after vaccination started. The KoCo19 cohort tracks the pandemic progress in the Munich general population for over two years, setting it apart in Europe.

Methods Recruitment occurred during the initial pandemic wave, including 5313 participants above 13 years from private households in Munich. Four follow-ups were held at crucial times of the pandemic, with response rates of at least 70%. Participants filled questionnaires on socio-demographics and potential risk factors of infection. From Follow-up 2, information on SARS-CoV-2 vaccination was added. SARS-CoV-2 antibody status was measured using the Roche Elecsys® Anti-SARS-CoV-2 anti-N assay (indicating previous infection) and the Roche Elecsys® Anti-SARS-CoV-2 anti-S assay (indicating previous infection and/or vaccination). This allowed us to distinguish between sources of acquired antibodies.

Results The SARS-CoV-2 estimated cumulative sero-prevalence increased from 1.6% (1.1-2.1%) in May 2020 to 14.5% (12.7-16.2%) in November 2021. Underreporting with respect to official numbers fluctuated with testing policies and capacities, becoming a factor of more than two during the second half of 2021. Simultaneously, the vaccination campaign against the SARS-CoV-2 virus increased the percentage of the Munich population having antibodies, with 86.8% (85.5-87.9%) having developed anti-S and/or anti-N in November 2021. Incidence rates for infections after (BTI) and without previous vaccination (INS) differed (ratio INS/BTI of 2.1, 0.7-3.6). However, the prevalence of infections was higher in the non-vaccinated population than in the vaccinated one. Considering the whole follow-up time, being born outside Germany, working in a high-risk job and living area per inhabitant were identified as risk

†Ronan Le Gleut and Michael Plank shared first authorship.

†Andreas Wieser, Michael Hoelscher, Christiane Fuchs and Noemi Castelletti shared last authorship.

*Correspondence:
Noemi Castelletti
noemi.castelletti@med.uni-muenchen.de
Full list of author information is available at the end of the article

factors for infection, while other socio-demographic and health-related variables were not. Although we obtained significant within-household clustering of SARS-CoV-2 cases, no further geospatial clustering was found.

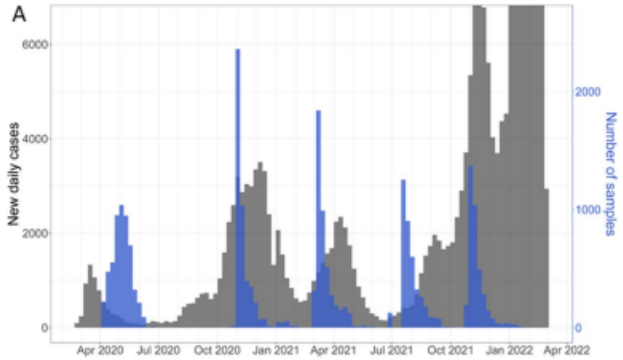
Conclusions Vaccination increased the coverage of the Munich population presenting SARS-CoV-2 antibodies, but breakthrough infections contribute to community spread. As underreporting stays relevant over time, infections can go undetected, so non-pharmaceutical measures are crucial, particularly for highly contagious strains like Omicron.

Keywords COVID-19, SARS-CoV-2, Population-based cohort study, Sero-prevalence, Sero-incidence, Vaccination status, Breakthrough infections, ORCHESTRA

Background

SARS-CoV-2 became pandemic mid-March 2020, within three months after the first report on 31st of December, 2019 in the city of Wuhan, Hubei province, China [1, 2]. In Germany, the first COVID-19 cases were observed in the municipality of Munich in late January 2020 [3]. Since then, the number of infections has been one of the predominant topics for political and social life [4, 5]. Looking at the pandemic in Munich in the time-frame between February 2020 and April 2022, four waves of infection can be identified (Fig. 1A):

- First wave: late January – mid June 2020
- Second wave: mid June 2020 – mid February 2021;



B

Vaccination	Past infection: Yes	Past infection: No
Yes	• Positive anti-N • Positive anti-S • Positive response to questionnaire item on vaccination	• Negative anti-N
Yes		• Positive anti-S
No	• Positive anti-N • Positive anti-S	• Negative anti-N
No	• Negative response to questionnaire item on vaccination	• Negative anti-S

Fig. 1 Epidemic evolution in Munich with description of the sample analysis. **A** Black: number of new daily SARS-CoV-2 cases officially reported by the Robert Koch Institute (RKI). Blue: number of blood/DBS samples of the KoCo19 collected daily. **B** Description of the lab analysis. With anti-N, anti-S and the response to the questionnaire item on vaccination it was possible to define the participants as: infected and vaccinated, infected and non-vaccinated, non-infected and vaccinated and non-infected and non-vaccinated. Blue shaded regions denote a negative response while orange regions a positive one

- Third wave: mid February 2021 – end July 2021;
- Fourth wave: end of July 2021 – after the end of the analysed period.

In the first wave, the main non-pharmaceutical interventions applied were to reduce contacts in the whole city of Munich followed by a lifting of the restrictions with still severe contact reductions. During this early phase of the pandemic, PCR tests were scarce good, and we suspect that only few chance finds entered the official statistics. In the second wave, contacts between people were reduced from June to October 2020, followed by stronger regulations, including FFP2 mask obligation. At the end of December 2020, only twelve months after the start of the pandemic, effective vaccines were introduced in Germany [6], preventing infection or at least reducing symptoms [7]. In parallel, the test capacity increased: starting in July 2020, the Bavarian state (including Munich) provided access to free PCR tests for all citizens, even without symptoms without a limit per person [8]. Antigen rapid tests became available nationwide for institutions like nursing homes or schools towards the end of 2020. By contact tracing more asymptomatic infected individuals could be identified [9–11]. In the third wave, the lock-down from the previous wave still continued with the so-called "emergency brake" starting in mid-April 2021: stronger contact reduction, night-time curfew and closure of many stores [12]. During this wave, the first new virus variant of SARS-CoV-2 was observed [13]: in early March 2021, the Alpha variant (B.1.1.7 variant) was detected in more than 40% of tested positive cases in Germany [14]. From early 2021 on, the testing capacity was further increased nationwide, and antigen test became available for home use [15, 16]. Such low-threshold access to testing supposedly facilitated detecting asymptomatic cases, which entered the official numbers after PCR confirmation. The fourth wave of the pandemic started in Munich with almost all cases classified as Delta (B.1.617.2) variant. Further relaxations were possible in the summer breaks from July 2021: more visitors at outdoor and cultural events, restaurants could stay open longer, mask rules were relaxed, bars could reopen [17, 18]. In October 2021, even clubs were allowed to open again [19].

Decisions on non-pharmaceutical interventions were mostly taken under the guidance of official case reports, which were shown to underestimate the true case numbers especially at the beginning of the pandemic, when testing capacity was still low [20]. In order to gain a better understanding of the true case numbers, we started the prospective Munich COVID-19 cohort (KoCo19) in April 2020 including 5313 participants living in private households. In this population-based cohort study we measured SARS-CoV-2 antibody prevalence at the following times of the pandemic (Fig. 1A):

- May 2020 at the peak of the first wave in Germany,
- December 2020, at the beginning of the second wave,
- March 2021, at the peak of the third wave and at the beginning of the vaccination campaign for the general population,
- August 2021, at the end of the third wave with around 68% of the general population 14 years or older being vaccinated against SARS-CoV-2,
- November 2021, in the middle of the fourth wave and before the spread of the Omicron variant started in Germany.

To the best of our knowledge, KoCo19 is the SARS-CoV-2 cohort with the longest follow-up time in the world. On December 1st, 2020, the KoCo19 cohort joined the ORCHESTRA (Connecting European Cohorts to Increase Common and Effective Response to SARS-CoV-2 Pandemic) project. During the whole pandemic, KoCo19 results were used to advise political decision making.

We here present the evolution of SARS-CoV-2 cumulative sero-positivity in the Munich general population 14 years and older over time. Furthermore, we report on risk factors for SARS-CoV-2 infection over time. The data described here were not published elsewhere.

Methods

Study population and field work

Baseline and follow-up questionnaires

A detailed description of the baseline study can be found in [20, 21]: We randomly sampled the Munich cohort of private households between April 5th and June 12th, 2020. Only household members 14 years and older who gave written informed consent were included in the cohort. For participants younger than 18 years, informed consent was obtained from the parents as well as the participants themselves.

Analyses use information from baseline individual and household questionnaires and from individual follow-up questionnaires. The different questionnaires were already described in detail [20], and included information on: socio-demographics, country of birth, smoking status, chronic conditions, general health, household size, living area per inhabitant, household type, housing type, self-estimated health-related risk taking behaviour, personal contacts, number and intensity of leisure time activities before the pandemic (in February 2020), number and intensity of leisure time activities two weeks prior to the follow-up questionnaire. Starting from Follow-up 2, we also asked about SARS-CoV-2 vaccination including

the number of vaccinations, type of vaccine and date of vaccination.

Baseline and follow-ups SARS-CoV-2 antibody study

At recruitment, a serum sample was gathered for 5313 household members 14 years and older. Thereafter, four antibody follow-ups were conducted in December 2020 [20], March 2021, August 2021 and November 2021 (Fig. 1A). Follow-ups were performed by sending out boxes with a self-sampling kit to take a capillary blood sample (dry blood spot; DBS). A detailed description of the DBS analysis procedure can be found in [22]. When self-DBS collection was impossible, participants were invited to give serum and DBS at our study centre.

For the measurements at baseline [23] and Follow-up 1, only the Elecsys® Anti-SARS-CoV-2 anti-N (Roche) (hereafter called Ro-N-Ig) assay was used for antibody detection after infection. From Follow-up 2 on, in addition, also the Elecsys® Anti-SARS-CoV-2 anti-S (Roche) (hereafter called Ro-RBD-Ig) assay was applied. This was necessary to distinguish antibodies due to infection (i.e., anti-S and anti-N present) and antibodies only due to vaccination (i.e., only anti-S present) (Fig. 1B).

For the measurement with full blood sampling, an optimised cut-off of 0.4218 for Ro-N-Ig was applied to indicate sero-positivity [23]. Estimates of sensitivity and specificity of blood Ro-N-Ig compared to reverse-transcription polymerase chain reaction (RT-PCR) were used to adjust the prevalence.

Taking full blood samples as ground truth, sensitivity and specificity of the DBS anti-N method were 99.2% and 98.7%, respectively, applying a cut-off of 0.105 [22]. Based on our internal validation cohort (data not shown here), only samples with Ro-RBD-Ig larger than or equal to 0.115 were considered positive (regarding anti-S) for vaccination and/or infection. Similarly, the DBS anti-S method had sensitivity and specificity of 96.6% and 97.8%, respectively. Since sensitivity and specificity of both tests turned out high, no additional adjustment for sensitivity and specificity was applied. The cut-offs for blood samples, as well as DBS samples, along with their sensitivity and specificity, were determined based on cohorts randomly selected using serology rather than symptom severity. This approach ensured that the assays are suitable for detecting milder community infections [22, 23].

Using the serological values in combination with questionnaire information, we were able to classify participants into the following groups (Fig. 1B):

- Non-vaccinated, non-infected: negative in both anti-S and anti-N antibodies;
- Vaccinated, non-infected: positive in anti-S and negative in anti-N antibodies;
- Non-vaccinated, infected: positive in both anti-S and anti-N antibodies, negative response to the questionnaire item on vaccination;
- Vaccinated and infected: positive in both anti-S and anti-N antibodies, positive response to questionnaire item on vaccination.

Statistics

All statistical analyses were performed using the softwares R (version 4.1.3, R Development Core Team, 2021) and Python (version $\geq$ 3.8.5).

After observed sero-conversion, antibody levels were imputed positive in all follow-ups, independently of the actual results of the round or in case of missingness („ever positiveness ", Fig. 2A). We thus disregard potential anit-N waning. Our definition allows us to estimate the cumulative sero-prevalence in the considered population, which in turn we take as a proxy for cumulative infections and compare to the official number of positive cases reported by the authorities, neglecting reinfections. For simplicity, we in the following suppress the word "cumulative" as a specification of the estimated sero-prevalence. In order to estimate the population prevalence, sero-prevalence estimates (adjusted and unadjusted for the sensitivity and specificity of the test) were computed using a weighting scheme. First, sampling weights for each participant at baseline were calculated according to the sampling design of the cohort [21]. These weights were then corrected for the attrition observed at each follow-up, modelling the underlying non-response mechanism [24]. The resulting weights were finally calibrated on the updated Munich structure at each round regarding age, sex, country of birth, presence of children in the household and single member households distributions [25]. For the last three follow-ups (March, August and November 2021), information on the vaccination status of the participants was assessed via questionnaires. The missing values (30% for Follow-up 2, 27% for Follow-up 3 and 8% for Follow-up 4) were imputed via multiple imputation ($m = 100$) crossing for each round the vaccination status with the information on the immune response (Ro-N-Ig and Ro-RBD-Ig results). The probability p of being vaccinated was estimated for each of the four anti-N and anti-S combinations for each of the imputed datasets and each Follow-up 2 to 4, see e.g. the values of one imputed dataset for Follow-up 4 in Table 1. The results for Follow-up 3 are comparable to these ones. At the beginning of the vaccination campaign (Follow-up 2), the probabilities to be vaccinated were lower, especially for anti-S and

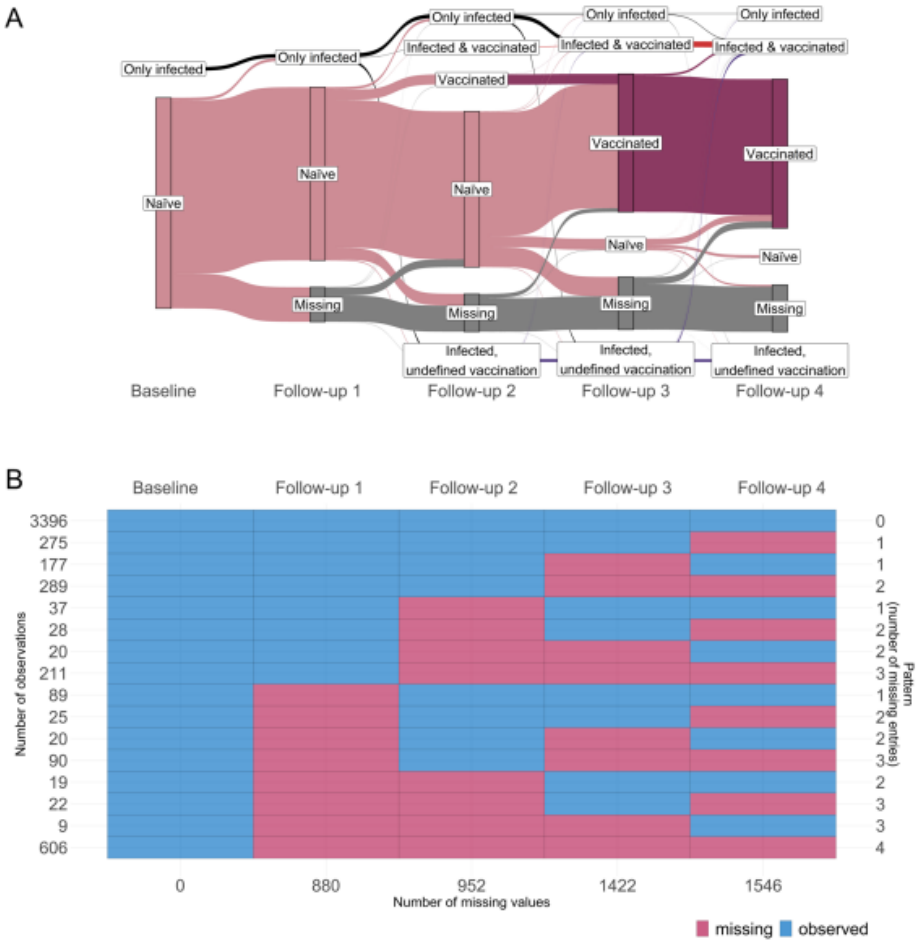


Fig. 2 Cohort description based on the ever-positive principle, i.e. anti-N sero-positivity remains for all rounds after sero-conversion, independently of other blood results or if missing. **A** Change of serological status of participants: only infected (anti-N ever positive and stated to be non-vaccinated in the questionnaire), naïve (anti-N and anti-S always negative), vaccinated (only anti-S ever positive), infected & vaccinated (in previous round only anti-S positive, or stated to be vaccinated in the questionnaire), infected without information on vaccination (infected, undefined vaccination) and non-responders/missing. **B** Observed responder behaviours. Left legend: number of participants. Right legend: number of missing rounds. Bottom legend: number of missing samples per round

anti-N positive ($p = 0.06$) with mostly only infected (and non vaccinated) persons.

The imputation was performed using a Bernoulli distribution with probability p for each participant with missing information.

Considering both Ro-RBD-Ig results and the questionnaire data, in the last two follow-ups 93% and 97%, respectively, of the participants could be assumed vaccinated. In contrast, the city of Munich reported that approximately 68% and 76%, respectively, of the

Table 1 Estimated probabilities to be vaccinated used for the imputation of the vaccination status during Follow-up 4

		Anti-N	
		Positive	**Negative**
Anti-S	Positive	$p = 0.94$	$p = 0.99$
	Negative	$p = 0$	$p = 0.19$

Anti-S negative may occur after vaccination in case of a delayed or an absence of antibody response. Moreover, Ro-RBD-Ig (anti-S) with a cut-off at 0.115 does not provide 100% sensitivity and specificity

population older than 14 years have been vaccinated [26]. The calibration of the cohort results is hence of crucial importance. The variance associated with the calibrated sero-prevalence estimates was computed using linearisation [25] and residual [25, 27] techniques. This variance accounts for the uncertainty due to the different stages of the sampling design (selection of the constituencies and of the households), the non-response mechanism [28] and the calibration process. As a sensitivity analysis, unweighted sero-prevalence estimates were also computed together with their uncertainty. The variance was determined by a nonparametric cluster bootstrap procedure that accounts for household clustering [29]. The sero-prevalence estimates were calculated in each of the 5000 bootstrap samples (sampling of households with replacement), and the variance of these 5000 estimates provided the uncertainty of the unweighted estimates. Finally, the variability associated with the multiple imputation procedure was added to the variance of the (weighted and unweighted) sero-prevalence estimates following the approach detailed in Honaker et al. (2011) [30]. In short, the final variance estimate V is a combination of the average of the variance estimates $V_j, j = 1, \ldots, m$ (described above) over the m replications and the variance of the m sero-prevalence estimates $\theta_j, j = 1, \ldots, m$:

$$V = \frac{1}{m}\sum_{j=1}^{m} V_j + S^2\left(1 + \frac{1}{m}\right), \text{with } S^2 = \frac{1}{m-1}\sum_{j=1}^{m}\left(\theta_j - \bar{\theta}\right)^2$$

The final sero-prevalence estimates were obtained using the means of the m estimates, and 95% confidence intervals were computed assuming a normal distribution.

Breakthrough infections (BTI) are defined as newly infected participants after vaccination. The corresponding SARS-CoV-2-related serological spectrum is hence given by: anti-N negative but anti-S positive in the past and anti-N positive for a given next round (Fig. 1B). Accordingly, newly anti-N positive cases without anti-S antibodies in the previous rounds were defined as infections of naïve subjects (INS). While these estimates could be adjusted for the sensitivity and specificity of the test, we report in the results Sect. 95% confidence intervals (CI) for the ratio INS/BTI without adjustment. Indeed, the calculation of the variance requires information at the individual level (enabling accounting for the sampling design, the non-response, the calibration and the multiple imputation), while the adjustment of the incidence rates is done directly on the estimates.

Of interest were also risk factors for infection, with the aim to model when, in the course of the pandemic period, the infection (anti-N positiveness) occurred. Right censoring was adopted for anti-N negative participants at the end of the observation period, Follow-up 4. An extended Cox regression model [31, 32] was applied to assess which baseline risk factors increase or decrease the risk of infection. Since positivity of individuals in one household might depend on each other (resulting in a potential high intra-cluster correlation [33]), the Cox regression model follows the count process formulation of Anderson and Gill [31] to adjust for intra-household clustering in the data obtaining robust standard error estimates.

The non-response mechanism (Fig. 2B) over the different rounds of interrogation was studied using a logistic regression. The missingness in the explanatory variables was corrected by multiple imputation with $m = 5$ replications (Table 2). Due to a high number of missing values on the income (Supplemental Figure S1), a sensitivity analysis was performed considering complete cases for all covariates, except for the income where an indicator variable for missingness was used (Supplemental Table S1). The results are similar between the two analyses.

In both the risk factor analysis and the non-response mechanism analysis, for explanatory variables with two categories, a constraint to zero for one category (e.g. females vs. males) was used. For covariates with three and more categories, a sum-to-zero constraint (i.e. compare each category to the average) was applied.

Results

Cohort development

Since anti-S becomes positive after vaccination but also after infection, the definition of being vaccinated for infected persons was obtained using the questionnaires when available (Fig. 1B). When describing the changes of antibody statuses over time, historical information needs to be taken into account. Figure 2A applies the definition of „ever positiveness " (see Supplemental Figure S2 for an alternative serological description) and considers the following major categories: only infected (anti-N ever positive, and vaccination excluded based on other information), naïve (anti-N and anti-S never positive), vaccinated (only anti-S ever positive), and infected & vaccinated (anti-N positive after anti-S positive, or anti-N

Table 2 Non-response mechanism at the different follow-ups using multiple imputation

Variable	Categories	Follow-up 2			Follow-up 3			Follow-up 4		
		OR	95% CI	*p*-value	OR	95% CI	*p*-value	OR	95% CI	*p*-value
Sex	Male	0.81	[0.66; 0.98]	*	0.97	[0.82; 1.15]		0.83	[0.69; 0.99]	*
Age *(years)*	14–19	0.82	[0.49; 1.37]		0.59	[0.36; 0.97]	*	0.61	[0.36; 1.05]	
	20–34	0.59	[0.45; 0.76]	***	0.55	[0.43; 0.69]	***	0.62	[0.49; 0.78]	***
	35–49	0.86	[0.67; 1.11]		1.02	[0.81; 1.30]		0.89	[0.70; 1.15]	
	50–64	1.47	[1.15; 1.88]	**	1.41	[1.13; 1.76]	**	1.57	[1.24; 1.98]	***
	65–79	1.87	[1.36; 2.58]	***	2.01	[1.46; 2.75]	***	1.28	[0.95; 1.71]	
	80 +	0.88	[0.57; 1.35]		1.06	[0.69; 1.63]		1.48	[0.96; 2.28]	
Birth country	Not Germany	0.98	[0.76; 1.27]		0.59	[0.47; 0.74]	***	0.63	[0.50; 0.79]	***
Level of education	In school	1.00	[0.58; 1.73]		0.88	[0.52; 1.50]		1.00	[0.57; 1.76]	
	< 12 years	0.94	[0.69; 1.27]		1.10	[0.83; 1.46]		0.93	[0.69; 1.24]	
	≥ 12 years	1.06	[0.78; 1.45]		1.03	[0.77; 1.37]		1.08	[0.78; 1.48]	
Employment status	Employed	1.07	[0.86; 1.32]		1.06	[0.87; 1.30]		0.98	[0.78; 1.22]	
	Self employed	0.89	[0.65; 1.23]		0.85	[0.65; 1.11]		0.90	[0.68; 1.20]	
	Unemployed	0.75	[0.57; 0.99]	*	1.27	[1.00; 1.62]		1.18	[0.90; 1.54]	
	Others	1.40	[0.84; 2.32]		0.87	[0.58; 1.31]		0.96	[0.59; 1.58]	
Risk employment	Yes	0.85	[0.63; 1.14]		1.10	[0.86; 1.41]		1.05	[0.82; 1.34]	
Smoking status	Non smoker	1.00	[0.87; 1.16]		1.17	[1.03; 1.33]	*	0.96	[0.84; 1.09]	
	Past smoker	0.92	[0.78; 1.09]		1.01	[0.87; 1.19]		1.00	[0.87; 1.15]	
	Current smoker	1.08	[0.91; 1.29]		0.84	[0.72; 0.98]	*	1.05	[0.89; 1.23]	
General health	Not good	0.61	[0.42; 0.88]	**	0.84	[0.60; 1.18]		0.59	[0.41; 0.85]	**
	Good	0.92	[0.73; 1.15]		1.08	[0.91; 1.28]		0.90	[0.75; 1.08]	
	Very good	1.28	[1.05; 1.54]	*	1.03	[0.88; 1.21]		1.19	[0.99; 1.44]	
	Excellent	1.39	[1.11; 1.76]	**	1.07	[0.86; 1.33]		1.57	[1.25; 1.97]	***
Respiratory allergies	Yes	0.92	[0.71; 1.19]		1.39	[1.10; 1.74]	**	0.83	[0.67; 1.02]	
Diabetes	Yes	1.37	[0.83; 2.28]		0.78	[0.48; 1.29]		0.81	[0.51; 1.30]	
CVD	Yes	1.10	[0.75; 1.60]		1.16	[0.87; 1.54]		1.15	[0.83; 1.58]	
Obesity	Yes	0.86	[0.50; 1.50]		0.89	[0.60; 1.31]		1.01	[0.67; 1.51]	
Cancer	Yes	0.87	[0.53; 1.43]		0.98	[0.58; 1.67]		1.02	[0.64; 1.63]	
Lung disease	Yes	0.93	[0.59; 1.45]		0.81	[0.58; 1.14]		0.97	[0.69; 1.36]	
Skin allergies	Yes	1.12	[0.81; 1.55]		0.98	[0.76; 1.27]		1.18	[0.90; 1.54]	
Autoimmune disease	Yes	1.28	[0.74; 2.22]		0.97	[0.68; 1.40]		1.34	[0.86; 2.08]	
Household type	Single	1.23	[0.93; 1.62]		1.25	[0.96; 1.62]		0.94	[0.73; 1.21]	
	Couple	1.24	[1.03; 1.49]	*	1.10	[0.94; 1.29]		1.19	[1.01; 1.40]	*
	Family	0.85	[0.69; 1.06]		0.86	[0.70; 1.06]		0.89	[0.73; 1.10]	
	Others	0.77	[0.61; 0.98]	*	0.85	[0.68; 1.06]		1.00	[0.78; 1.29]	
Household income *(Euro)*	≤ 2500	0.84	[0.67; 1.05]		0.81	[0.63; 1.04]		0.94	[0.75; 1.17]	
	2501–4000	1.01	[0.78; 1.30]		0.91	[0.76; 1.10]		0.92	[0.78; 1.08]	
	4001–6000	1.13	[0.95; 1.33]		1.16	[0.92; 1.46]		1.09	[0.92; 1.28]	
	6001 +	1.05	[0.76; 1.44]		1.16	[0.94; 1.44]		1.07	[0.87; 1.32]	
Living area/inhabitant *(sqm/individual)*	≤ 30	1.13	[0.92; 1.38]		0.97	[0.81; 1.17]		0.96	[0.80; 1.16]	
	31–40	1.03	[0.86; 1.23]		0.88	[0.75; 1.03]		1.02	[0.86; 1.21]	
	41–55	0.91	[0.74; 1.10]		1.27	[1.06; 1.51]	*	1.10	[0.91; 1.33]	
	56 +	0.95	[0.74; 1.22]		0.92	[0.74; 1.15]		0.92	[0.75; 1.15]	
Building type *(nb of apartments)*	1–2	1.24	[1.02; 1.51]	*	0.90	[0.76; 1.08]		1.11	[0.92; 1.33]	
	3–4	0.91	[0.70; 1.19]		1.48	[1.14; 1.91]	**	1.03	[0.80; 1.31]	
	5 +	0.88	[0.75; 1.04]		0.75	[0.64; 0.88]	***	0.88	[0.75; 1.03]	
Seropositivity in the previous rounds	Negative	4.52	[3.78; 5.40]	***	5.42	[4.74; 6.19]	***	5.27	[4.63; 6.01]	***
	Positive	2.01	[1.48; 2.72]	***	1.88	[1.54; 2.30]	***	1.90	[1.57; 2.31]	***
	Missing	0.11	[0.09; 0.13]	***	0.10	[0.08; 0.12]	***	0.10	[0.09; 0.12]	***

Variables with 2 categories have contrasts with one category set to 0. For variables with 3 and more categories, constraint sum-to-zero contrasts was applied
OR odds ratio
*p-value: *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$*

positive with respective questionnaire information). From Follow-up 2 on, participants started moving from the naïve to the vaccinated status, which became the most prominent stage in Follow-ups 3 and 4. The status of non-responders is labelled as missing: 64% (3396/5313) of the participants gave blood in all rounds, 11% (578/5313) / 8% (401/5313) / 6% (332/5313) had exactly one/two/three rounds missing, and 11% (606/5313) dropped out for all four follow-ups after the baseline measurement (Fig. 2B). Some non-responders still answered back in subsequent round(s), thus moving away from stage missing. Overall, the response rate was satisfactory (83% Follow-up 1; 82% Follow-up 2; 73% Follow-up 3; 71% Follow-up 4; Fig. 2B), especially considering the duration of the cohort.

Non-responder analyses

The non-response mechanism for the Follow-up 1 was previously presented [20]. We show the results for the last three follow-ups (Table 2). Females and participants between 50 and 79 years were more likely to take part to the follow-ups, while young participants (age < 35 years old) together with participants with a migration background were less likely to participate. People who reported a bad general health condition tended to drop out of the cohort while those with excellent health continued answering to the survey. Couples were slightly more likely to provide blood samples than other household types. Members of a household with a low or medium-to-low income were less likely to take part in the survey in comparison to households with a medium-to-high or high income, even though the differences were not significant (see Supplemental Table S1 for sensitivity analysis). During Follow-up 2, households in buildings with 1–2 apartments tended to answer more often, while during Follow-up 3, those living in buildings with 3–4 apartments answered more often. Households in buildings with 5 or more apartments answered less often. Participants not taking part in one previous round of interrogation were less likely to take part in the next rounds. Having at least one positive anti-N serological result in the previous rounds lead to a lower response rate in the next follow-ups in comparison to always having negative anti-N results in the past. All other covariates investigated in the non-response mechanism (level of education, employment status, smoking status, etc.) showed no or negligible association to the response behaviour.

SARS-CoV-2 sero-prevalence, underreporting factor and sero-incidence over time

The blue estimate in Fig. 3A shows the calibrated cumulative sero-prevalence (adjusted for sensitivity and specificity) in private households for the Munich population 14 years and older:

- Baseline: 1.6% (1.1 – 2.1%),
- Follow-up 1: 4.1% (3.3%—4.9%), and after adjustment for vaccination status
- Follow-up 2: 7.3% (6.1—8.5%),
- Follow-up 3: 12.4% (10.7—14.1%),
- Follow-up 4: 14.5% (12.7—16.2%).

Without adjustment for vaccination status for the Follow-ups 3 and 4, the sero-prevalence would have been significantly lower: 8.5% (7.2-9.8%) for August 2021 and 10.5% (9.1-11.9%) for November 2021. Indeed, the proportion of vaccinated persons is greater in the cohort in comparison to the general Munich population. Therefore, the calibration on the vaccination status increases the weight of the participants who are not vaccinated. The sero-prevalence being greater in the non-vaccinated population (see below and Fig. 3C), the overall sero-prevalence, including both vaccinated and non-vaccinated, also increases with the calibration.

The official number of positive cases is reported in pink in Fig. 3A for the general population of Munich (including institutions like nursing homes and potential reinfections). Considering that the KoCo19 cohort is limited to private households and that the estimated sero-prevalence does not account for multiple infections, a comparison of this estimate with the official number over time allows us to estimate a lower bound for the underreporting factor (with the false assumption that all cases reported by the authorities occurred in private households and neglecting reinfections). The estimated underreporting factor changed over the rounds:

- Baseline: 3.4 (2.4 – 4.4),
- Follow-up 1: 1.3 (1.0 – 1.6),
- Follow-up 2: 1.8 (1.5 – 2.1),
- Follow-up 3: 2.3 (2.0 – 2.6),
- Follow-up 4: 2.2 (2.0—2.5).

Figure 3B depicts the sero-incidence (adjusted for sensitivity and specificity), i.e. the percentage of new infections between two consecutive rounds:

- Follow-up 1: 2.0% (1.4—2.7%),
- Follow-up 2: 3.1% (2.3—3.9%),
- Follow-up 3: 3.2% (2.5—3.9%),
- Follow-up 4: 2.4% (1.4—3.4%),

with the time interval between Follow-ups 3 and 4 being rather short (three months).

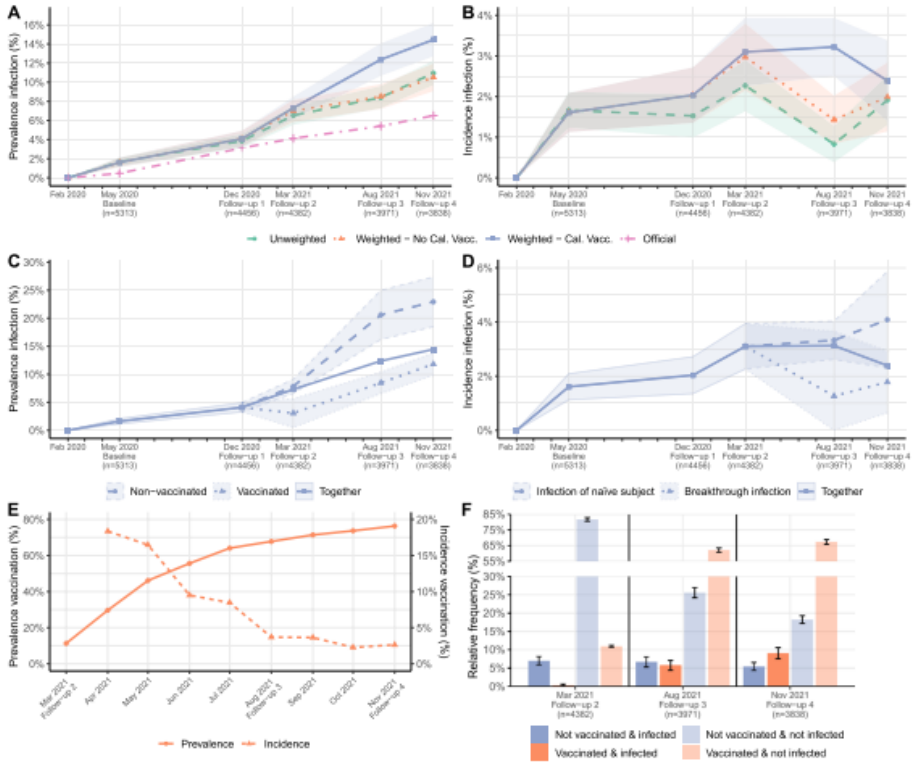


Fig. 3 **A** Weighted and unweighted cumulative anti-N sero-prevalence in private households and official numbers of cases reported by the authorities for the Munich population older than 13 years. **B** Weighted and unweighted anti-N sero-incidence. **C** Anti-N sero-prevalence estimates calibrated on the number of vaccinated people split according to the vaccination status of the same round. **D** Calibrated estimates for the infection of naïve subjects and breakthrough infections. **E** Prevalence and incidence of vaccination in Munich (official numbers). **F** Relative frequencies according to the infection and vaccination status

Breakthrough infections in the Munich population

To better understand the effect of the vaccination campaign (see also next section), the calibrated cumulative sero-prevalence was split between vaccinated versus non-vaccinated people (Fig. 3C):

- Follow-up 2: 3.1% (0.5% - 5.6%) versus 7.8% (6.6 – 9.1%),
- Follow-up 3: 8.5% (6.6 – 10.4%) versus 20.6% (16.2 - 25.0%) and
- Follow-up 4: 11.8% (9.8 - 13.8%) versus 22.9% (18.5 - 27.4%).

The sero-prevalence of the vaccinated group is lower compared to the non-vaccinated group.

Figure 3D compares the adjusted (for sensitivity and specificity) incidence rates for BTI versus INS over the rounds:

- Follow-up 3: 1.3% (0 - 3.7%) versus 3.3% (2.6 - 4%) and
- Follow-up 4: 1.8% (0.6 - 2.9%) versus 4.1% (2.3 - 5.9%).

In August and November 2021, incidence rates of INS were greater than the ones of BTI. Significant

differences between unadjusted INS and BTI incidence rates (INS/BTI) could however not be achieved:

- Follow-up 3: ratio of 2.8 (0 - 7.7) and
- Follow-up 4: 2.1 (0.7 - 3.6).

The low sample sizes led to low power and may thus have implied the non-significant findings: In Follow-up 2, the low number of vaccinated persons led to high uncertainty in the estimation of BTI in Follow-up 3; vice versa, in Follow-up 3, the low number of non-vaccinated persons led to high uncertainty in the estimation of INS in Follow-up 4.

The vaccination campaign in the Munich population

The introduction of vaccination quickly changed the SARS-CoV-2-related serological spectrum of the Munich population. The percentage of the Munich population presenting antibodies against the virus (either anti-S after infection and/or vaccination and/or anti-N antibodies after infection) increased fast over time:

- Follow-up 2: 11.2% (9.6 - 12.8%),
- Follow-up 3: 74.2% (72.6 – 75.8%),
- Follow-up 4: 86.8% (85.8 - 87.9%).

Even though the cumulative sero-prevalence and the sero-incidence seemed to be higher among the non-vaccinated population compared to the vaccinated population (Fig. 3C and D), BTI contributed relevantly to the community spread, considering that the size of the population of vaccinated people was much larger than the non-vaccinated one during the last rounds of interrogation (Fig. 3E). Figure 3F illustrates this effect in more detail. The proportion of people vaccinated and infected increased over time, up to Follow-up 4 where this proportion was significantly greater than the one of infected and non-vaccinated people. This figure also shows that the proportion of the population without any antibodies related to SARS-CoV-2 (non-vaccinated and non-infected) was decreasing over time, while the share of people vaccinated and non-infected increased (cf. Fig. 2A).

Risk factors for SARS-CoV-2 sero-prevalence

The results of the risk factor analysis can be found in Fig. 4. The extended Cox regression model suggests that being born outside Germany (hazard ratio (HR) 1.36, 95% confidence interval (CI) 1.01–1.85) and having a job with a high potential of contact to COVID-19 cases (HR 1.31, 95% CI 1.00–1.70) were risk factors for SARS-CoV-2 sero-positivity. Living area of 30–40 square meters per inhabitant presented a slightly higher risk of infection (HR 1.27, 95% CI 1.01–1.59), while for 40–55 square meters per inhabitant the risk decreased (HR 0.74, 95% CI 0.57–0.97), compared to the average Hazard of all categories of living area. All other socio-demographic (sex, age, level of education, employment status, building type, household income) and health-related variables (smoking status, general health status, different diseases and drug intakes) were not identified as risk factors for infection.

Household and neighbourhood clustering of SARS-CoV-2 cases

SARS-CoV-2 transmission within households was found to be highly significant for baseline [33] and Follow-up 1 [20] analyses and was confirmed until Follow-up 4 (Supplemental Figure S3). While the overall picture obtained in recent rounds showed a lower-than-expected mean variance at 500 m as well, we now could not find sufficient proof of spatial clustering beyond household level, especially if one adjusted *p*-values for multiple testing.

Discussion

We present the development of the SARS-CoV-2 pandemic in the municipality of Munich. To estimate the real number of SARS-CoV-2 infections, the members of the prospective KoCo19 cohort were asked five times to give their blood for study purposes between spring 2020 and fall 2021. SARS-CoV-2 antibodies generated by silent or symptomatic infections and/or vaccination could hence be measured. We could show that the sero-prevalence drastically increased over time, from 1.6% during the baseline to 14.5% in Follow-up 4, with a relevant under-reporting bias. Risk factors for SARS-CoV-2 sero-positivity, such as being born outside of Germany, living area per inhabitant and working in a job with high potential of contact with COVID-19, could be identified together with household clustering.

Sero-prevalence was still low towards the end of the first pandemic wave and increased drastically in every follow-up. Comparison of our results with official numbers reveals an underreporting factor that changes over time. These changes might result from different testing policies as well as different variants of the virus. The estimates present lower bounds of the true under-reporting factor, since our study focused on private households whereas the official number of reported cases included institutions (like nursing homes) as well. Moreover, potential reinfections counted in the official numbers were here neglected. Indeed, our study focuses on the pandemic from its beginning to the Delta variant,

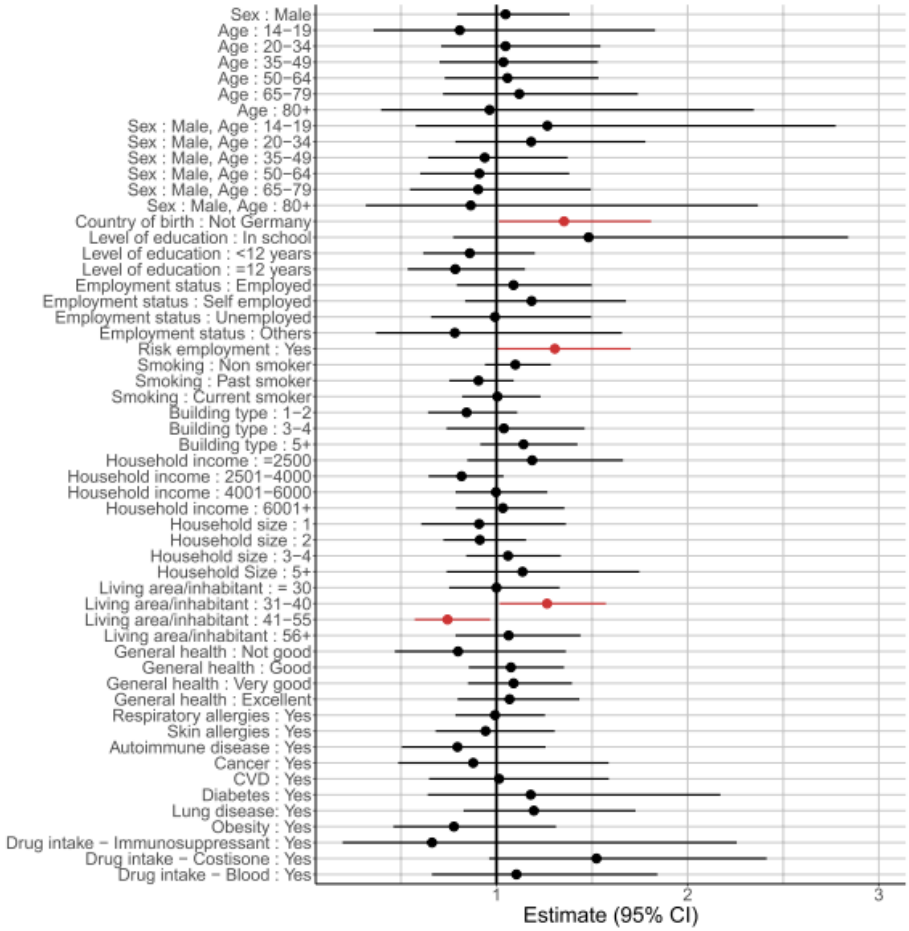


Fig. 4 Association between potential risk factors and SARS-CoV-2 sero-positivity taking into account time between baseline and Follow-up 4; events are thus right-censored. Results are based on multiple imputation. The main individual level risk factors were country of birth outside Germany and being employed in a job more in contact with the epidemic. Living in an apartment with a living area of 30–40 square meters per inhabitant revealed a slightly higher risk, while for 40–55 square meters per inhabitant the hazard ratio decreased

before the spread of the Omicron variant. Therefore, the low number of reinfections did not play a major role during this period [34–36].

In our data it was possible to separate infection of naïve subjects from breakthrough infections in low- and high-incidence time periods. In all follow-ups, our results indicate a contribution of breakthrough infections to the spread of SARS-CoV-2. The findings presented here, based on serology, contribute to current knowledge so far derived from PCR test results. The number of breakthrough infections detected based on PCR tests that were either done routinely, because of symptoms or among case contacts [37, 38] might miss an important number of silent infections,

especially as vaccinated individuals tend to have less pronounced symptoms. In our cohort, only a small part was fully vaccinated until March 2021 (Follow-up 2), given the vaccination scheme in Germany at that time. This resulted in a wide confidence interval for breakthrough infections during the next follow-up. During August 2021 (Follow-up 3), almost the complete cohort got vaccinated and therefore, the estimation uncertainty for breakthrough infections during Follow-up 4 decreased. 99.4% of the people stating vaccination in the questionnaire sero-converted in anti-S, indicating a good efficacy of the vaccinations. In concordance with other studies [39, 40], a considerable proportion of breakthrough infections was detected. Our results as well as other studies suggest that vaccination lowers the risk of infection [41]. Moreover, the share of infected persons (sero-prevalence) was shown to be greater in the non-vaccinated population in comparison to the vaccinated one. The sero-incidence of (most likely asymptomatic) infections among vaccinated people in the population was lower than the one in non-vaccinated people; however, the difference was statistically non-significant. BTIs might thus relevantly contribute to the community spread, considering also the fact that the vaccinated population was much larger compared to the non-vaccinated one. This might be even more relevant for highly transmissible variants like Omicron.

With an increasing prevalence of vaccination in the population, silent infections or persons presenting only mild symptoms are common. In this context, population-based sero-prevalence studies are important to estimate the true population prevalence. A couple of German cross-sectional population-based sero-prevalence studies were published especially during the first and second wave of the pandemic [42–44]. To our knowledge, all these studies stopped by mid 2021, leaving our cohort as the only one.

In our first analysis [33], an increased (albeit not statistically significant) risk of infection of having a job with a high potential of contact to COVID-19 cases could be found. With this analysis the risk factor became statistically significant, which is in line with other studies [45–47]. The World Health Organisation reported that among the COVID-19 cases reported worldwide, 14% belong to the group of healthcare workers, whereas in most countries this group represents less than 3% of the general population [48].

Participants with a living area between 31 and 40 square meters per inhabitant showed a significantly increased risk for infection, while the risk of the group with a living area between 41 and 55 square meters per inhabitant significantly decreased. Considering the number of household members, we found that 56% (76%) of the households with 31 - 40 (41—55) squared meters per inhabitant also have only one or two household members. Knowing that a larger household size implies more possible infectious contacts [49–51] suggests that the risk also depends on the household composition: Less members are associated to lower risk of infection. Household size is included in the model but does not show any significant effect, also not as interaction term, although the risk of infection seems to become higher with more household members (Fig. 4). This might be due to the fact that the variables household size, living area per inhabitant and building type all describe the living situation, with difficulties in separating the risk effects. Nevertheless, no multicollinearity issues were detected for this analysis.

Beside the two aforementioned risks for infection and being born outside Germany, no other socio-demographic or health-related risk factors were identified in our study. These results should rather be seen as exploratory than confirmatory, considering that we made no adjustment for multiple testing.

Major strengths of our study are its population-based approach, the appropriate weighting of results for the general Munich population, the high number of participants, the thorough validation of the assays used, and the use of validated questionnaire items. The overall response to the study was high compared to other population-based epidemiological studies in Germany (64% of the participants gave specimens in all rounds) [52]. While most participants completed the questionnaire online or on paper, we also provided the alternative of telephone interviews, which helped increasing participation. A relevant limitation of our study is the exclusion of children and residents not living in private households. While in general, people with migration background are less likely to participate in population-based studies, the lack of translated questionnaires further limited the number of migrants participating in our study [21]. To increase response, blood samples were collected at participants' homes or via mail with the DBS introduction and not at a centralized testing facility. Although until now a lot of research has been done for the COVID-19 pandemic, definitions like correlate of protection and long COVID symptoms are still not fully understood. Therefore, we aim to continue our longitudinal prospective representative cohort.

Conclusion

Despite the vaccination campaign, SARS-CoV-2 sero-prevalence in the Munich general population increased drastically towards the end of 2021, but was still below 20%. The estimated number of infected persons was nevertheless at least twice as high as the official number

reported by the authorities during the second half of 2021. Workers with a high potential of contact to infected persons experienced an increased risk of infection. Breakthrough infections still contribute to the community spread, thus we conclude that non-pharmaceutical interventions are still relevant, especially in the presence of highly transmissible variants like Omicron.

Abbreviations

anti-N	Anti-Nucleocapsid antibodies
anti-S	Anti-Spike antibodies
BTI	Breakthrough infections
CI	Confidence interval
DBS	Dry blood spot
HR	Hazard ratio
INS	Infections of naïve subjects
KoCo19	Representative COVID-19 Cohort Munich
PCR	Polymerase chain reaction
Ro-N-Ig	Elecsys® Anti-SARS-CoV-2 anti-N (Roche)
Ro-RBD-Ig	Elecsys® Anti-SARS-CoV-2 anti-S (Roche)
RT-PCR	Reverse transcription polymerase chain reaction
SARS-CoV-2	SARS corona virus 2

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12879-023-08435-1.

Additional file 1: Figure S1. Missing pattern in the baseline questionnaire. Bottom middle: variable analysed for missing information. Bottom left: bar chart depicting numbers of missing information for that variable. Bottom right: description of intersection pattern between variables (all possible combinations of the variables for which a missing information was given, from left to right e.g. only income information missing, income & living & household type information missing, all variables missing, etc.). Top: bar chart depicting the numbers of participants that did not give information for that intersection pattern.

Additional file 2: Figure S2. Cohort description based on current lab result (in contrast to ever-positivity as in Figure 2). Change of serological status of participants: only infected (anti-N positive and stated to be non-vaccinated in the questionnaire), naïve (anti-N and anti-S negative), vaccinated (only anti-S positive), infected & vaccinated (anti-N positive and in previous round only anti-S positive, or anti-N positive and stated to be vaccinated in the questionnaire), infected without information on vaccination status (infected, undefined vaccination) and non-responders/missing.

Additional file 3: Figure S3. Proximity cluster analysis at Follow-ups 2 to 4. The grey points and curves show the distribution of mean within-cluster variances for 10,000 random permutations of cluster assignments. The horizontal lines show the observed values. Cluster variables are households, buildings, and geospatial clusters of different sizes. Household membership was left invariant when considering buildings and geospatial clusters. p-values indicate the one-sided probability of observing smaller than observed values under random cluster assignments. Results indicate within-household clustering and suggest neighbourhood transmission only in the cluster with 500m.

Additional file 4: Table S1. Non-response mechanism at the different follow-ups using complete cases and indicator of missingness for income.

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KoCo19/ORCHESTRA Study group: Mohamed Ibraheem Mohamed Ahmed, Emad Alamoudi, Jared Anderson, Valeria Baldassarre, Maximilian Baumann, Marc Becker, Franziska Bednarski, Marieke Behlen, Olimbek Bemirayev, Jessica Beyerl, Patrick Bitzer, Rebecca Böhnlein, Isabel Brand, Anna Brauer, Vera Britz, Jan Bruger, Franziska Bünz, Friedrich Caroli, Josephine Coleman, Lorenzo Contento, Alina Czwienzek, Flora Deák, Maximilian N. Diefenbach, Paulina Diepers, Anna Do, Gerhard Dobler, Jürgen Durner, Tabea Eser, Ute Eberle, Judith Eckstein, Philine Falk, Manuela Feyereisen, Volker Fingerle, Stefanie Fischer, Jonathan Frese, Felix Forster, Günter Fröschl, Otto Geisenberger, Mercè Garí, Marius Gasser, Sonja Gauder, Raffaela Geier, Kristina Gillig, Christof Geldmacher, Keisha Gezgin, Leonard Gilberg, Kristina Gillig, Philipp Girl, Elias Golschan, Vitus Grauvogl, Jessica Michelle Guggenbuehl Noller, Elena Maria Guglielmini, Pablo Gutierrez, Anselm Haderer, Celina Halfmann, Lena Hartinger, Timm Haselwarter, Jan Hasenauer, Alejandra Hernandez, Luca Heller, Arlett Heiber, Matthias Herrmann, Leah Hillari, Stefan Hillmann, Christian Hinske, Janna Hoeflin, Tim Hofberger, Michael Höfinger, Larissa Hofmann, Sacha Horn, Kristina Huber, Christian Janke, Lilian Karger, Ursula Kappl, Antonia Keßler, Zohaib Khan, Charlotte Kiani, Isabel Klugherz, Norah Kreider, Johanna Kresin, Arne Kroidl, Pratik Kunder, Magdalena Lang, Clemens Lang, Silvan Lange, Ekaterina Lapteva, Michael Laxy, Reiner Leidl, Leopold Liedl, Felix Lindner, Xhovana Lucaj, Elisabeth Lucke, Fabian Luppa, Alexandra Sophie Nafziger, Alexander Maczka, Petra Mang, Alisa Markgraf, Paula Matcau, Rebecca Mayrhofer, Anna-Maria Mekota, Dafni Metaxa, Emily Mohr, Hannah Müller, Katharina Müller, Nathalia Nascimento, Kasimir Niermeyer, Sophia Nikolaides, Leonie Pattard, Claire Pleimelding, Michel Pletschette, Viona Poll, Stephan Prückner, Kerstin Puchinger, Konstantin Pusl, Elba Raimúndez, Julius Raschka, Jakob Reich, Christina Reinkemeyer, Camilla Rothe, Viktoria Ruci, Elmar Saathoff, Nicole Schäfer, Paul Schandelmaier, Benedikt Schluse, Annika Schneider, Lara Schneider, Sophie Schultz, Mirjam Schunk, Lars Schwettmann, Josefin Sedlmeier, Linda Sintu-Sempta, Alba Soler, Peter Sothmann, Katharina Strobl, Aida Strüber, Laura Strüber, Jeni Tang, Fabian Theis, Verena Thiel, Eva Thumser, Niklas Thur, Julian Ullrich, Vincent Vollmayr, Emilia Von Lovenberg, Jonathan Von Lovenberg, Carsten Vos, Julia Waibel, Claudia Wallrauch, Nikolas Weigl, Roman Wölfl, Julia Wolff, Pia Wullinger, Tobias Würfel, Patrick Wustrow, Sabine Zange, Eleftheria Zeggini, Anna Zielke, Thorbjörn Zimmer, Thomas Zimmermann, Anna Zielke, Lea Zuche.

Authors' contributions

M.H. is the principal investigator of this study and obtained most necessary funds. M.H. and K.R. conceived the study with input from L.O., I.K., and A.W. Sample collection was led by S.W., M.P., C.R., I.N., C.J., and M.H. The laboratory set-up and sample processing were led by R. R.-A., I.P., and A.W. Data acquisition and data management were coordinated by N.C., with contributions of F.R., and S.W. Data was cleaned and prepared by N.C. Statistical analyses and data visualization were performed by N.C. and R.L.G. with contributions from A.B., P.P. and Y.S. C.F. led the statistical analyses. N.C., C.F. and R.L.G. conceptualized the result presentation with input from P.P. and K.R. The manuscript was primarily written by N.C., C.F. and R.L.G., with significant contributions from M.P. All authors have read and agreed to the published version of the manuscript.

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Availability of data and materials
Our data are accessible to researchers upon reasonable request to the corresponding author taking data protection laws and privacy of study participants into account. To facilitate reproducibility and reuse, the analysis and figure generation code has been made available on GitHub (https://github.com/koco19/epi3) and will be uploaded to ZENODO for long-term storage.

Declarations

Ethics approval and consent to participate
The study was conducted in accordance with good clinical (GCP) and epidemiological practice (GEP) standards as well as the Declaration of Helsinki in its most recent form (as amended by the 64th WMA General Assembly, Fortaleza, Brazil, in October 2013). The study protocol was approved by the Institutional Review Board of the Medical Faculty at Ludwig Maximilian University Munich, Germany (opinion dated 31 March 2020; number 20–275; opinion date amendment: 10 October 2020), prior to study initiation. Informed consent was obtained from all study participants prior to study inclusion.

Consent for publication
Not applicable.

Competing interests
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Author details
[1]Institute of Computational Biology, Helmholtz Munich, German Research Centre for Environmental Health, 85764 Neuherberg, Germany. [2]Core Facility Statistical Consulting, Helmholtz Munich, German Research Centre for Environmental Health, 85764 Neuherberg, Germany. [3]Division of Infectious Diseases and Tropical Medicine, University Hospital, LMU Munich, 80802 Munich, Germany. [4]Robert Koch Institute, Nordufer 20, 13353 Berlin, Germany. [5]Institute and Outpatient Clinic for Occupational, Social and Environmental Medicine, University Hospital, LMU Munich, 80336 Munich, Germany. [6]Centre for International Health (CIH), University Hospital, LMU Munich, 80336 Munich, Germany. [7]Comprehensive Pneumology Centre (CPC) Munich, German Centre for Lung Research (DZL), 89337 Munich, Germany. [8]Centre for Mathematics, Technische Universität München, 85748 Garching, Germany. [9]Life and Medical Sciences Institute, University of Bonn, 53115 Bonn, Germany. [10]German Centre for Infection Research (DZIF), Partner Site, Munich, Germany. [11]Fraunhofer Institute for Translational Medicine and Pharmacology ITMP, Immunology, Infection and Pandemic Research, 80799 Munich, Germany. [12]Max Von Pettenkofer Institute, Faculty of Medicine, LMU Munich, 80336 Munich, Germany. [13]Faculty of Business Administration and Economics, Bielefeld University, 33615 Bielefeld, Germany. [14]Institute of Radiation Medicine, Helmholtz Munich, German Research Centre for Environmental Health, 85764 Neuherberg, Germany.

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Clinical and immunological benefits of full primary COVID-19 vaccination in individuals with SARS-CoV-2 breakthrough infections: A prospective cohort study in non-hospitalized adults

Martina Prelog [a,1], Samuel D. Jeske [b,1], Claudia Asam [c,1], Andre Fuchs [d,1], Andreas Wieser [e], Christine Gall [f], Monika Wytopil [g], Sandra M. Mueller-Schmucker [g], Stephanie Beileke [g], Mehmet Goekkaya [h], Elisabeth Kling [i], Christof Geldmacher [e,j], Raquel Rubio-Acero [e], Michael Plank [e], Catharina Christa [b], Annika Willmann [b], Martin Vu [b], Sebastian Einhauser [k], Manuela Weps [c], Benedikt M.J. Lampl [l,m], Giovanni Almanzar [a], Kimia Kousha [a], Valeria Schwägerl [n], Bernhard Liebl [o], Beatrix Weber [p], Johannes Drescher [q], Jörg Scheidt [p], Olaf Gefeller [f], Helmut Messmann [d], Ulrike Protzer [b,r], Johannes Liese [n], Michael Hoelscher [e,j], Ralf Wagner [c,k], Klaus Überla [g], Philipp Steininger [g,*], on behalf of the CoVaKo Study Group

[a] Pediatric Rheumatology / Special Immunology, Department of Pediatrics, University Hospital Würzburg, Würzburg, Germany
[b] Institute of Virology, Technical University of Munich, School of Medicine, Munich, Germany
[c] Institute of Clinical Microbiology and Hygiene, University Hospital Regensburg, Regensburg, Germany
[d] Internal Medicine III - Gastroenterology and Infectious Diseases, University Hospital of Augsburg, Augsburg, Germany
[e] Division of Infectious Diseases and Tropical Medicine, University Hospital, Ludwig-Maximilians-Universität (LMU) Munich, Munich, Germany
[f] Department of Medical Informatics, Biometry and Epidemiology, Friedrich-Alexander-Universität Erlangen-Nürnberg, Erlangen, Germany
[g] Institute of Clinical and Molecular Virology, Universitätsklinikum Erlangen, Friedrich-Alexander-Universität Erlangen-Nürnberg, Erlangen, Germany
[h] Environmental Medicine, Faculty of Medicine, University of Augsburg, Institute of Environmental Medicine Helmholtz Zentrum München, German Research Center for Environmental Health, Augsburg, Germany
[i] Institute of Laboratory Medicine and Microbiology University Hospital Augsburg, Augsburg, Germany
[j] German Centre for Infection Research (DZIF), Partner Site Munich, Munich, Germany
[k] Institute of Medical Microbiology and Hygiene, Molecular Microbiology (Virology), University of Regensburg, Regensburg, Germany
[l] Regensburg Department of Public Health, Division of Infection Control and Prevention, Regensburg, Germany
[m] Department of Epidemiology and Preventive Medicine, University of Regensburg, Regensburg, Germany
[n] Pediatric Infectious Diseases, Department of Pediatrics, University Hospital Würzburg, Würzburg, Germany
[o] Bavarian Health and Food Safety Authority (LGL), Oberschleißheim, Germany
[p] Institute for Information Systems, University of Applied Sciences Hof, Hof, Germany
[q] smartlytic GmbH, Hof, Germany
[r] Institute of Virology, Helmholtz Munich, Munich, Germany, and German Center for Infection Research, Munich partner site

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ABSTRACT

Background: SARS-CoV-2 variants of concern (VOC) may result in breakthrough infections (BTIs) in vaccinated individuals. The aim of this study was to investigate the effects of full primary (two-dose) COVID-19 vaccination with wild-type-based SARS-CoV-2 vaccines on symptoms and immunogenicity of SARS-CoV-2 VOC BTIs.
Methods: In a longitudinal multicenter controlled cohort study in Bavaria, Germany, COVID-19 vaccinated and unvaccinated non-hospitalized individuals were prospectively enrolled within 14 days of a PCR-confirmed SARS-CoV-2 infection. Individuals were visited weekly up to 4 times, performing a structured record of medical data and viral load assessment. SARS-CoV-2-specific antibody response was characterized by anti-spike-(*S*)- and anti-nucleocapsid-(N)-antibody concentrations, anti-S-IgG avidity and neutralization capacity.
Results: A total of 300 individuals (212 BTIs, 88 non-BTIs) were included with VOC Alpha or Delta SARS-CoV-2 infections. Full primary COVID-19 vaccination provided a significant effectiveness against five symptoms

* Corresponding author at: Institute of Clinical and Molecular Virology, University Hospital Erlangen, Schlossgarten 4, 91054, Erlangen, Germany.
E-mail address: philipp.steininger@uk-erlangen.de (P. Steininger).
[1] These authors contributed equally to this work.

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(relative risk reduction): fever (33 %), cough (21 %), dysgeusia (22 %), dizziness (52 %) and nausea/vomiting (48 %). Full primary vaccinated individuals showed significantly higher 50 % inhibitory concentration (IC_{50}) values against the infecting VOC compared to unvaccinated individuals at week 1 (269 vs. 56, respectively), and weeks 5–7 (1,917 vs. 932, respectively) with significantly higher relative anti-S-IgG avidity (78% vs. 27 % at week 4, respectively).

Conclusions: Full primary COVID-19 vaccination reduced symptom frequencies in non-hospitalized individuals with BTIs and elicited a more rapid and longer lasting neutralization capacity against the infecting VOC compared to unvaccinated individuals. These results support the recommendation to offer at least full primary vaccination to all adults to reduce disease severity caused by immune escape-variants.

1. Introduction

Immune-escape variants of SARS-CoV-2 contribute to the high incidence of breakthrough infections (BTIs) in COVID-19 vaccinated individuals [1,2]. During our study period in Germany, the majority of the adult population had received a full primary vaccination course by two doses of wild-type-based COVID-19 mRNA or vector vaccines, while the circulating SARS-CoV-2 variants Alpha (B.1.1.7) and Delta (B.1.617.2), which were classified as variants of concern (VOCs) at the time of study recruitment, were associated with increasing numbers of BTIs [3,4]. Effectiveness of primary vaccination has been demonstrated for protection from severe COVID-19 including clinically significant organ dysfunction, hospitalization or death [5,6]. However, for non-hospitalized individuals with mild COVID-19, there is still need for further knowledge on symptom profile, viral dynamics and development of hybrid immunity after BTIs [7–14]. Most countries recommend basic immunity for all individuals ≥18 years and define basic immunity not only by three COVID-19 vaccine doses but also by two-dose primary vaccination and one BTI based on immunogenicity data including antibody avidity and neutralizing activity also against heterologous VOCs [15,16].

Therefore, the primary aim of this prospective, longitudinal, multicenter controlled cohort study was to compare the clinical and immunological course of Alpha and Delta BTIs after full primary vaccination in direct comparison to non-BTIs in a cohort of non-hospitalized adults from the general population in Bavaria, Germany.

2. Methods

2.1. Study design and participants

This prospective longitudinal multicenter controlled cohort study was designed by the COVID-19 Vaccine Consortium (CoVaKo) and was implemented at six study centers in Bavaria, Germany (University Hospitals of Erlangen, Augsburg, Munich [LMU and TUM], Regensburg and Würzburg) covering a population of approximately 4.8 million inhabitants. Participants were recruited in cooperation with the Bavarian Health and Food Safety Authority and local public health departments (Supplementary Methods). Data from April 13, 2021 until November 25, 2021 were analyzed, which covered the third (prevalent VOC Alpha) and fourth (Delta) SARS-CoV-2 waves in Germany [17]. Non-hospitalized adults (age ≥18 years) with a newly diagnosed (≤14 days) PCR-confirmed SARS-CoV-2 infection were consecutively enrolled and allocated into three groups according to their COVID-19 vaccination status including wild-type-based SARS-CoV-2 mRNA vaccines (Comirnaty, BioNTech/Pfizer or Spikevax, Moderna) and vector vaccines (Vaxzevria, AstraZeneca or JCOVDEN, Janssen): 1) full primary vaccinated individuals (group F) with two vaccinations regardless of vaccine type with ≥14 days between second vaccination and SARS-CoV-2 detection, 2) partially vaccinated individuals (group P) with either one vaccination or two vaccinations <14 days before SARS-CoV-2 detection, and 3) unvaccinated individuals (group U).

2.2. Data and specimen collection

For longitudinal analysis, a primary visit and up to three follow-up visits at weekly intervals were performed. A structured record of medical data was applied, and vital parameters, a nasopharyngeal swab, and serum were collected at each visit (Table 1 and Supplementary Methods). Symptom severity was classified in four categories based on symptoms, vital parameters and hospitalization status (Supplementary Table 1). Study data were documented using a web-based application, designed and hosted by the University of Applied Sciences Hof, Germany.

2.3. Viral load and antibody measurements

The SARS-CoV-2-RNA concentration in nasopharyngeal specimens was quantified in International Units per mL (IU/mL) by real-time RT-PCR. VOCs Alpha (B.1.1.7) and Delta (B.1.617.2) were identified by RT-PCR-based variant screening assays (Supplementary Methods). The SARS-CoV-2-specific antibody response was analyzed by determining concentrations of antibodies targeted against spike (*S*)-antigen or nucleocapsid (N)-antigen using the Roche Cobas Elecsys Anti-SARS-CoV-2 S (anti-S Ab; wild-type [Wuhan strain] receptor binding domain [RBD], binding antibody units [BAU]/mL) and Anti-SARS-CoV-2 (anti-N Ab, wild-type, cut-off index [COI]) chemiluminescence immunoassay tests (Roche Diagnostics, Penzberg, Germany). Anti-S-IgG avidity was analyzed by adaptation of the IgG-agile-SARS-CoV-2 ELISA (wild-type S; Institute Virion\Serion, Würzburg, Germany) as described [15,18,19]. Surrogate neutralizing antibodies (surNAb) against the wild-type RBD were detected using the iFlash 2019-nCoV neutralization antibody assay (YHLO Biotech, Shenzen, China). The neutralizing capacity against the homologous (infecting Alpha or Delta) VOC was determined using a lentiviral pseudotype neutralization assay, as described [20].

2.4. Statistical analysis

Statistical analyses were carried out using R Version 4.2.2. (R Foundation for Statistical Computing, Vienna, Austria) (Supplementary methods) [21]. Due to the explorative design of the study, no sample size calculation was performed. For analysis of viral load and antibody response, the study visit measurements were classified by weeks from the initial positive SARS-CoV-2 PCR result. Differences were considered significant at $p < 0.05$.

3. Results

3.1. Participants' characteristics

In total, 308 non-hospitalized adult individuals with PCR confirmed SARS-CoV-2 infection were enrolled and 300 (212 BTIs, 88 non-BTIs) were eligible for analysis (Fig. 1 and Supplementary Table 2). The demographic and clinical baseline data are summarized in Table 1 and Supplementary Table 3.

Table 1
Demographic and clinical characteristics of the individuals at study inclusion.

	Unvaccinated (U)	Partially vaccinated (P)	Full primary vaccinated (F)	p value (U vs. P)	p value (P vs. F)	p value (U vs. F)
Number of individuals	88	52	160			
Demographic characteristics						
Age, mean ± SD (years)	38 ± 12	38 ± 16	42 ± 15	0.945	0.162	0.031
Sex – no. (%)						
Female	55 (62%)	21 (40%)	96 (60%)	0.014	0.016	0.786
Male	33 (38%)	31 (60%)	64 (40%)	0.014	0.016	0.786
BMI, mean ± SD (kg/m^2)	25 ± 5	25 ± 6	25 ± 5	0.641	0.899	0.358
SARS-CoV-2 diagnosis						
Days between PCR diagnosis and study enrollment (visit 1), mean ± SD	7.7 ± 2.8	6.6 ± 3.1	6.5 ± 3.1	0.047	0.827	0.003
Days between onset of symptoms (if any) and PCR diagnosis, mean ± SD	2.5 ± 1.7	2.3 ± 1.5	2.2 ± 2.2	0.665	0.732	0.321
Symptoms before SARS-CoV-2 testing – no. (%)						
Asymptomatic	39 (44%)	33 (63%)	73 (46%)	0.036	0.037	0.894
General Symptoms						
Fever	23 (26%)	5 (10%)	25 (16%)	0.027	0.363	0.064
Fatigue	32 (36%)	7 (13%)	46 (29%)	0.003	0.028	0.253
Respiratory Symptoms						
Cough	19 (22%)	6 (12%)	35 (22%)	0.172	0.111	1
Shortness of breath	4 (5%)	1 (2%)	2 (1%)	0.651	0.572	0.190
Runny nose	18 (20%)	8 (15%)	55 (34%)	0.508	0.009	0.029
Sore throat	16 (18%)	11 (21%)	47 (29%)	0.664	0.286	0.067
Hoarseness	1 (1%)	2 (4%)	4 (2%)	0.555	0.637	0.658
Neurologic Symptoms						
Headache	26 (30%)	6 (12%)	36 (22%)	0.021	0.109	0.225
Hyposmia	6 (7%)	1 (2%)	12 (8%)	0.258	0.194	1
Dysgeusia	6 (7%)	2 (4%)	10 (6%)	0.710	0.734	1
Dizziness	6 (7%)	3 (6%)	6 (4%)	1	0.692	0.355
Gastrointestinal Symptoms						
Nausea / Vomiting	9 (10%)	1 (2%)	0 (0%)	0.091	0.245	< 0.001
Diarrhea	3 (3%)	0 (0%)	4 (2%)	0.295	0.574	0.701
COVID-19 vaccination regimen and previous SARS-CoV-2 infections						
Vector	–	25 (48%)	–			
mRNA	–	19 (37%)	–			
Vector/Vector	–	0 (0%)	8 (5%)			
mRNA/mRNA	–	5 (10%)	146 (91%)			
Vector/mRNA	–	3 (6%)	5 (3%)			
Days between last vaccination and PCR diagnosis, mean ± SD	–	38.1 ± 38.4	103 ± 59	n.a.	< 0.001	n.a.
Self-reported previous SARS-CoV-2 infection – no. (%)	0 (0%)	3 (6%)	0 (0%)	0.049	0.014	1
SARS-CoV-2 VOC – no. (%)						
Alpha (B.1.1.7)	8 (9%)	15 (29%)	28 (18%)	0.004	0.111	0.090
Delta (B.1.617.2)	67 (76%)	23 (44%)	121 (76%)	< 0.001	< 0.001	1
not available[a]	13 (15%)	14 (27%)	11 (7%)	0.119	< 0.001	0.070

[a] Variant of concern (VOC) could not be identified due to a very low or negative viral load. Abbreviations: BMI, body mass index; no., number; SD, standard deviation.

3.2. Clinical course of COVID-19 symptoms

At the first two visits in total 23 (8 %) of the individuals negated any symptoms. Among asymptomatic individuals at the time of initial positive SARS-CoV-2 PCR, 2 (5 %) of U and 11 (15 %) of F remained asymptomatic during the observation time. The most prevalent symptoms at the first two study visits were fatigue (78 %), cough (75 %) and hyposmia (72 %) in U, and fatigue (74 %), runny nose (71 %) and cough (59%) in F (Table 2). F were significantly less likely to show fever, cough, dysgeusia, dizziness and nausea/vomiting in comparison to U with a relative risk reduction (RRR) between 21 % and 52 %. Overall, the portions of F and P in the category "mild" of the symptom severity scale was substantially higher in comparison to U (Table 2 and Supplementary Table 4). At the final study visit (28.2 ± 3.7 days after the initial positive SARS-CoV-2 PCR), 48 (58 %) of U and 92 (59 %) of F still reported at least one symptom. The most common symptoms at that time were fatigue, cough, hyposmia, and dysgeusia with frequencies ranging from 19% to 33% without significant differences between U and F.

3.3. SARS-CoV-2 viral load kinetics

To study the impact of vaccination on SARS-CoV-2 replication in the upper respiratory tract, we analyzed viral load (Fig. 2a) and duration of viral shedding using nasopharyngeal swab specimens. Between week 1 and 2 after SARS-CoV-2-PCR diagnosis, the viral load decreased by a factor of 320 in F and of 224 in U. In week 2, the median viral load of F in comparison to U was significantly lower (250 [interquartile range, IQR 0–5880] vs. 630 [IQR 0–25,210] IU/mL, $p = 0.041$). In F, 8% had successful viral clearance compared to none in U in week 1 ($p = 0.018$). At week 2, 51 % of F compared to 28% of U showed viral clearance ($p = 0.002$) and 78 % of F compared to 69% in U had a viral load below 10,000 IU/mL, corresponding to a Ct-value of above approximately 30 in our quantitation. At week 1, the viral load correlated inversely with homologous neutralization titers in U and F (Fig. 3). We could not detect any significant correlations between viral load kinetics and symptom frequencies for U and F.

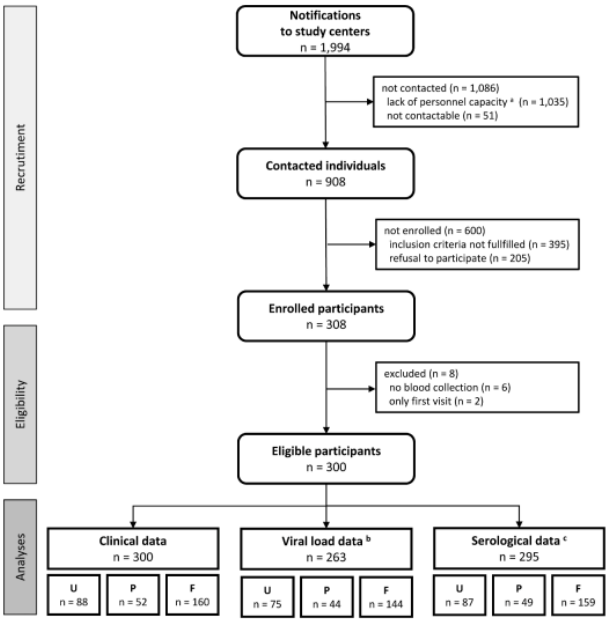

Fig. 1. Overview of the CoVaKo (COVID-19 Vaccine Consortium) study population and analyses.
Individuals were assigned to three groups according to their vaccination status: unvaccinated (U), partially vaccinated (P) and full primary vaccinated (F). [a]Notifications could not be processed at times when study centers had no sufficient personnel capacity for additional study visits. [b]Viral load data are not available from one study center since a non-quantitative SARS-CoV-2 PCR was performed at that site. [c]Number of individuals with results of at least one serological assay. Variant-specific homologous neutralization titers were only measured for individuals with identified Alpha or Delta variant of concern. Avidity assays could be performed only in samples with anti-spike antibody reactivity above the ELISA detection cut-off.

3.4. Temporal dynamics of the antibody response against SARS-CoV-2

With the exception of one individual, all F were initially anti-S (wild-type RBD) Ab positive and displayed a high median anti-S Ab concentration of 6887 BAU/mL (IQR 2549–11,623) in week 1 (Fig. 2b). In contrast, in 73% of U no anti-S Abs were detected in week 1 while the median anti-S Ab concentration of antibody positive individuals in U was as low as 7.2 BAU/mL (IQR 1.4–28). In F, a marked increase of anti-S Ab within two weeks was observed. Median anti-S Ab concentration reached the peak in week 3 (37,934 BAU/mL, IQR 12,380–66,861) and stayed high until weeks 5–7. This was contrasted by a delayed and only slight increase of anti-S Ab concentration in U, which was characterized by the highest median anti-S Ab concentration of 48.8 BAU/mL (IQR 13.6–213) in weeks 5–7. In comparison to F, this peak concentration was lower by a factor of 777. Anti-S Ab correlated with both surNAb and homologous neutralization titers at weeks 1 to 4 in all groups.

The majority of U (82%) and F (86%) exhibited no anti-N Ab response at week 1 (Fig. 2c). Subsequently, U showed a more rapid and significantly higher anti-N response throughout weeks 2 to 4 compared to F (median anti-N Ab concentration at week 4, 35.7 COI [IQR 6.1–73.6] vs. 9.1 COI [IQR 2.4–22.0], respectively; $p = 0.001$).

F revealed a significantly higher relative avidity index (RAI) compared to U at all time points (Fig. 2d). At week 1, 75 % of F showed a high RAI of >60 %, whereas it was equally low in U and P throughout the observation period. At week 4, the median RAI was 78 % for F and 27% for U, respectively. In P, RAI at weeks 1 and 2 correlated with the time since vaccination (Fig. 3).

P and F exhibited significantly higher surNAb against wild-type RBD from week 1 on when compared to U (Fig. 2e). At weeks 5–7, all individuals in F showed detectable surNAb at a median concentration of 735 AU/mL while only 44 % of U showed detectable surNAb at a low median concentration of 18 AU/mL.

VOC-specific homologous neutralization titers revealed significantly increased titers for F compared to U at week 1 ($p = 0.029$) and increased until week 4 in all groups (Fig. 2f). However, a trend towards decreasing neutralizing titers was visible for P and U in weeks 5–7, emphasized by a significantly lower median titer for U compared to F at weeks 5–7 ($p = 0.013$). In F and P, a positive correlation between surNAb and homologous neutralization titers was observed (Fig. 3). Both surNAb and homologous neutralization titers positively correlated with RAI at week 1.

4. Discussion

This prospective controlled multicenter cohort study emphasized the effectiveness of full primary wild-type-based COVID-19 vaccination for reducing symptom frequency in non-hospitalized individuals with Alpha or Delta BTIs. Our findings also showed that BTIs elicit neutralizing antibodies with high avidities against immune escape variants, such as Alpha and Delta, already at early stages after infection.

Full primary COVID-19 vaccination mitigated disease course by significantly reducing fever, cough, dysgeusia, dizziness, and nausea/vomiting with RRR ranging from 21 to 52 %. The reduced frequencies of cough and fever in F might be interpreted as an indicator of less severe respiratory tract and systemic infection, while the observed effectiveness

Table 2
Comparison of clinical characteristics between unvaccinated and full primary vaccinated individuals.

	Visits 1 and 2[a] Unvaccinated	Full primary vaccinated	RRR [CI]	p value	Visit 4 Unvaccinated	Full primary vaccinated	RRR [CI]	p value
Number of individuals	88	160			83	155		
Hospitalized individuals	0 (0%)	1 (1%)	not calculable	1	0 (0%)	0 (0%)	not calculable	1
General symptoms								
Fever	36 (41%)	44 (28%)	33% [4%;53%]	0.034	1 (1%)	0 (0%)	not calculable	0.355
Fatigue	69 (78%)	119 (74%)	5% [−9%;18%]	0.537	19 (23%)	51 (33%)	−44% [−126%;9%]	0.105
Musculoskeletal pain	46 (52%)	64 (40%)	23% [−1%;42%]	0.082	4 (5%)	9 (6%)	−20% [−279%;62%]	1
Respiratory symptoms								
Cough	66 (75%)	95 (59%)	21% [6%;34%]	0.018	17 (20%)	30 (19%)	6% [−61%;44%]	1
Shortness of breath	18 (20%)	29 (18%)	11% [−50%;48%]	0.735	6 (7%)	14 (9%)	−25% [−213%;50%]	0.808
Runny nose	51 (58%)	113 (71%)	−22% [−49%;1%]	0.05	9 (11%)	11 (7%)	35% [−52%;72%]	0.465
Sore throat	40 (45%)	74 (46%)	−2% [−35%;23%]	1	1 (1%)	6 (4%)	−221% [−2524%;61%]	0.427
Hoarseness	19 (22%)	29 (18%)	16% [−41%;50%]	0.507	4 (5%)	2 (1%)	73% [−43%;95%]	0.190
Neurologic symptoms symptom								
Headache	58 (66%)	94 (59%)	11% [−9%;27%]	0.279	6 (7%)	23 (15%)	−105% [−384%;13%]	0.098
Hyposmia	63 (72%)	94 (59%)	18% [1%;32%]	0.054	27 (33%)	31 (20%)	39% [4%;60%]	0.059
Dysgeusia	59 (67%)	84 (52%)	22% [4%;36%]	0.032	24 (29%)	27 (17%)	40% [3%;63%]	0.070
Dizziness	31 (35%)	27 (17%)	52% [25%;69%]	0.002	3 (4%)	3 (2%)	46% [−159%;89%]	0.669
Gastrointestinal symptoms								
Nausea / Vomiting	18 (20%)	17 (11%)	48% [4%;72%]	0.038	0 (0%)	3 (2%)	not calculable	0.554
Diarrhea	20 (23%)	29 (18%)	20% [−32%;52%]	0.407	0 (0%)	2 (1%)	not calculable	0.540
Abdominal pain	12 (14%)	11 (7%)	50% [−10%;77%]	0.108	2 (2%)	5 (3%)	−34% [−575%;73%]	1
Vital parameters								
Respiratory rate ≥ 30/min	0 (0%)	1 (1%)	not calculable	1	0 (0%)	0 (0%)	not calculable	1
Peripheral oxygen saturation < 92%	0 (0%)	0 (0%)	not calculable	1	0 (0%)	0 (0%)	not calculable	1
Blood pressure systolic < 90 mmHg or diastolic < 60 mmHg	2 (2%)	2 (1%)	45% [−284%;92%]	0.617	1 (1%)	0 (0%)	not calculable	0.355
Heart rate > 125/min	0 (0%)	0 (0%)	not calculable	1	0 (0%)	0 (0%)	not calculable	1
Body temperature ≥ 38.0 °C	2 (2%)	6 (4%)	−65% [−700%;66%]	0.716	1 (1%)	3 (2%)	−61% [−1420%;83%]	1
Symptom severity scale[b]				0.012				0.063
Asymptomatic	3 (3%)	13 (8%)			48 (58%)	72 (46%)		
Mild	11 (12%)	43 (27%)			13 (16%)	43 (28%)		
Moderate	72 (82%)	101 (63%)			21 (25%)	40 (26%)		
Severe	2 (2%)	3 (2%)			1 (1%)	0 (0%)		

[a] Symptoms recorded at visit 1 and 2 are summarized by reporting whether the symptom occurred at least at one visit in order to assess the maximum symptom severity.
[b] Two-group-comparisons were performed by using the overall chi-squared test on hierarchical categories with p values from Monte Carlo simulation. Abbreviations: CI, 95 % confidence interval; relative risk reduction, RRR.

against dysgeusia and dizziness possibly reflects less neurological manifestations in vaccinated individuals as found by others [22–26]. In contrast, we could not detect any association between symptom frequency and SARS-CoV-2 viral load in nasopharyngeal swabs. However, viral load was inversely correlated with the initial homologous neutralization titer in U and F. While the viral load was only slightly reduced in F as compared to U within the first two weeks after PCR diagnosis, F demonstrated a significantly higher viral clearance rate already in the first week. These findings are consistent with previous studies describing a faster decline in viral load in vaccinated individuals during the early symptomatic phase possibly impacting transmission [7,27]. Notably, from week 2 on the majority of both U and F showed a viral load below 10,000 IU/ml, corresponding to a Ct-value of >30, which is reported to indicate a marked decrease in virus isolation success and infectivity [28].

Our results emphasized the development of superior hybrid immunity in the early stages upon BTI. We could clearly show that F had significantly higher RAI from the beginning, whereas U and P exhibited low RAI over the whole observation period. [15]. These findings indicate that primary COVID-19 vaccination might induce long-term affinity maturation which can be further enhanced by the third S-antigen contact via BTI [15,29]. There may be two reasons why a portion of F nevertheless showed only low or moderate S-specific antibody avidity at week 1: first, the time period between primary vaccination and BTI was too short for complete avidity maturation; second, the result of the RAI measurement was a mixture of high-affinity vaccine-induced antibodies and low-affinity infection-induced antibodies within the pool of S-specific antibodies after BTI. Thus, there may occur a competition in the repertoire of expanding B cell-clones either primed by vaccine-encoded wild-type S-antigen or VOC-encoded S-antigen. Already from the first week, F and P exhibited significantly higher surNAb concentrations against vaccine-matching wild-type RBD in comparison to U with continuous increase during the observation time. We also found a more rapid onset of the homologous (to the infecting VOC) neutralizing antibody response in F and P as compared to U. Although differences between the groups became smaller over the first weeks, F maintained a higher homologous neutralization titer in the later convalescence phase in comparison to U. Overall, the differences in neutralizing antibody response between F and U were more marked for the surNAb concentrations than for the homologous neutralization titers. This may imply that neutralizing antibody production is more pronounced against

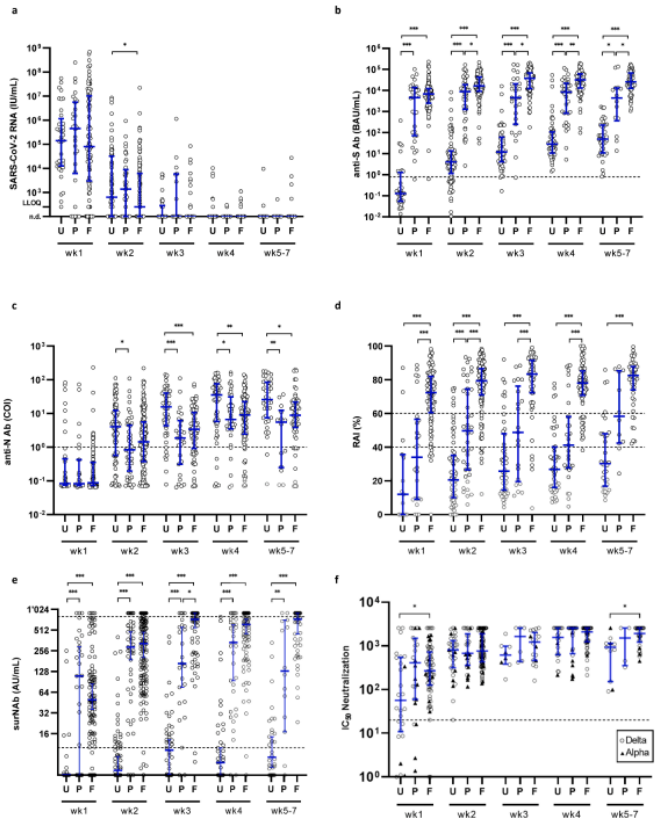

Fig. 2. Comparison of SARS-CoV-2 viral load and antibody responses depending on the vaccination status.
Dynamic of viral load in the upper respiratory tract and antibody responses against SARS-CoV-2 in unvaccinated (U), partially vaccinated (P) and full primary vaccinated (F) individuals at week 1 (wk1), 2 (wk2), 3 (wk3), 4 (wk4) and weeks 5 to 7 (wk5–7) after PCR diagnosis. Circles represent the results from the individuals. Median and interquartile ranges are indicated by blue horizontal lines. Data were analyzed by Kruskal-Dunns test and significance levels were adjusted by Bonferroni correction for multiple tests. Statistically significant differences are indicated by p values (* $p<0.05$, ** $p<0.005$, *** $p<0.001$). **a**, SARS-CoV-2 RNA concentration (International Units [IU]/mL) in a total of 741 nasopharyngeal specimens (no. of samples, 205 [U], 132 [P] and 404 [F], respectively). The lower limit of quantitation (LLOQ) was 250 International Units per mL (IU/mL). Negative PCR results are displayed as not detected (n.d.). **b**, Antibody concentration against the spike antigen (anti-S Ab) quantified as binding arbitrary units (BAU)/mL for 908 samples (266 [U], 154 [P] and 488 [F], respectively) and **c**, antibody concentration against the nucleocapsid antigen (anti-N Ab) given as cut-off index (COI) for in total 935 samples (279 [U], 155 [P] and 501 [F], respectively). Dashed lines indicate the cut-off for each assay. **d**, relative avidity index (RAI;%) of IgG antibodies against the S-antigen in 749 serologically positive samples (204 [U], 130 [P] and 415 [F], respectively) with >15 IU/mL in the IgG agile SARS-CoV-2 ELISA. Dashed lines indicate the cut-offs of low (RAI <40%), moderate (RAI 40–60%) and high (RAI >60%) avidity. **e**, Surrogate neutralizing antibody (surNAb) concentrations (antibody units [AU]/mL) against the receptor binding domain (RBD) of the S-antigen determined in the chemiluminescence immunoassay surrogate neutralization test for 809 samples (229 [U], 148 [P] and 432 [F], respectively). The upper limit of quantification (ULOQ, 800 AU/mL) and the lower limit of quantitation (LLOQ, 10 AU/mL) are shown by dashed lines. **f**, Serum 50% inhibitory concentration (IC_{50}) values for the homologous infection-neutralization capacity against the Alpha variant (no. of samples, 23 [U], 37 [P] and 60 [F], respectively) and Delta variant (no. of samples, 80 [U], 51 [P] and 227 [F], respectively). The assay cut-off is indicated by the dashed line. Circles and triangles represent the results from Delta- and Alpha-infected individuals, respectively.

homologous VOC antigens compared to wild-type S-antigen in unvaccinated individuals. These observations were also described for Omicron infections after which cross-neutralization for wild-type was lower in unvaccinated individuals whereas vaccinated individuals showed higher neutralization capacity against the infecting VOC [30,31]. Finally, we observed a differential immune response against non-vaccine-encoded antigens between BTIs and non-BTIs, since individuals of F showed a delayed and reduced antibody generation against the non-vaccine-encoded N-antigen in comparison to U. This may be explained by an early immune recall against S-antigen in vaccinated individuals and predominant expansion of S-specific memory B-cell clones relatively limiting the N-specific de novo B-cell response. An enhanced S-specific but attenuated N-specific response has also been shown for CD4+*T*-cells [32].

However, our study has some limitations. Since it was an exploratory analysis, there might be the risk of false-positive differences due to

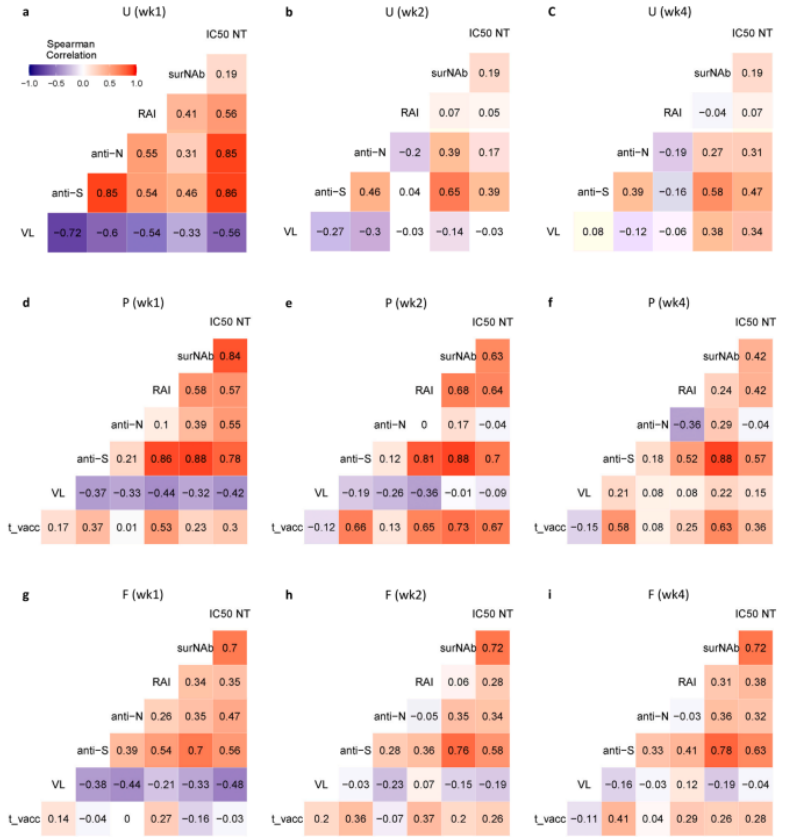

Fig. 3. Heatmaps of SARS-CoV-2 viral load, antibody response and time since vaccination.
Heatmaps illustrate correlations between SARS-CoV-2 viral load (VL), anti-spike antibodies (anti-S Ab), anti-nucleocapsid antibodies (anti-N Ab), relative avidity index (RAI) of anti-S-IgG, surrogate neutralizing antibodies (surNAb) and 50% inhibitory concentration neutralization titers (IC_{50} NT) in unvaccinated (U; a-c), partially vaccinated (P; d-f) and full primary vaccinated (F; g-i) individuals at week 1 (wk1), week 2 (wk2) and week 4 (wk4), respectively. For vaccinated individuals, correlations for time since last vaccinations (t_{vacc}) are additionally presented.

comparison of multiple parameters between BTIs and non-BTIs and of false-negative results due to an insufficient number of participants. There may be recruitment bias since severely diseased individuals may have not been able to participate. Symptom reporting might be biased by psychological factors which may lead to overestimation of symptoms in vaccinated individuals or underreporting of symptoms in unvaccinated individuals or vice versa [33]. SARS-CoV-2 viral load was longitudinally monitored in the upper respiratory tract, but there is only a limited correlation between viral RNA concentration and infectious virus [13, 34–36]. Since the study period was before emergence of Omicron as predominant circulating variant, only Alpha and Delta infections were investigated. Therefore, clinical and immunological differences between BTIs and non-BTIs by other immune escape variants cannot be inferred in general. This study does not allow any conclusion on the effectiveness of hybrid immunity since only BTIs in individuals with vaccine-induced immunity were included. Longevity of antibody responses has not been analyzed and does not allow deduction of booster recommendations.

To our knowledge, this study is one of the largest prospective studies on Alpha/Delta BTIs in individuals after full primary COVID-19 vaccination with comprehensive investigation of clinical, virological and serological data in direct comparison to non-BTIs. The prospective design with weekly follow-up visits allowed a detailed analysis of the clinical course and the kinetics of viral load and antibody response.

In conclusion, our study demonstrates that full primary COVID-19 vaccination reduces symptom frequency in non-hospitalized adults with Alpha or Delta BTIs. In accordance to previous data emphasizing that three antigen exposures enhance the neutralizing capacities also against immune escape variants [15], the present study clearly showed that hybrid immunity acquired by full primary vaccination and subsequent VOC BTI resulted in high anti-S-IgG avidity and neutralizing activity against possible immune escape variants. These data provide further evidence for clinical and immunological benefits of full primary COVID-19 vaccination against SARS-CoV-2 immune escape variants and support recommendations to offer at least two vaccinations to all adults.

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Ethical approval

The study was approved by the Ethics Committee of the Friedrich-Alexander-Universität Erlangen-Nürnberg, Germany (vote 46_21 B) and adopted by the local ethics committees of all other study centers. The study complies with the 1964 Declaration of Helsiniki and its later amendments. The study was registered in the German Clinical Trials Register (DRKS00024739, date of registration 04 March 2021, prospectively registered). Written informed consent was obtained from all participants upon study enrollment.

CRediT authorship contribution statement

Martina Prelog: Conceptualization, Formal analysis, Investigation, Methodology, Resources, Supervision, Visualization, Writing – original draft, Writing – review & editing. **Samuel D. Jeske:** Investigation, Methodology. **Claudia Asam:** Investigation, Visualization, Writing – review & editing. **Andre Fuchs:** Investigation, Methodology, Writing – original draft, Writing – review & editing. **Andreas Wieser:** Investigation, Methodology. **Christine Gall:** Formal analysis, Visualization, Writing – review & editing. **Monika Wytopil:** Investigation, Writing – review & editing. **Sandra M. Mueller-Schmucker:** Investigation, Writing – review & editing. **Stephanie Beileke:** Investigation, Writing – review & editing. **Mehmet Goekkaya:** Investigation, Writing – review & editing. **Elisabeth Kling:** Investigation. **Christof Geldmacher:** Investigation. **Raquel Rubio-Acero:** Investigation. **Michael Plank:** Investigation. **Catharina Christa:** Investigation. **Annika Willmann:** Investigation. **Martin Vu:** Investigation. **Sebastian Einhauser:** Investigation, Writing – review & editing. **Manuela Weps:** Investigation. **Benedikt M.J. Lampl:** Investigation. **Giovanni Almanzar:** Investigation. **Kimia Kousha:** Investigation. **Valeria Schwägerl:** Investigation. **Bernhard Liebl:** Investigation. **Beatrix Weber:** Data curation. **Johannes Drescher:** Data curation. **Jörg Scheidt:** Data curation. **Olaf Gefeller:** Formal analysis, Writing – review & editing. **Helmut Messmann:** Funding acquisition, Resources, Supervision. **Ulrike Protzer:** Funding acquisition, Resources, Supervision, Conceptualization. **Johannes Liese:** Conceptualization, Funding acquisition, Resources, Supervision. **Michael Hoelscher:** Funding acquisition, Resources, Supervision. **Ralf Wagner:** Conceptualization, Funding acquisition, Methodology, Resources, Supervision, Writing – review & editing. **Klaus Überla:** Conceptualization, Funding acquisition, Methodology, Project administration, Resources, Supervision, Writing – review & editing. **Philipp Steininger:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Supervision, Visualization, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: MPr receives honoraria for scientific talks from Abbvie, BioNTech, Chugai-Roche, GSK, Esanum, Janssen, Novartis, Moderna, MSD, Pfizer, Sanofi, and SOBI, for consultant tasks from Abbvie, BioNTech, GSK, Janssen, Novartis, and Pfizer, travel scholarships from Chugai-Roche, GSK, Novartis, and Pfizer and support for investigator-initiated research from Baxter, Chugai-Roche, Galapagos, GSK, MSD, Moderna, Novartis, Pfizer and SOBI. UP received personal fees from Abbott, Abbvie, Arbutus, Gilead, GSK, J&J, MSD, Roche, Sanofi, Sobi, and Vaccitech. UP is co-founder, share-holder and board member of SCG Cell Therapy Inc.. The other authors have no competing interests to declare.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jcv.2023.105622.

Appendix

Members of the CoVaKo Study Group

Prof. Dr. Helmut Messmann, Dr. Andre Fuchs, Dr. Alanna Ebigbo, Dr. Christoph Römmele, Maximilian Ullrich, Marie Freitag, Prof. Dr. Claudia Traidl-Hoffmann, Mehmet Goekkaya, Aline Metz, Corinna Holetschek, Prof. Dr. Avidan Neumann, Elisabeth Kling, Prof. Dr. Reinhard Hoffmann, Mihail Pruteanu, PD Dr. Thomas Wibmer, Dr. Susanne Rost, Prof. Dr. Klaus Überla, Dr. Philipp Steininger, Dr. Monika Wytopil, Stephanie Beileke, Dr. Sandra M. Müller-Schmucker, Dr. Klaus Korn, Tamara Hastreiter, Kirsten Fraedrich, Debora Obergfäll, Dr. Frank Neumann, Dr. Claudia Kuhn, Dr. Katja Günther, Dr. Elke Friedrich, Prof. Dr. Michael Hoelscher, PD Dr. Andreas Wieser, PD Dr. Christof Geldmacher, Christian Janke, Michael Plank, Jessica Guggenbühl, Christina Reinkemeyer, Ivan Noreña, Dr. Noemi Castelletti, Dr. Raquel Rubio Acero, M. I.M. Ahmed, Paulina Diepers, Tabea M. Eser, Anna Fuchs, Olga Baranov, Bernadette Bauer, Danni Wang, Ivana Paunovic, Prof. Dr. Ulrike Protzer, Samuel D. Jeske, Catharina Christa, Kathrin Tinnefeld, Martin Vu, Annika Willmann, Dr. Hedwig Roggendorf, Dr. Nina Körber, Dr. Tanja Bauer, PD Dr. Sabine Gleich, Prof. Dr. Ralf Wagner, Dr. Claudia Asam, Sebastian Einhauser, Manuela Weps, Antonia Senninger, Dr. George Carnell, Prof. Jonathan Luke Heeney, Antonia Ebner, Maria José de Schultz, Cedric Rajes, Aya Al Wafai, David Brenner, Laura Sicheneder, Melanie Berr, Anja Schütz, Dr. Stilla Bauernfeind, Prof. Dr. Dr. André Gessner, Prof. Dr. Barbara Schmidt, Daniela Biermeier, PD Dr. Benedikt M.J. Lampl, Ulrich Rothe, Dr. Ute Gleißner, Dr. Susanne Brückner, Michaela Treml, Holger Schedl, Dr. Beate Biermaier, Markus Achatz, Dr. Daniela Hierhammer, Johanna Englhardt, Werner Scheidl, Dr. Sivaji Jeyaraman, Dr. Barbara Schutt, Prof. Dr. Johannes Liese, Prof. Dr. Martina Prelog, PD Dr. Giovanni Almanzar, Valeria Schwägerl, Dr. Julia Bley, Tim Vogt, Kimia Kousha, Lars Ziegler, Astrid Stein, Franziska Förg, Dr. med. Johann Löw, Barbara Finkenberg, Dennis Pollak, Alexander Zamzow, Dr. Nicole Eberbach, Lara Balkie, Tanja Kretzschmann, Matthias Gehrig, Matthias Bandorf, Kilian Keck, Dr. Jan Allmanritter, Shahid Rafique, Mona Finster, Dr. med. Ingo Baumgart, Sabine Heumüller-Klug, Hans-Jürgen Koglin, Prof. Dr. Olaf Gefeller, Dr. Christine Gall, Prof. Dr. Annette B. Pfahlberg, Isabelle Kaiser, Prof. Dr. Jörg Scheidt, Johannes Drescher, Yannic Siebenhaar, Dr. Florian Wogenstein, Dr. Dirk Reinel, Prof. Dr. Beatrix Weber, Fabian Zarzitzky, Prof. Dr. Bernhard Liebl, Prof. Dr. Caroline Herr, Dr. Katharina Katz, Prof. Dr. Dr. Andreas Sing and Dr. Alexandra Dangel.

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7. Paper III

diagnostics

MDPI

Brief Report

Impact of Omicron Variant Infection on Assessment of Spike-Specific Immune Responses Using the EUROIMMUN Quan-T-Cell SARS-CoV-2 Assay and Roche Elecsys Anti-SARS-CoV-2-S

Mohamed I. M. Ahmed [1,2,*,†], Michael Plank [1,†], Noemi Castelletti [1,3,4], Paulina Diepers [1], Tabea M. Eser [1,2], Raquel Rubio-Acero [1], Ivan Noreña [1], Christina Reinkemeyer [1], Dorinja Zapf [5], Michael Hoelscher [1,2,3], Christian Janke [1], Andreas Wieser [1,2,3,6,‡], Christof Geldmacher [1,2,3,†] and on behalf of the KoCo19/ORCHESTRA Study Group [‡]

[1] Division of Infectious Diseases and Tropical Medicine, University Hospital, LMU Munich, 80802 Munich, Germany
[2] German Center for Infection Research (DZIF), Partner Site Munich, 81675 Munich, Germany
[3] Fraunhofer Institute for Translational Medicine and Pharmacology ITMP, Immunology, Infection and Pandemic Research, 80799 Munich, Germany
[4] Institute of Radiation Medicine, Helmholtz Munich, German Research Centre for Environmental Health, 85764 Neuherberg, Germany
[5] Institute for Experimental Immunology, Affiliated to EUROIMMUN Medizinische Labordiagnostika AG, 23560 Lübeck, Germany
[6] Max Von Pettenkofer Institute, Faculty of Medicine, LMU Munich, 80336 Munich, Germany
* Correspondence: mohamed.ahmed@lrz.uni-muenchen.de or mohamed.ahmed@med.uni-muenchen.de
† These authors contributed equally to this work.
‡ Membership of KoCo19/ORCHESTRA Study Group is provided in the Acknowledgements.

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Abstract: The currently prevailing variants of SARS-CoV-2 are subvariants of the Omicron variant. The aim of this study was to analyze the effect of mutations in the Spike protein of Omicron on the results Quan-T-Cell SARS-CoV-2 assays and Roche Elecsys anti-SARS-CoV-2 anti-S1. Omicron infected subjects (($n = 37$), vaccinated ($n = 20$) and unvaccinated ($n = 17$)) were recruited approximately 3 weeks after a positive PCR test. The Quan-T-Cell SARS-CoV-2 assays (EUROIMMUN) using Wuhan and the Omicron adapted antigen assay and a serological test (Roche Elecsys anti-SARS-CoV-2 anti-S1) were performed. Using the original Wuhan SARS-CoV-2 IGRA TUBE, in 19 of 21 tested Omicron infected subjects, a positive IFNy response was detected, while 2 non-vaccinated but infected subjects did not respond. The Omicron adapted antigen tube resulted in comparable results. In contrast, the serological assay detected a factor 100-fold lower median Spike-specific RBD antibody concentration in non-vaccinated Omicron infected patients ($n = 12$) compared to patients from the pre Omicron era ($n = 12$) at matched time points, and eight individuals remained below the detection threshold for positivity. For vaccinated subjects, the Roche assay detected antibodies in all subjects and showed a 400 times higher median specific antibody concentration compared to non-vaccinated infected subjects in the pre-Omicron era. Our results suggest that Omicron antigen adapted IGRA stimulator tubes did not improve detection of SARS-CoV-2-specific T-cell responses in the Quant-T-Cell-SARS-CoV-2 assay. In non-vaccinated Omicron infected individuals, the Wuhan based Elecsys anti-SARS-CoV-2 anti-S1 serological assay results in many negative results at 3 weeks after diagnosis.

Keywords: SARS-CoV-2; spike-specific immune response; omicron; breakthrough infections

1. Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is the causative agent of the current COVID-19 pandemic; with more than half a billion infected individuals and more than 6 million deaths. The virus was first detected in December 2019 in Wuhan,

China, and rapidly spread across the world. The currently prevailing variants of the virus are subvariants of the Omicron variant (B.1.1.529), which evolved by July 2022 into BA.1, BA2-5 and BQ1.1, that dominate the pandemic. Omicron differs from previous variants of concern in regard to its infectiousness and the more than 30 different mutations within the Spike protein [1]. However, Omicron and its subvariants are substantially less pathogenic compared to the original Wuhan or Delta strains [2].

Current diagnostic techniques involved in the detection of acute SARS-CoV-2 infections utilize nose or throat swabs, followed by direct viral RNA detection using RT-PCR techniques or direct detection of specific SARS-CoV-2 antigens. Antigen tests have proven to be useful; however, they lack in sensitivity, especially with lower viral loads [3]. To prove past infections with SARS-CoV-2, serologic tests, such as the Roche Elecsys anti-SARS-CoV-2 anti-S1 or anti-nucleocapsid, are used. These tests detect antibodies against SARS-CoV-2 proteins, such as Spike or Nucleocapsid, and can be used with good sensitivity and specificity at high throughput [4]. However, such techniques often still use Wuhan wild type virus antigens. Spike-specific T-cell responses against SARS-CoV-2 appear to be less affected by Spike mutations in Omicron variants [5], which is relevant for immunodiagnosis of infection and for characterization of adaptive immunity in convalescent patients, vulnerable populations (e.g., immunologically impaired individuals or the elderly population) and/or vaccinated subjects [6,7].

The Quan-T-Cell SARS-CoV-2 assay is a commercially available interferon gamma release assay (IGRA) which quantifies interferon-γ (IFNγ), which is specifically released by T-cells upon in vitro restimulation with specific peptides of the Spike antigen [3]. SARS-CoV-2-specific T-cells producing IFNγ contribute to immune protection from severe disease in humans [8] and are essential for vaccine-induced protection upon SARS-CoV-1 infection in mice and non-human primates (NHP) [9–12]. The benefits of IGRA assays in general are their relative simplicity in comparison to ELISPOT and intracellular cytokine staining, making them suitable for assessment of T-cell responses even in resource-limited settings.

This Quan-T-Cell SARS-CoV-2 assay was developed by using N-terminal Spike peptides based on the Wuhan strain in antigenic regions. These regions are affected by mutations occurring in the Omicron variant, which may affect assay accuracy [13]. Indeed, reduced test sensitivity for Omicron variant samples has been demonstrated for several rapid-tests or serology assays when compared to Wuhan and delta variant samples [14].

The aim of this study was to determine whether the currently marketed Quan-T-Cell SARS-CoV-2 from EUROIMMUN could be tailored and improved by adapting the antigenic cocktail with Omicron variant peptides. In order to achieve this, we tested the CE-IVD certified "Wuhan" based stimulator tube against an updated version containing antigens based on the Omicron Spike protein and compared the measured IFNγ concentration in the supernatant in a head-to-head comparison. Furthermore, we also tested to what degree Omicron infections, breakthrough infections (BTI) and non-breakthrough infections (non BTI), influence the results of the serological Roche Elecsys anti-SARS-CoV-2 anti-S assay in direct comparison to samples from patients infected during earlier phases of the pandemic, when the Wuhan strain still dominated.

2. Materials and Methods

2.1. Study Population

The study participants were recruited from the KoCo19-Immu cohort (Project number: 20-371), which is a prospective study started in 2020 and conducted in Munich, Germany. On 1 December 2020, the KoCo19-Immu cohort joined the ORCHESTRA (Connecting European Cohorts to Increase Common and Effective Response to SARS-CoV-2 Pandemic) project.

KoCo19-Immu aims to identify and characterize factors that influence the clinical course and further transmission of SARS-CoV-2 infection. The 37 participants of this KoCo19-sub-study were recruited from December 2021 until the end of March 2022. The general inclusion and exclusion criteria for the KoCoImmu study are based on the ones

of the KoCo19 study which have been described in detail previously [15]. Additionally, there were specific criteria used for the purpose of the Omicron subgroup. Only outpatient BTI (vaccinated followed by infection, n = 20) and non-BTI (first time infected, n = 17) were recruited. Potential participants were questioned prior to the visit and only the ones who reported no previous SARS-CoV-2 infection were included. Furthermore, for the purpose of this analysis, only subjects that were confirmed SARS-CoV-2 PCR positive by routine laboratory diagnostics were considered. Subjects were either reported by the health authorities as confirmed Omicron cases or had a confirmation of Omicron infection by the initial PCR test or a high likelihood of an Omicron infection, which was indicated by testing positive for specific mutation markers. No PCR confirmation or detection of a different virus variant were exclusion criteria. Recruitment into the study followed 3 weeks after the SARS-CoV-2 PCR positive diagnosis. Missing necessary samples were classified as exclusion criteria. Samples from acutely SARS-CoV-2 infected subjects recruited during the early phase of the pandemic (May 2020–January 2021) were matched according to the time since diagnosis for serological comparisons with the Omicron cases.

2.2. Quan-T-Cell SARS-CoV-2 Interferon Gamma Release Assay

Blood samples for testing in the Quan-T-Cell SARS-CoV-2 Interferon gamma release assay could be obtained and processed from 26 of all subjects with a SARS-CoV-2 infection by the Omicron strain; out of these, 16 were BTI and 10 were non BTI cases. Samples were collected after a median of 21 days (Range: 11–55) and 28 days (Range: 6–37) after diagnosis for BTI and non BTI, respectively. The participants had a median age of 51 (Range: 25–81) in the BTI group and 50 (Range: 24–59) in the non BTI group. The majority of the study subjects were female (77%, 20/26).

Heparin tubes were used to collect 6 mL of fresh whole blood. A volume of 500 µL was then stimulated overnight (16–18 h) at 37 °C and 5% CO_2 in the SARS-CoV-2 IGRA BLANK (negative control), STIM (positive control using mitogen), and TUBE (antigens based on the SARS-CoV-2 Wuhan Spike protein) tubes (Quan-T-Cell SARS-CoV-2, EUROIMMUN, Ref: ET 2606-3003) and the SARS-CoV-2 IGRA Omicron (antigens based on the SARS-CoV-2 Omicron Spike protein) tube (EUROIMMUN). Following incubation, the cells were centrifuged at 12,000× g for 10 min and the plasma collected and frozen at −80 °C for later IFNy analysis using the Quan-T-Cell-ELISA kit (EUROIMMUN, Ref: EQ 6841-9601) on the fully automated EUROIMMUN Analyzer I (EUROIMMUN).

Background subtraction was performed and, thereafter, IFNy concentrations from the two different measurements were classified into three different categories: (i) negative (<0.1 IU/mL), (ii) borderline (0.1–0.2 IU/mL) and (iii) positive (>0.2 IU/mL). These cut-offs were taken from the CE-IVD certified kit for the SARS-CoV-2 IGRA TUBE. The limit of detection for the Quan-T-Cell SARS-CoV-2 assay was provided by the manufacturer and is 18.44 IU/mL. Similar cut-off values were used for the Omicron tube, as no official cut-offs were available from the manufacturer at the time of the study. Measurements were categorized as invalid if the negative control was >0.4 IU/mL or the positive control was <0.4 IU/mL after background subtraction. Samples with a detection level above the maximum linear range value were placed with values greater than the largest IFNy value detectable after extrapolation (>8 IU/mL).

2.3. Roche Elecsys Anti-SARS-CoV-2 S Measurement

EDTA-plasma samples were used to perform the serologic assays. Samples from 24 subjects were included, 11 BTI and 13 non BTI. Venous samples were taken in 3 mL EDTA plasma tubes (Sarstedt, Nümbrecht, Germany) and mixed by inverting several times. The cell pellet was removed by centrifugation (for 10 min, 2500 rpm) and the plasma was transferred into 2 mL individually barcoded screw cap tubes (Sarstedt, Nümbrecht Germany). We performed the serologic assessment as recommended by the manufacturer. In brief, values are given in Units/mL, the positivity threshold is set to 0.8 as recommended. Values above the linear range specified by the manufacturer (250 U/mL) were diluted as

recommended in the manual until the measurements reached linear range again. The final concentration in these cases was calculated using the dilution factor and the measured units. Complete descriptions of the assays used for this analysis have been already published and can be found [16].

2.4. Statistical Analysis

The complete dataset was cleaned and locked prior to the conduction of any analyses that were performed in R (version 4.0.5, R Development Core Team, 2021). Overall testing was performed with a Kruskal–Wallis test, while differences between groups were accessed via Mann–Whitney testing. The Graph visualizing the IFNy release (Figure 1) was created in GraphPad Prism Version 6.0.

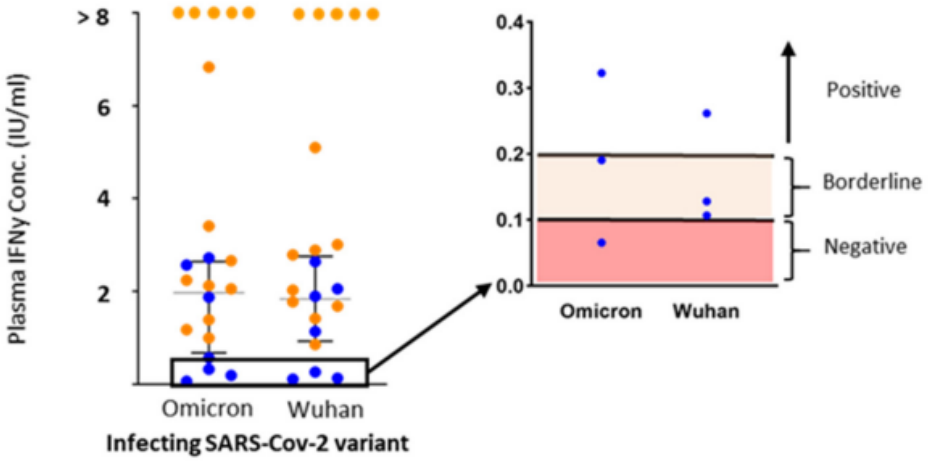

Figure 1. Interferon gamma release upon in vitro restimulation with SARS-CoV-2 Wuhan and Omicron variant antigens. IFNy release was tested in the interferon gamma release assay after overnight stimulation with Omicron Spike variant antigen and Wuhan Spike variant antigen (*x*-axis) in individuals with breakthrough infection (BTI, orange circle, n = 14) and non BTI (blue circle, n = 7). A zoomed image (black box) shows the subjects that fell within the regions considered as a negative response (<0.1 IU/mL, shaded red) to SARS-CoV-2, and borderline results (0.1–0.2 IU/mL, shaded orange).

Continuous variables were plotted as boxplots (Figure 2).

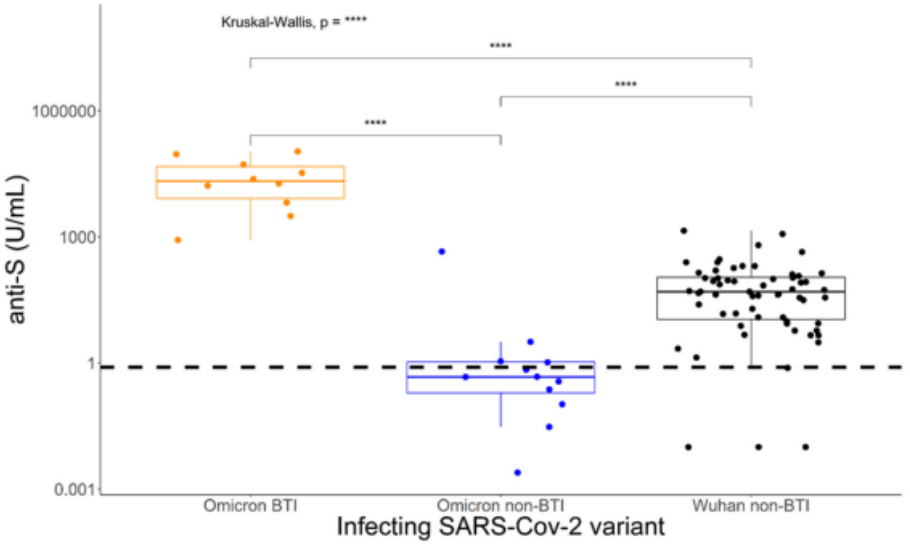

Figure 2. Induction of Wuhan Spike-Receptor Binding Domain-specific antibody concentrations differ between Omicron and Wuhan non-breakthrough infections and Omicron breakthrough infections. Patients were tested at 2–5 weeks after PCR diagnosis of SARS-CoV-2 infection with the Roche Elecsys anti-S assay, which incorporates the receptor binding region of the Wuhan wild type virus. Statistical analyses were performed using the Mann–Whitney U test. **** $p < 0.0001$.

3. Results

In order to determine whether the SARS-CoV-2 IGRA TUBE was able to detect SARS-CoV-2 infection similarly to the SARS-CoV-2 IGRA Omicron tube, the plasma concentration of IFNy was measured. This was performed 6–55 days after the infection, after overnight stimulation of whole blood with the respective antigens. Out of the 26 subjects, 2 had to be excluded from the analysis; 1 subject had an invalid negative control (IFNy > 0.4 IU/mL), while 1 subject had an invalid positive control (IFNy < 0.4 IU/mL). Further, three subjects had a missing Omicron tube resulting in $n = 21$ complete datasets. When it comes to the original SARS-CoV-2 IGRA TUBE, two measurements fell within the borderline range of IFNy detection (0.1–0.2 IU/mL), while SARS-CoV-2 in the rest of the measurements ($n = 19$) was detected (>0.2 IU/mL) (Figure 1). However, when it comes to the SARS-CoV-2 IGRA Omicron tube, 1 measurement was categorized as negative to SARS-CoV-2, 1 measurement was in the borderline range, while 19 measurements were considered SARS-CoV-2 positive (Figure 1). Next, we wanted to determine whether the IFNy production from the Omicron tube might be of higher concentration due to similarity with the strain causing the infection. For this analysis, only subjects that had values in the linear range for both tubes were included ($n = 16$). There was no significant difference in the IFNy production between both tubes ($p = 0.99$) (Figure 1). Furthermore, the ratio of the median difference in IFNy concentration between the Omicron and the IGRA TUBE was 1.04 (IQR: 0.82–1.2).

Next, we compared the influence of Omicron versus Wuhan infection on detection of SARS-CoV-2-specific antibodies using the Roche Elecsys anti-S assay at 2–5 weeks after infection. This assay detects Spike-specific antibodies to the RBD region using a truncated S1 with the original Wuhan antigen sequence. In non-vaccinated (non BTIs) Omicron infected patients ($n = 12$), the assay detected a factor 100-times lower median Spike-specific RBD antibody concentration ($p < 0.0001$) compared to patients at matched time points

after diagnosis during May 2020–January 2021 (Figure 2). In contrast, for patients who had been vaccinated against SARS-CoV-2 before Omicron infection (n = 10) (BTIs), the Roche Elecsys anti-SARS-CoV-2 anti-S1 assay detected a 400-times higher median specific antibody concentration ($p < 0.0001$) compared to patients referring to the beginning of the pandemic.

4. Discussion

With frequent emergence of new SARS-CoV-2 virus variants, accumulating especially immune evasion mutations in the Spike protein, it is of utmost importance to ensure that current diagnostic tools are up to date and still functional in the detection of the most recent and prevalent virus strains. Furthermore, determining potential shortcomings early is important as the development or update of these diagnostic tools is a lengthy process. In this study, we have decided to analyze the Quan-T-Cell SARS-CoV-2 assay produced by EUROIMMUN. They contain an IGRA stimulator TUBE, containing peptide antigens derived from the Spike protein of the original Wuhan virus strain. By using samples from Omicron infected patients, we compared this original stimulator tube with an updated version of the stimulator tube containing Omicron variant based peptides.

EUROIMMUN provided both tubes, in order to determine whether the stimulator tube within the kit needs to be adapted to the Omicron variant. Our results show that the original stimulator with the Wuhan antigen was able to detect all the Omicron infected subjects. Furthermore, the concentrations of IFNy produced by the SARS-CoV-2-specific immune response were similar between both stimulator tubes. This can be explained by the T-cells recognizing a variety of epitopes in antigenic regions more conserved within the Spike protein, compared to the more variable RBD region, which contains the majority of Spike-specific antibody escape mutations impacting on RBD recognition. This is consistent with previous results using other techniques, such as intracellular cytokine staining or virus neutralization assays [5]. Nevertheless, this study has some limitations. For example, the relatively small sample size and the different group sizes. This was due to the difficulties to quickly identify and recruit participants—particularly unvaccinated individuals. Future studies should avoid these limitations by including a sample size calculation in the process of the study design and focus on an even recruitment in both groups. In addition, the Roche Elecsys anti-SARS-CoV-2 anti-S1 assay is based on the original Wuhan virus Spike antigen and was not adapted to include Omicron Spike version.

In conclusion, our results suggest that the current Wuhan antigen based Quant-T-Cell-SARS-CoV-2 kit detects T-cell response to the currently prevalent Omicron variants with similar results to tubes with an Omicron adapted antigen. The data suggest that different test systems show variable performance when used on patients infected with different variants of SARS-CoV-2. An Omicron only infection induces an antibody response that better recognizes Omicron-Spike variants over the Wuhan variants, while Spike-specific T-cell responses are much less affected [5,17–19]. As a consequence, commercial diagnostic assays using Wuhan-based antigen for quantifying the Spike-specific antibody response upon Omicron infection may underestimate the variant-specific response magnitude, while assays that detect T-cell responses are much less affected. Using the tests presented here, we can conclude that unvaccinated Omicron first time infected patients for example will show weak and very delayed seroconversions in the Elecsys anti S1 assay, while the IGRA with wild type or Omicron peptides is positive much earlier.

Thus, depending on the clinical question, medical doctors and laboratories need to know the performance characteristics of their test systems in regard to the history of the patient to draw the right conclusions.

Furthermore, the results of our research also suggest that the original Quan-T-Cell kits can still be used in the current Omicron phase of the pandemic. Potential applications of the T-cell analysis could, for example, be to provide immunological insight into the disease dynamics over a longer timeframe, compared to, for example, PCR tests. Furthermore, it can be used to check the immune status and compare breakthrough infections and

non-breakthrough infected patients. The assay may also be used to detect asymptomatic infections. Comparing the serologic results of the groups, all Omicron BTI-subjects are far above the positivity threshold. However, infection- and vaccination-naive individuals responded in a much weaker fashion than what was observed in the control group of patients infected with the Wuhan strain. Actually, in the anti-S1 response shown here, after 20–40 days, only 4 out of 12 are above the positivity threshold provided by the manufacturer at the investigated timepoint. Almost all of the patients had seroconverted against Nucleocapsid at the same time point (data not shown). This demonstrates that a detectable serological response was also found, confirming the diagnosis and data obtained with the IGRA assays. However, the measured response in the Roche Anti-S ELECSYS assay, using a truncated S1 as a target structure, are considerably lower compared to the values observed in Wuhan strain infected subjects [20]. Whether this observation is primarily due to differences in binding to the antigen used, or due to less immune- stimulation in the commonly milder Omicron variant infections as compared to the Wuhan strain is unclear and cannot be elucidated within this study.

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Informed Consent Statement: Informed consent was obtained from all participants prior to any study activities.

Data Availability Statement: Further information and data can be requested via mail to the corresponding authors and is not available online due to privacy reasons.

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Conflicts of Interest: D.Z. is employed by EUROIMMUN, a manufacturer of diagnostic reagents and co-owner of patents related to serological assays for the diagnosis of SARS-CoV-2 and the detection of immunity as a result of vaccination. EUROIMMUN and Roche provided kits and machines for analyses at discounted rates. The authors declare no conflict of interest.

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9. Figures and Tables

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